

Analysis of the *Borrelia burgdorferi* Cyclic-di-GMP-Binding Protein PlzA Reveals a Role in Motility and Virulence[∇]

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Received 21 January 2011/Returned for modification 13 February 2011/Accepted 18 February 2011

The cyclic-dimeric-GMP (c-di-GMP)-binding protein PilZ has been implicated in bacterial motility and pathogenesis. Although BB0733 (PlzA), the only PilZ domain-containing protein in *Borrelia burgdorferi*, was reported to bind c-di-GMP, neither its role in motility or virulence nor its affinity for c-di-GMP has been reported. We determined that PlzA specifically binds c-di-GMP with high affinity (dissociation constant [K_d], 1.25 μ M), consistent with K_d values reported for c-di-GMP-binding proteins from other bacteria. Inactivation of the monocistronically transcribed *plzA* resulted in an opaque/solid colony morphology, whereas the wild-type colonies were translucent. While the swimming pattern of mutant cells appeared normal, on swarm plates, mutant cells exhibited a significantly reduced swarm diameter, demonstrating a role of *plzA* in motility. Furthermore, the *plzA* mutant cells were significantly less infectious in experimental mice (as determined by 50% infectious dose [ID_{50}]) relative to wild-type spirochetes. The mutant also had survival rates in fed ticks lower than those of the wild type. Consequently, *plzA* mutant cells failed to complete the mouse-tick-mouse infection cycle, indicating *plzA* is essential for the enzootic life cycle of *B. burgdorferi*. All of these defects were corrected when the mutant was complemented in *cis*. We propose that failure of *plzA* mutant cells to infect mice was due to altered motility; however, the possibility that an unidentified factor(s) contributed to interruption of the *B. burgdorferi* enzootic life cycle cannot yet be excluded.

Cyclic dimeric-GMP (c-di-GMP) [bis(3',5')-cyclic diguanylic acid] is a ubiquitous bacterial second messenger. c-di-GMP is produced from 2 molecules of GTP by the activity of diguanylate cyclase and is degraded by phosphodiesterase enzymes. The activities of these cyclase and phosphodiesterase enzymes are often regulated by changing physiological conditions. c-di-GMP has been implicated in the regulation of diverse bacterial properties, including biosynthesis of adhesins and exopolysaccharide matrix for biofilm formation, different forms of motility, long-term survival, and response to environmental stresses, in addition to production of virulence factors and to virulence (14, 29, 39, 76, 88, 96). These cellular functions regulated by c-di-GMP often utilize multiple receptors and signaling mechanisms. Several types of c-di-GMP receptors have been reported, including PilZ, PelD, I-site effectors, FleQ, and Clp (29, 30, 40, 76). Although many of the downstream mechanisms remain to be identified, studies indicate that c-di-GMP can impact cellular signaling at multiple levels, including transcription, translation, posttranslation, protein activity, protein secretion, and protein stability (7, 15, 17, 30, 41, 48, 50, 57, 58, 70, 86, 93).

One c-di-GMP receptor, the PilZ protein, was originally identified *in silico* as a putative conserved sequence based on

the binding of c-di-GMP to the BcsA1 protein, a component of the cellulose synthase complex of *Acetobacter xylinus* (2, 69, 94). The “PilZ domain,” named after the PilZ protein from *Pseudomonas aeruginosa*, is required for the assembly of functional type 4 pili (T4P) (1). Recently, proteins containing PilZ domains have been shown to bind c-di-GMP in several species of bacteria (see references 29 and 76 and references therein). Strong affinity of c-di-GMP for PilZ domains has been observed, with dissociation constants (K_d) in the range of 1 nM to 2 μ M, and this strong affinity is consistent with the low concentration of c-di-GMP in cells (29, 37, 76, 78). Furthermore, PilZ proteins from a number of bacteria have been implicated in the regulation of specific cellular processes, such as motility, polysaccharide synthesis, biofilm formation, and virulence of animal and plant pathogens (5, 13, 15, 30, 41, 48, 60, 70). The roles of PilZ appear to be different in different bacterial species. For example, as mentioned above, PilZ of *P. aeruginosa* (PA2960) is necessary for T4P biogenesis and twitching motility. A *pilZ* knockout mutant is defective in the secretion of PilA polymers to the bacterial surface, though intracellular PilA pools are not affected (1). On the other hand, a PilZ ortholog knockout mutant of *Neisseria meningitidis* is piliated but is not able to produce bacterial aggregates (11); a *Xanthomonas campestris* pv. *campestris* PilZ knockout mutant has slightly reduced T4P-dependent motility on semisolid Eiken agar (47); and a *pilZ* mutation in *Xanthomonas axonopodis* pv. *citri* resulted in increased swarming motility relative to the parental wild-type cells (27). A PilZ domain-containing protein, Alg44, was shown to upregulate alginate production in *P. aeruginosa*

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∇ Published ahead of print on 28 February 2011.

(48). Furthermore, in *Escherichia coli* (and *Salmonella enterica*), the reduced motility of a phosphodiesterase mutant is alleviated by a *pilZ* mutation (58, 70), indicating a role of PilZ in bacterial motility. Specifically, the *E. coli* PilZ protein, YcgR, was found to control motility by directly interacting with and altering flagellar switch protein FliG, FliM, or motor protein MotA function in response to c-di-GMP (3, 7, 17, 57). In *Vibrio cholerae*, a mutation in genes encoding PilZ proteins also resulted in altered motility (60). These observations suggest that signaling by PilZ could be modulated differently in different species.

Although c-di-GMP may have different roles in different species of bacteria, little is known about this signaling system in *Borrelia burgdorferi*, the Lyme disease spirochete. The spirochete resides in disparate host environments, including *Ixodes* ticks and mammalian hosts. During transmission between hosts, *B. burgdorferi* modulates gene expression in response to environmental factors, such as nutrient availability, pH, CO₂, temperature, host factors, and immune response (10, 55, 65, 74, 90, 91). c-di-GMP levels, which are controlled by a two-component system, are also believed to be involved in bacterial adaptation to different host environments (84, 88, 95). While many species of bacteria were reported to contain multiple enzymes responsible for controlling the levels of c-di-GMP (22), bioinformatics, as well as experimental analysis, indicates that *B. burgdorferi* utilizes only a limited number of enzymes to modulate c-di-GMP: a single diguanylate cyclase, two phosphodiesterases, and a single c-di-GMP-binding PilZ homolog designated PlzA (2, 21, 22, 66, 71, 87). *B. burgdorferi* diguanylate cyclase (BB0419/Rrp1) was shown to regulate a diverse set of genes, many of which were reported to be associated with *B. burgdorferi* virulence, implying a role of c-di-GMP in pathogenesis (66, 71). We recently demonstrated that BB0363 is a phosphodiesterase which specifically hydrolyzed c-di-GMP into pGpG (87). Furthermore, a *bb0363/pdeA* mutant exhibited impaired motility, and those mutant cells were unable to infect experimental mice, irrespective of the size or route of inoculum (needle or tick), demonstrating a role of c-di-GMP in *B. burgdorferi* motility and virulence (87).

Recently, PlzA was shown to specifically bind c-di-GMP, and the expression of *plzA* was shown to be modulated under different environmental conditions (21). While PilZ has different functions in different bacteria, a role for the lone c-di-GMP-binding protein PlzA has not been demonstrated in *B. burgdorferi*. Here we demonstrate that PlzA binds c-di-GMP *in vitro* with strong affinity. The deletion of *plzA* reduces motility and infectivity in experimental mice. The mutant cells exhibit lowered viability in ticks. Consequently, the *plzA* cells are unable to complete the mouse-tick-mouse infection cycle, consistent with a role for PilZ in bacterial pathogenesis. Furthermore, by constructing a *plzA* mutant in the *pdeA* mutant background, where the cellular c-di-GMP level is 2× higher than that in the wild-type cells, we demonstrate that PlzA regulates *B. burgdorferi* motility in a different manner from in *E. coli* and that elevated c-di-GMP in the *B. burgdorferi pdeA* mutant regulates motility by a mechanism independent of PlzA. A mechanism of altered motility and infectivity is proposed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Low-passage, virulent *B. burgdorferi* strain B31-A3-68 *Δbbe002* was used as the wild type throughout the study (a kind gift from R. Rego and P. Rosa, Rocky Mountain Laboratories, NIH) (63). This strain is a derivative of A3-68 (63), lacking circular plasmid 9 (cp9) and linear plasmid 56 (lp56), with the *bbe002* gene inactivated with a *P_{regB}-aadA* cassette to increase transformation frequency (36, 63). B31-A3-68 *Δbbe002* is a derivative of strain B31 (16). The genome of the virulent B31 strain has been sequenced and was found to contain a total of 21 plasmids with 12 linear and 9 circular plasmids, in addition to the 960-kb linear chromosome (12, 20). *B. burgdorferi* was cultured in liquid Barbour-Stoenner-Kelly (BSK-II) medium as described below; plating BSK medium was prepared with 0.6% agarose (52). Swarm plate assays were carried out with 0.35% agarose in plating BSK medium; approximately 1×10^6 cells in a volume of 5 μ l were spotted onto plates containing plating BSK medium diluted 1:10 in Dulbecco's phosphate-buffered saline without divalent cations. Because *B. burgdorferi* is a slow-growing organism with an 8- to 12-h generation time, swarm plates were incubated for 5 to 7 days (52, 53). The paired Student's *t* test (Minitab, Inc.) was used to compare wild-type and *plzA* mutant cell swarm diameters.

BB0733 recombinant protein expression. Recombinant BB0733 protein (PlzA) was expressed with an N-terminal 6×His (His₆) fusion tag. To express BB0733 protein in *E. coli*, the coding sequence was amplified by PCR using the primers (5'→3' throughout) R-BB0733-F (CTTTTATCTAGAAAATAAGAG) and T-BB0733-R (CTTTGATTAATTGAAATAATC). The amplified DNA fragment was ligated into an *E. coli* expression vector pTrc His TOPO (Invitrogen, Inc.) by TA cloning. The vector was then digested and sequenced to confirm its integrity. *E. coli* cells containing *pb0733-TRC* were induced with 0.25 mM isopropyl β -D-1-thiogalactopyranoside at 30°C for 4 h and Ni²⁺ affinity purified under native conditions. Purified recombinant BB0733 protein was resolved by SDS-PAGE to determine protein concentration and purity.

c-di-GMP-binding assays by equilibrium dialysis. Equilibrium dialysis was performed as described previously (70). Briefly, DispoBiodialyzer cassettes (The Nest Group, Southborough, MA) contain 2 chambers separated by a 5-kDa cutoff membrane. Sixty microliters of c-di-GMP (2 to 50 μ M) was injected into one chamber, and 60 μ l (10 μ M) of test protein BB0733 or positive control *E. coli* c-di-GMP-binding PilZ receptor YcgR (70) was injected into the other chamber; the c-di-GMP and test proteins were contained in a mixture of 300 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 50 mM sodium phosphate (pH 7.4). Following gentle rocking at room temperature for 24 h to reach equilibrium, samples from both sides of the cassettes were removed, boiled for 4 min, centrifuged, and filtered through a 0.22- μ m-pore microfilter (Millipore Ultrafree MC). c-di-GMP concentrations in both samples were determined via reverse-phase high-performance liquid chromatography (HPLC) on a Supelcosil LC-18T column using the same buffer and gradient program described in reference 71, and c-di-GMP was quantified at 254 nm. The binding constant was determined with Graph Pad Prism 5 software.

RT-PCR. Exponentially growing *B. burgdorferi* wild-type cells (2×10^7 cells ml⁻¹) were treated with RNAProtect followed by total RNA isolation with the RNeasy minikit (Qiagen, Inc.). Contaminating DNA in the RNA samples was removed by RNase-free Turbo DNase I (Ambion, Inc.) digestion for 3 h at 37°C followed by RNeasy minipurification. For reverse transcription-PCR (RT-PCR), cDNA was prepared from 1 μ g RNA with Superscript III reverse transcriptase according to the manufacturer's protocol (Invitrogen, Inc.). The RT-PCR primer sequences were a1 (TGTTAAATATATAAGCAGCTTATGCG), a2 (GCGGGCAATACTGTATCTTCTTGA), b1 (TTGAGATGAGATAGGCTCTTATACC), b2 (GCGGGCAATACTGTATCTTCTTGA), c1 (TTGAGT GCTTCTGCTTATTCTTCT), and c2 (GATACGAAAATACTTATCACAAAAGC). A Bio-Rad iScript cDNA synthesis kit and an iCycler detection system were used to measure *bb0733* transcript levels according to the manufacturer's instructions. The gene coding for *B. burgdorferi* enolase was used as a reference gene (54, 87). The gene-specific primers were RT-enolase-F (TGGAGCGTACAAAGCCAACATT), RT-enolase-R (TGAAAAACCTCTGCTGCCATTC), Bb0733-rt-F (TTGAGATGAGAAATAGGCTCTTATACC), and Bb0733-rt-R (GCGGGCAATACTGTATCTTCTTGA). The relative level of expression was calculated by the threshold cycle ($2^{-\Delta\Delta CT}$) method (46, 79, 87).

Construction of a *plzA* (*bb0733*) mutant. Targeted inactivation of *bb0733* (a 786-bp gene) was achieved by homologous recombination using a promoterless kanamycin (Pi-Kan) cassette (87). PCR amplification of *bb0733*, construction of the inactivation plasmids, and electroporation of linear *bb0733*-Pi-Kan DNA into competent cells were carried out as described previously (52, 53, 73, 87). Briefly, *bb0733* DNA was PCR amplified with the GoTaq PCR system (Promega, Inc.) and primers BB0733-KO-F (AGTTTTGCATTTGGAAGCT

GGA) and BB0733-KO-R (ATCCCCAAAGTGCCTCATG). The amplified DNA was ligated into the pGEM-T Easy vector (Promega, Inc.), yielding *pb0733*-Easy. Pl-Kan was similarly PCR amplified from *P_{flgB}*-Kan (8) with the primers PrLs-Kan AfIII-F (CTTAAGTAGTAAAAGCAAT) and PrLs-Kan AfIII-R (CTTAAGTTAGAAAACTCAT) (restriction sites are in boldface). The AfIII-restricted Pl-Kan DNA was then inserted into the AfIII sites within *bb0733* (located 167 and 676 bp from the ATG start codon) of the *pb0733*-Easy vector, yielding *pb0733*-Pl-Kan. This deletion-insertion was *in frame*. Restriction mapping indicated that the direction of transcription of Pl-Kan was the same as that of *bb0733*. *bb0733*-Pl-Kan was digested and separated from *pb0733*-Pl-Kan vector by NotI digestion, precipitated, and electroporated into the wild-type competent cells (52). Mutants were selected on solid growth medium containing 200 $\mu\text{g ml}^{-1}$ kanamycin plus 100 $\mu\text{g ml}^{-1}$ streptomycin. Resistant colonies were analyzed by PCR for the presence of the Pl-Kan cassette with the primers PrLs-Kan AfIII-F and -R as well as primers BB0733-KO-F and -R. PCR-positive mutants were further examined for their plasmid contents by using 21 sets of primers to detect 21 linear and circular plasmids (100).

Complementation of the *bb0733* (*plzA*) mutant. To complement *bb0733::Pl-Kan*, the promoter of *bb0733* (*P_{plzA}*; 400 bp upstream from the ATG start codon of the *bb0733/plzA* gene) and the coding sequence was PCR amplified by PCR with primers 733 comp-F-SpeI (GCGCCACTAGTTGTGATAATAGAAAAGCTAACTGCGAAATA) and 733-comp-R-NotI (GCGCGCGGCCGCAAAATACAA CCAAGAAGACTTAAAC). The PCR product was ligated into pGEM-T Easy. The *bb0733* promoter-*bb0733* (*P_{bb0733}-bb0733/P_{plzA}-plzA*) DNA was then inserted into suicide vector pXLF14301 (99) by SpeI-NotI restriction digestion, yielding pXLFBB0733. Fifty micrograms of the resultant plasmid was electroporated into the *bb0733::Pl-Kan* competent cells. Transformants were selected in plating BSK medium containing 100 $\mu\text{g ml}^{-1}$ streptomycin, 200 $\mu\text{g ml}^{-1}$ kanamycin plus 40 $\mu\text{g ml}^{-1}$ gentamicin. Resistant transformants were analyzed by PCR for the presence of kanamycin (Kan) and gentamicin (Gen) genes and an intact *bb0733* gene. Positive clones were further examined for their plasmid content by PCR.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting by an enhanced chemiluminescent detection method (GE Healthcare) were carried out as previously reported (23). *B. burgdorferi* strain B31-A3-68 *Abbe002* (wild-type), *plzA*, or *plzA-pdeA* cells were cultured to 5×10^7 cells ml^{-1} . Cells were pelleted, resuspended in phosphate-buffered saline (PBS), and equalized to protein content with the Bradford assay reagent (Bio-Rad, Inc.). Samples were boiled, and approximately 5 μg of cell lysate was loaded per lane. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane and Western blotted with monoclonal FlaB (A. Barbour), polyclonal MotB (J. Carroll), FliG1 and FliG2 (C. Li), FliM (D. Blair), CheY3, and FliL antisera. Specific monoclonal or polyclonal reactivity to *B. burgdorferi* FlaB, MotB, FliG1, FliG2, FliM, FliL, and CheY3 has been reported previously (42, 43, 51, 53, 54, 72; M. A. Motaleb et al., unpublished data).

Experimental mouse-tick-mouse infection model of *B. burgdorferi*. Six-week-old female C3H/HeN mice were purchased from Charles River Laboratories, North Carolina, and housed in the East Carolina University animal facility at the Brody School of Medicine according to the institutional guidelines for the care and use of laboratory animals. For infection via needle, 5×10^2 to 5×10^6 *in vitro*-grown spirochetes were injected subcutaneously as described previously (16, 35, 64). The number of spirochetes was determined with a Petroff-Hausser chamber, and each clone was verified for its retention of the lp25, lp28-1, and lp36 plasmids. Mice were bled 2 weeks postinfection for immunoblot analysis of mouse sera against *B. burgdorferi* antigens to determine infectivity, as described previously (35, 80, 87). Reisolation of *B. burgdorferi* from mouse skin, bladder, and joint tissues was performed 6 weeks postinfection to assess the ability of spirochetes to infect mice as described previously (16, 26, 83, 87). Mouse tissues were placed in BSK-II growth medium and incubated for up to 35 days; the presence of spirochetes was determined by dark-field microscopy. The 50% infectious dose (ID_{50}) was calculated as described below (35, 62).

For tick infection studies, naïve *Ixodes scapularis* larvae were purchased from Oklahoma State University. Two independent experiments were performed with ticks. Ticks were kept at 23 to 24°C under a 14-h-light/10-h-dark photoperiod in a humidified chamber with 85 to 90% relative humidity. Approximately 100 larval ticks were fed to repletion on spirochete-infected mice for 5 to 7 days, allowed to fall off, and collected. One subset of larvae was dissected 7 days after repletion, and the isolated midguts were analyzed by indirect immunofluorescence assays (IFAs) for the presence of spirochetes (83, 87). A second subset of fed larvae was surface sterilized with 3% H_2O_2 , followed by 70% ethanol, crushed in BSK-II medium, and plated to determine the number of CFU per tick. A third subset of larvae was crushed, and genomic DNA was extracted with the DNeasy blood and tissue kit (Qiagen, Inc.). Quantitative PCR (qPCR) was performed to

quantitate the level of the tick actin gene by using primers ActinT-F (GATCA TGTTGAGACCTTCA) and ActinT-R (CGATACCCGTGGTACGA) versus the level of *B. burgdorferi* *flaB* by using primers FlaB qRT-F (TTGCTGATCA AGCTCAATATAACCA) and FlaB qRT-R (TTGAGACCCTGAAAGTGAT GC), as described previously (97, 99). The remaining fed larvae were allowed to molt to nymphs. The infected nymphs were then fed to repletion on naïve 6-week-old female C3H/HeN mice, allowed to fall off, and collected. Subsets of nymphs were processed for IFA or plated to determine the number of CFU per tick as described above. Six weeks postinfection, mouse sera were collected for immunoblot analysis, and tissues were collected for reisolation of *B. burgdorferi*.

Determination of ID_{50} and statistical analysis. The dose required to infect 50% of the mice inoculated was experimentally determined for the wild-type, *plzA* mutant, and complemented *plzA⁺* strains as described previously (35, 62). The data from the ID_{50} infection experiment and the single-dose infection experiment for each strain were combined for the estimations of the 50% infectious dose. Comparison between strain ID_{50} values was made by using a generalized linear model with a probit link function. This method is also known as probit regression (35), and in it we assume identical slopes in the response/log-dose relationship but different intercepts for each strain. Graphically those assumptions manifest themselves as dose-response curves with lateral shifts corresponding to the changes in intercept. Additionally, an overdispersion parameter was fit in order to accommodate greater homogeneity in infection rates than would otherwise be permitted by the model. All calculations were carried out with JMP V9 software (SAS Institute, Inc., Cary, NC).

Artificial inoculation of ticks by immersion studies. Tick immersion studies were performed as described previously (87). Briefly, approximately 150 tick larvae were artificially infected by immersion (in duplicate) in equal-density exponential-phase *B. burgdorferi* cultures (5×10^7 cells/ml), as described previously (4, 59, 83, 87). Ticks were fed to repletion on separate naïve mice for 5 to 7 days, allowed to fall off, and collected. Subsets of larvae were dissected 7 to 10 days after repletion, and the crushed ticks were analyzed by immunofluorescence assay (IFA) for the presence of spirochetes (83, 87). Fed larvae were treated as described above to determine the number of CFU per tick. Reisolation of spirochetes from mouse ears, joints, and bladders was performed as described above.

IFAs. Ticks were dissected in 25 μl PBS-5 mM MgCl_2 in Teflon-coated microscope slides, mixed by pipetting, and then air dried (4, 87). To avoid quenching by hemin in the blood, dissected tick contents were 10-fold serially diluted. Slides were blocked with 0.75% bovine serum albumin (BSA) in PBS-5 mM MgCl_2 and washed with PBS-5 mM MgCl_2 . Spirochetes were detected with a 1:100 dilution of goat anti-*B. burgdorferi* antisera labeled with fluorescein isothiocyanate (Kirkegaard & Perry Laboratories). Images were captured with a Zeiss Axio Imager M1 microscope coupled with an AxioCam MRc digital camera (Carl Zeiss, Inc.).

RESULTS

BB0733 binds c-di-GMP with high affinity. Bioinformatics BLAST analysis suggests that BB0733 is the only PilZ domain-containing protein in *B. burgdorferi* (PilZ domain; Pfam PF07238) (1, 2, 20). BB0733, which has been named “PlzA,” shares significant amino acid sequence similarity with orthologs in other *Borrelia* strains (21). Although PlzA shares poor homology with PilZ proteins of other species of bacteria, it contains the conserved residues of the “PilZ domain” (RXXXR; D/NZSXXG where “X” is any amino acid and “Z” is a hydrophobic residue) but lacks the so-called “PilZ-N”-terminal domain (N-terminal domain of *E. coli* PilZ protein YegR [PF07317]) (2, 70) and therefore is considered a “stand-alone” c-di-GMP effector protein (21). Recently, using X-ray cross-linking studies, Freedman et al. confirmed that PlzA specifically binds c-di-GMP and that the conserved arginine (R) residues of $^{160}\text{RXXXR}$ (number based on amino acid residues of BB0733 protein) (20) is critical for c-di-GMP binding (21). However, a dissociation constant (K_d) of PlzA for c-di-GMP was not determined (21). To determine the K_d of PlzA, synthetic c-di-GMP (38, 41) and recombinant, purified His₆-PlzA (Fig. 1) were used in equilibrium dialysis, as described previ-

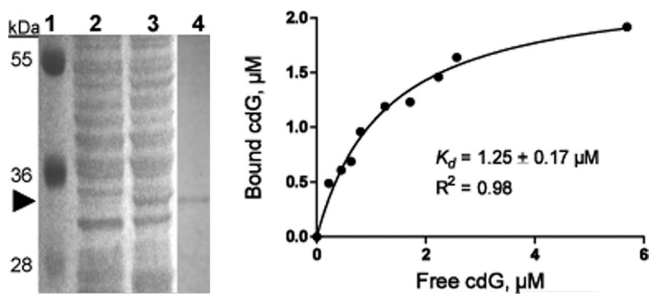


FIG. 1. (Left panel) SDS-PAGE analysis of expressed His₆-BB0733/PlzA recombinant protein from *E. coli*. Lane 1, protein size marker with sizes in kDa; lanes 2 and 3, uninduced and induced *E. coli* lysates, respectively; lane 4, His₆-PlzA protein after purification in an Ni²⁺-agarose column. An arrowhead indicates the His₆-PlzA (~32-kDa) protein band. (Right panel) Plot of equilibrium binding between PlzA and synthetic c-di-GMP (cdG). Equilibrium dialysis was performed as described above, and the binding constant was determined with GraphPad Prism 5 (one site, specific binding). Results with GTP, cAMP, or cGMP were not shown since no binding with His₆-PlzA was detected.

ously (70). An *E. coli* c-di-GMP-binding PilZ protein, YcgR, was used as a positive control (70). Figure 1 shows that His₆-PlzA is highly purified and binds c-di-GMP; His₆-PlzA did not bind other nucleotides tested (cyclic AMP [cAMP], GTP, and cyclic GMP [cGMP]; data not shown) confirming the findings reported by Freedman et al. (21). The dissociation constant of PlzA was determined to be $1.25 \pm 0.17 \mu\text{M}$, indicating high affinity for c-di-GMP, consistent with K_d values (1 nM to 2 μM) reported for c-di-GMP-binding proteins from other bacteria (29, 37).

Inactivation and complementation of *plzA* in *B. burgdorferi*.

B. burgdorferi genome sequence analysis predicted that the genes upstream (*bb0732*) and downstream (*bb0734*) of *bb0733* are transcribed in the opposite direction from *bb0733* (Fig. 2). Using RT-PCR, we confirmed that *bb0733* is a monocistronic mRNA that is expressed in growing *B. burgdorferi* cells and that the *bb0732* and *bb0734* genes are not cotranscribed (data not shown). *bb0732* is annotated as a penicillin-binding protein that is predicted to be responsible for biosynthesis and degradation of murein sacculus and peptidoglycan of cell envelope, whereas *bb0734* encodes a Sua5/YciO/YrdC/YwC family protein of unknown function. In *Shewanella oneidensis* and the psychrophile *Colwellia psychrerythraea* 34H, the Sua5/YciO/YrdC/YwC family protein is a putative translation factor involved in translation, as well as ribosomal structure and biogenesis (28, 49).

The expression of *B. burgdorferi* PlzA was reported to be maintained throughout the enzootic cycle; however, its expression was significantly increased in ticks after a blood meal (21). A function of *plzA* has not been demonstrated. To investigate the function of PlzA during the enzootic life cycle of *B. burgdorferi*, we inactivated *plzA* with a promoterless kanamycin cassette (PI-Kan) by allelic exchange, as described previously (87). Electroporation of the *plzA*::PI-Kan linear DNA (Fig. 2) into the wild-type competent cells yielded ~10 kanamycin-resistant clones. PCR analysis confirmed that a 509 bp internal fragment of *plzA* is replaced by the 872-bp PI-Kan cassette in all clones, confirming inactivation of *plzA* (Fig. 3, left panel).

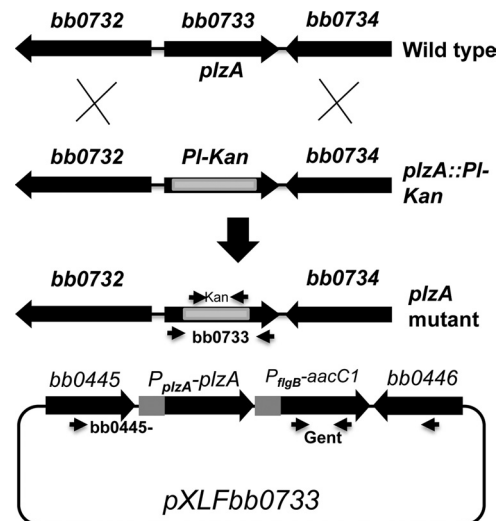


FIG. 2. Inactivation and complementation of *bb0733* (*plzA*). (Top) In-frame insertion of the PI-Kan cassette within the *bb0733* gene by homologous recombination resulted in the creation of *plzA* mutant. Approximate locations of *kan* and *bb0733* primer sets are shown by thin arrows that were used to confirm the *bb0733* inactivation by PCR (Fig. 3). (Bottom) Complementation plasmid pXLFbb0733 containing the *bb0733* promoter (P_{plzA}) that drives the expression of the *bb0733* (*plzA*) gene was used to complement the *plzA* mutant in *cis* by genomic integration in a region where genes *bb0445* and *bb0446* converge. Approximate locations of the primer sets (*bb0445* and *bb0446* and gentamicin [Gent]) used to confirm the integration of P_{plzA} -*plzA*- P_{flgB} -*aacC1* are shown by thin horizontal arrows. PCR primer products of each of these primer set are shown in Fig. 3.

Characterization of several positive clones confirmed identical genotypes and phenotypes; one randomly chosen clone was used for subsequent analysis.

To restore the synthesis (and phenotype [see below]) of *plzA* at the wild-type level, we complemented the *plzA* mutant by in *cis* genomic integration where genes *bb0445* and *bb0446* converge (Fig. 2, bottom). The insertion of P_{plzA} -*plzA* was downstream of both the *bb0445* and *bb0446* genes and less likely to affect their expression, as described previously (44, 99). We chose to complement the mutant in *cis*, since the shuttle vectors developed so far to complement *B. burgdorferi* mutants are multicopy plasmids (89) that result in overexpression of the target gene (43, 53, 77, 89). Accordingly, we introduced suicide vector pXLFbb0733 (Fig. 2, bottom) containing an intact *plzA* gene under its own promoter (P_{plzA} -*plzA*) into the mutant cells. Positive clones were characterized by PCR by using kanamycin, gentamicin, and *bb0445*-*bb0446* gene-specific primers (Fig. 3, middle panel). PCR analysis confirmed that P_{plzA} -*plzA* integrated within the *bb0445*-*bb0446* region, as expected (Fig. 2 and 3). To detect the expression of *plzA* in the wild-type, *plzA* mutant, and complemented *plzA* (*plzA*⁺) strains, we employed qPCR using gene-specific primers. Real-time PCR detected the *plzA* transcripts in wild-type and the complemented *plzA*⁺ cells but not in the *plzA* mutant cells (Fig. 3, right panel). Furthermore, the level of *plzA* transcripts synthesized in the complemented *plzA*⁺ cells was approximately 70% the level of wild-type cells, indicating the restoration of *plzA* synthesis in the complemented cells.

To confirm retention of *B. burgdorferi* endogenous plasmids

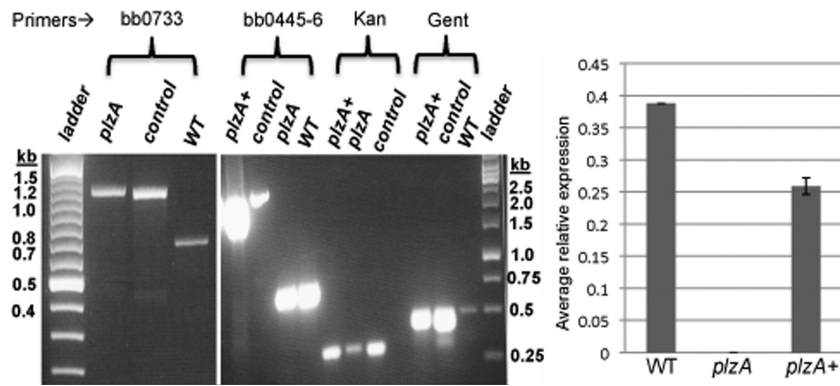


FIG. 3. Confirmation of *plzA* inactivation, complementation in *cis*, and restoration of *plzA* transcript synthesis. (Left panel) PCR was used to confirm the integration of PI-Kan within the *bb0733* (*plzA*) gene. The locations of PCR primers are shown in Fig. 2. An 892-bp DNA containing the PI-Kan cassette was inserted after deletion of a 509-bp fragment from the *plzA* gene. The size of PCR products for lanes containing *plzA* and the positive “control” DNA is 1,132 bp; the size for the wild type (WT) is 783 bp. A 100-bp ladder was used (Fermentas, Inc.). (Middle panel) Confirmation of the integration of *P_{plzA}-plzA-P_{flgB}-aacC1* between genes *bb0445* and *bb0446*. The PCR primers used to amplify a specific region are indicated at the top of the agarose gels. The sizes of all PCR products were amplified as expected. Controls in both gels were the plasmid DNAs that were used to inactivate *plzA* or complement the *plzA* mutant. A 1-kb DNA ladder (Fermentas, Inc.) was used in the middle panel. (Right panel) Real-time PCR detecting *bb0733* mRNA from the indicated strains. The bar represents the mean + standard error of the mean (SEM) from 2 independent experiments. Average relative expression of *bb0733* from each strain was calculated by the $2^{-\Delta\Delta CT}$ method. Enolase was used as an internal control (54, 87).

required for infectivity (56, 61) in the *plzA* mutant and complemented strains, PCR-based plasmid profiling using each of the plasmid-specific primers was employed (61, 100). As shown in Fig. 4, the plasmid profiles of the wild-type, *plzA* mutant, and complemented *plzA*⁺ strains were the same, confirming retention of all plasmids required for infectivity (see below).

***plzA* mutant is deficient in swarming motility.** Mutation of a gene encoding PilZ has been reported to generate various phenotypes: rough, rugose, opaque, or translucent bacterial

colony morphology and altered motility (2, 13, 27, 58, 60, 98). To determine if a mutation in *plzA* altered the *B. burgdorferi* swimming pattern or swarming motility (42, 53), the mutant cells were analyzed by microscopy and swarm plate motility assays in 0.35% agarose (52). Although cellular morphology and the swimming pattern (run, pause/flex, and reverse) (42, 53) of the *plzA* mutant cells were indistinguishable from those of the wild-type cells, the swarm diameter of *plzA* mutant cells was significantly reduced ($P = 0.001$) compared to that of the wild-type cells when the plates were incubated for 7 days (Fig. 5). Additionally, while the colony morphology of the wild-type cells was translucent, the mutant cells colonies were solid/opaque (which the figure was not clear enough to show). Both the swarming motility and colony morphology phenotypes were restored to wild type upon complementation (Fig. 5 and data not shown), indicating that the phenotypes associated with *plzA* cells are solely due to the mutation and not a secondary alteration elsewhere. Noticeable growth defects were not observed in *plzA* mutant cells, indicating the decreased-swarming phenotype was not due to a growth defect (not shown). These results indicate that *plzA* is essential for normal *B. burgdorferi* motility and colony morphology.

To determine if the deficient motility exhibited by *plzA* cells resulted from alteration of motility/chemotaxis protein synthesis, we performed Western blotting using specific antisera against the periplasmic flagellar protein FlaB; motor switch proteins FliM, FliG1, FliG2, and MotB; flagellar protein FliL; or the chemotaxis protein CheY3. As shown in Fig. 6, the levels of proteins detected by specific antisera in the *plzA* mutant cells were not altered compared with those of the wild-type cells. These results indicate that the reduced swarming observed in the *plzA* mutant cells was not due to a reduction in levels of FlaB, FliL, FliG1, FliG2, FliM, MotB, or CheY3 proteins.

***plzA* mutant cells are significantly attenuated in infecting mice.** In some bacteria, *pilZ* was shown to be required for the

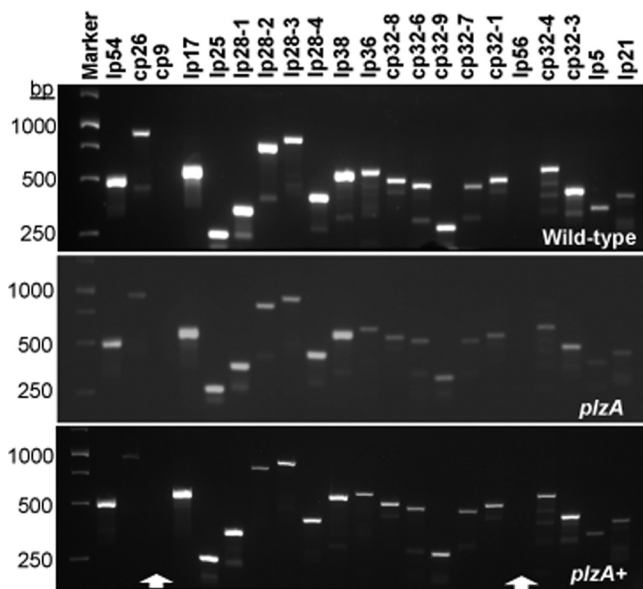


FIG. 4. PCR analysis confirmed that the wild-type, *plzA* mutant, and the complemented *plzA*⁺ cells retained endogenous plasmids required for infection of mice and ticks. Twenty-one sets of PCR primers were used to detect 12 linear (lp) and 9 circular (cp) *B. burgdorferi* plasmids (12, 100). Arrows indicate the cp9 and lp56 plasmids were lost from all strains.

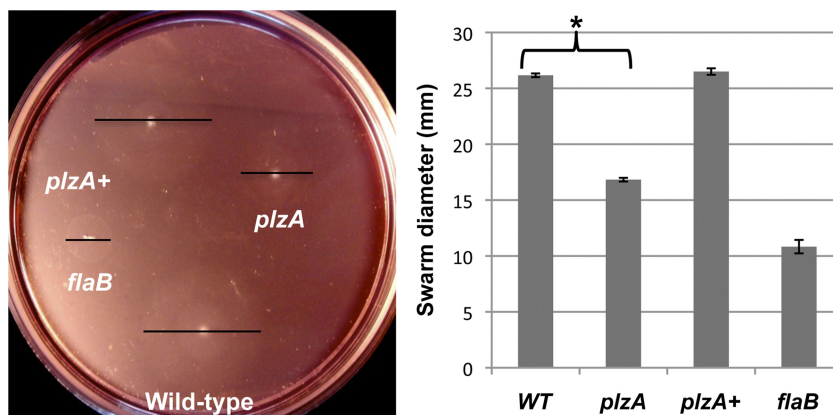


FIG. 5. Swarm plate motility assays confirmed that the *plzA* mutant was defective in motility. A representative swarm assay with *plzA* mutant cells incubated for 7 days is shown. Since the swarm diameters were not clearly visible, a black bar was placed on each swarm, which represents the size of their swarm diameters (left panel). Swarm diameters from 4 assays were measured in millimeters \pm standard deviations (right panel). A flagellinless, nonmotile *flaB* mutant was used as a control (51). *, significant difference ($P = 0.001$) between wild-type (WT) and *plzA* mutant cells.

host colonization/disease processes (47, 60). In order to evaluate the infection potential of the *plzA* mutant, groups of C3H/HeN mice were challenged with 10-fold-increasing doses of wild-type, *plzA*::Pl-Kan, or isogenic complemented *plzA*⁺ cells to determine the 50% infectious dose (ID₅₀). Two weeks postinoculation, the mice were bled and their sera were assessed for reactivity with *B. burgdorferi* antigen membrane protein A, also known as P39 (35, 80). Furthermore, to confirm the serology results, mice were sacrificed 6 weeks postinoculation, and spirochetes were reisolated from ear, bladder, and joint tissues. Serology results indicated that *plzA* mutant cells were attenuated in establishing an infection in mice (data not shown) and correlated well with reisolation of spirochetes from the tissues examined (Table 1). Whereas the *plzA*⁺ cells did

not show an increase in the ID₅₀ compared to the parental wild-type cells, the ID₅₀ for the *plzA* mutant was reproducibly more than 1 logarithm higher than that of the wild type (or complemented *plzA*⁺ strain), indicating a significant attenuation in virulence ($P = 0.0031$) (Table 1).

The *B. burgdorferi* *plzA* mutant strain is unable to complete the mouse-tick-mouse infection cycle. Because infection of and survival within the mammalian host represent only one facet of the *B. burgdorferi* enzootic life cycle, a more comprehensive evaluation of the behavior of these mutants in the tick vector was warranted. Naïve *Ixodes scapularis* larval ticks were allowed to feed on mice that were infected with mutant *plzA* (dose, 5×10^6 /mouse), wild-type, or complemented strains (dose, 5×10^4 /mouse). These doses were chosen to infect 100% of mice with wild-type, *plzA*, or complemented *plzA*⁺ cells (Table 1). Nine days after feeding on infected mice, a subsample of ticks were analyzed by immunofluorescence assays (IFAs) to determine the percentage of ticks infected with the different cells (Fig. 7 and data not shown). Spirochete loads in those fed ticks were determined by qPCR as well as by crushing ticks and plating on *B. burgdorferi* growth plates to determine the number of viable CFU per tick (Table 2). The majority of the fed larvae were allowed to molt to nymphs. As shown in Table 2, while almost 100% of naïve ticks that fed on wild-type- or the complemented *plzA*⁺ strain-infected mice became infected, only 55% of the ticks that fed on *plzA* mutant-infected mice were positive. Furthermore, the spirochete burden in the *plzA* mutant-infected ticks was >150-fold lower

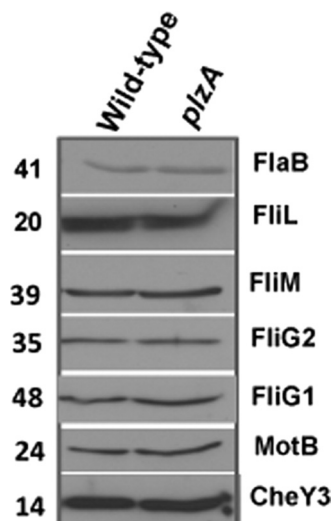


FIG. 6. The levels of prominent motility or chemotaxis proteins were not altered in the *plzA* mutant. Western blots using 5 μ g of cell lysates from the indicated strains were probed with antisera against the indicated motility and chemotaxis proteins. Specific antisera against the proteins used are indicated on the right side of each blot. The values on the left are molecular sizes in kilodaltons.

TABLE 1. *plzA* mutant cells are significantly attenuated in C3H/HeN mouse infection

Type of strain	No. of mice infected/total at dose of:					ID ₅₀
	5×10^2	5×10^3	5×10^4	5×10^5	5×10^6	
Wild type	0/8	3/8	10/10			8×10^3
<i>plzA</i> mutant	0/8	1/8	6/12	6/10	10/10	9×10^4
Complemented <i>plzA</i> ⁺	1/8	4/8	10/10			4×10^3

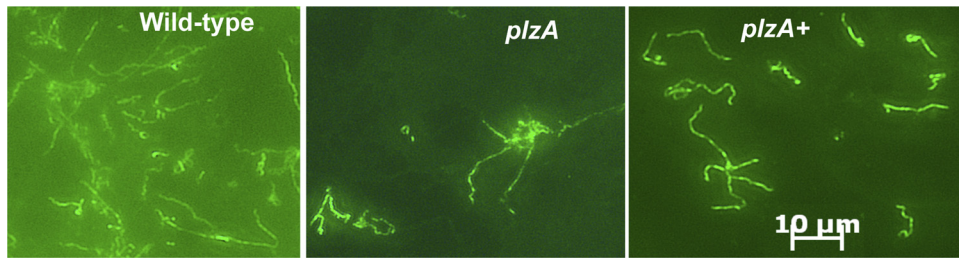


FIG. 7. Immunofluorescence assays (IFAs) of immersed larval ticks 7 days postfeeding. Spirochetes were detected in the wild-type, *plzA* mutant, and complemented *plzA*⁺ strains; however, this is the only tick in which several *B. burgdorferi* *plzA* mutant cells were detected. In nearly all ticks infected with *plzA* mutant spirochetes by immersion, no spirochetes were detected by IFA 7 days postfeeding (*n* = 15).

than that in the wild-type or isogenic complemented strains (Table 2). These results indicate that *plzA* mutant cells are deficient in ability to survive in ticks. Moreover, when the *plzA* strain-infected nymphs were fed on naïve mice, none of the mice became infected, whereas all naïve mice were infected with nymphs containing wild-type or the complemented *plzA*⁺ cells (Table 2). The failure of the *plzA* ticks to transmit the infection to naïve mice was not due to the number of nymphal ticks allowed to feed on mice. Since only 55% of *plzA* mutant ticks were positive (versus ~100% for the wild-type or the complemented *plzA*⁺ strain), we allowed 30 *plzA* mutant nymphs to feed on a naïve mouse versus only 10 nymphs for the wild-type or complemented *plzA*⁺ cells (Table 2). Together, these results clearly demonstrate that *B. burgdorferi* PilZ protein PlzA appears essential to complete the mouse-tick-mouse infection cycle.

***plzA* mutant cells are deficient in surviving in artificially infected ticks.** To better determine why *plzA* cells in infected mice are deficient in the ability to transmit the infection to naïve ticks, we artificially infected naïve larval ticks with wild-type, *plzA* mutant, and complemented *plzA*⁺ cells by immersion (Table 3). Tick immersion studies (83, 85, 87) allow for direct artificial tick infection and serve two purposes: (i) to optimally infect naïve ticks with the wild-type and mutant spirochetes and determine their colonization and survivability within the tick vector and (ii) to examine the spirochete’s potential to migrate from the arthropod host vector to the mammalian host. While ticks inoculated with wild-type or *plzA*⁺ cells were readily transmitted to and infected naïve mice, the *plzA* strain-infected ticks failed to transmit the infection to naïve mice (*n* = 2) (Table 3). We found that the *plzA* cells were severely defective in surviving in ticks that fed on the naïve mouse, although the tick’s infection rates were similar (80%) between the wild-type and mutant cells (Table 3). The number of viable *plzA* spirochetes was very low compared to the number of viable wild-type or complemented *plzA*⁺ spirochetes

(Table 3). These results indicated that a mutation in *plzA* resulted in cells that are deficient in surviving in fed ticks and, consequently, failed to transmit the infection from infected ticks to naïve mice (Tables 2 and 3).

DISCUSSION

c-di-GMP controls diverse cellular processes, including the motility and virulence of some bacteria (47, 60, 96). *B. burgdorferi* is an ideal organism with which to study c-di-GMP effects, in that there is no repetition of the genes that synthesize or hydrolyze c-di-GMP. The *B. burgdorferi* genome encodes a single protein for the GGDEF, EAL, or HD-GYP domains (22, 66, 71, 87); however, relatively little is known about how these proteins function mechanistically in different cellular process. Recently, we have shown that the EAL domain-containing phosphodiesterase BB0363 plays a role in motility and virulence of *B. burgdorferi* (87).

The c-di-GMP-binding PilZ domain proteins are important for mediating c-di-GMP signaling that controls bacterial motility and colony morphology and affects virulence. PlzA/BB0733 is the only recognizable PilZ domain-containing protein in *B. burgdorferi* (2), and recently it has been shown to specifically bind to c-di-GMP (21). The *K_d* value we determined for *B. burgdorferi* PilZ was 1.25 μM, which is consistent with the *K_d* values of other c-di-GMP-binding PilZ proteins (29, 37) and indicates strong affinity for c-di-GMP (Fig. 1). Cellular c-di-GMP concentrations, together with the effector components’ affinities for c-di-GMP, are crucial for triggering many c-di-GMP-dependent outputs (29).

Analysis of the *B. burgdorferi* genome as well as our RT-PCR results indicates that *plzA* is transcribed monocistronically and is separated from divergently transcribed upstream and downstream genes by 309 and 205 bp, respectively. Although genetic manipulations in *B. burgdorferi* virulent, low-passage strains are challenging and often result in failure (33, 66, 68), we con-

TABLE 2. Inability of *plzA* mutant cells to complete mouse-tick-mouse infection cycle

Type of strain	Dose	% of:		No. of viable spirochetes/tick	No. of nymphs/mouse	No. of mice infected/total
		Mice infected	Ticks positive			
Wild type	5 × 10 ⁴	100	95	3,947 ± 725	10	3/3
<i>plzA</i> mutant	5 × 10 ⁶	100	55	20 ^a	30	0/5
Complemented <i>plzA</i> ⁺	5 × 10 ⁴	100	100	3,006 ± 537	10	5/5

^a *plzA*-infected ticks had ≤20 spirochetes/tick (*n* = 10).

TABLE 3. Defective ability of *plzA* mutant to survive in ticks and to be transmitted to naïve mice based on tick immersion studies

Type of strain	% of fed ticks positive for <i>B. burgdorferi</i>	No. of spirochetes/tick	No. of mice infected/total
Wild type	80	3,100 ± 1,125	2/2
<i>plzA</i> mutant	80	330 ± 183	0/2
Complemented <i>plzA</i> ⁺	41	5,600 ± 4,869	2/2

constructed a *plzA* mutant from the virulent wild-type strain B31-A3-68 $\Delta bbe002$ that retained all of the endogenous plasmids (Fig. 4) required for mouse and tick infections (Tables 1 to 3) (63). *pilZ* mutants of different bacteria exhibit different and diverse phenotypes, including but not limited to the translucent, “rdar” (rough, dry, and rugose colony morphology) morphology and altered motility (60, 67, 70, 98). We discovered for the first time that the *B. burgdorferi* *plzA* mutant exhibited an opaque colony morphology consistent with other bacteria. The colony morphology is specific to the *plzA* mutation since complementation in *cis* resulted in restoration of the wild-type translucent colony morphology. Phase variation in other bacteria has been described to arise from programmed genetic rearrangements or variable expression patterns in an isogenic population (92). Molecular mechanisms underlying the translucent colony morphology exhibited by the *B. burgdorferi* *plzA* mutant are currently unknown and require further investigation. The *plzA* mutant cells’ run-pause-reverse swimming pattern was indistinguishable from that of the wild-type cells (42, 53). However, the swarming motility on 0.35% agarose was markedly reduced compared to that of the isogenic complemented or the parental wild-type cells when plates were incubated for 7 days (Fig. 5). (*B. burgdorferi* cells do not swarm on 0.5% agarose [our unpublished results].) We note that when swarm plates were incubated for 5 days, the swarm diameters of the mutant strain were only slightly smaller than those of the wild-type cells; these results suggest that PilZ has a positive effect on *B. burgdorferi* motility.

In other species of bacteria, PilZ was reported to control motility in response to changes in the level of c-di-GMP (13, 58, 70). Specifically, in *Caulobacter crescentus*, the c-di-GMP-binding PilZ protein, DgrA, when complexed with c-di-GMP, diminished FliL synthesis, altering motility (13). However, this is likely not to be the case in *B. burgdorferi*: the levels of motility and chemotaxis proteins FliL, FliG1, FliG2, FliM, MotB, FlaB, or CheY3 were not altered in the *plzA*, *pdeA*, or *plzA pdeA* double mutant, as shown below (Fig. 6 and data not shown). Furthermore, in *E. coli* (and *S. enterica*), a mutation in the phosphodiesterase *yhjH* gene reduces motility, while a *ycgR* (encoding a PilZ protein) mutant exhibits normal motility. Furthermore, the motility defect of the *yhjH* mutant was rescued by deleting *ycgR* (24, 58, 70), indicating that YcgR mediated the reduction in motility due to elevated c-di-GMP (*yhjH* mutant). In this case, the PilZ protein YcgR controls motility posttranslationally: in response to elevated c-di-GMP, YcgR was reported to interact with motor switch proteins FliM, FliG, or MotA, interfering with the electrostatic interaction between the motor and switch protein controlling motility (3, 7, 17, 57). To determine if PlzA can function in response to c-di-GMP to

regulate *B. burgdorferi* motility, we inactivated *plzA* in a phosphodiesterase (*pdeA/bb0363*) mutant background (87) where the cellular c-di-GMP level is 2× higher than that in the wild-type cells (our unpublished observation). We have demonstrated that *bb0363* (*pdeA*) mutants exhibit reduced motility and that those mutant cells failed to reverse, whereas the wild-type cells ran, paused, and reversed (87). *B. burgdorferi* cells do not swim well in their BSK-II growth medium, and analysis of their swimming pattern requires highly viscous medium, such as methylcellulose (25, 43, 52, 87). The *plzA* mutant constructed in the *pdeA* mutant background (elevated c-di-GMP) did not rescue the *pdeA* cells’ swimming behavior: the *plzA pdeA* double mutant cells’ swimming pattern was indistinguishable from the *pdeA* cells’ constant run-pause motility phenotype (87). These results suggest that PlzA regulates *B. burgdorferi* motility in a different manner than it does in *E. coli* and that elevated c-di-GMP in the *B. burgdorferi* *pdeA* mutant alters motility by a mechanism independent of PlzA. It is tempting to speculate that *B. burgdorferi* may possess an additional c-di-GMP-binding protein(s) that responds to elevated c-di-GMP in a manner similar to that reported in *E. coli* or *C. crescentus* to control motility (7, 13, 57, 87). Furthermore, our findings are consistent with the observations that PilZ proteins lacking the N-terminal YcgR domain are likely unable to interact with the flagellar switch FliG protein to control bacterial motility (17).

On the other hand, in some bacteria PilZ can act independent of c-di-GMP. For example, *V. cholerae* contains five PilZ domain proteins (60), but only PlzC and PlzD bind c-di-GMP (60). However, both *plzB* and *plzC* mutant cells were reported to exhibit decreased motility, and a *plzD* mutant displayed an increased swarming motility relative to wild-type *V. cholerae* (45, 60). In addition, a *V. cholerae* strain that produced elevated levels of c-di-GMP exhibited reduced motility due to diminished transcription of motility and chemotaxis genes (6, 60). However, as described above, the expression of *B. burgdorferi* motility or chemotaxis genes was not affected in *pdeA*, *plzA*, or *pdeA plzA* mutant cells.

While the motility (Fig. 5) of *plzA* mutant cells was attenuated relative to wild-type cells, the swimming pattern of the *plzA* cells was indistinguishable from that of the wild-type cells, and expression of motility and chemotaxis genes was also unaltered. These findings may suggest that assembly of the periplasmic flagella was not affected due to a mutation in *plzA* (96). We speculate that PlzA or a PlzA-regulated factor(s) may modulate the function of a motor protein, perhaps by modulating the MotB proton channel. A recent study with *E. coli* reported that amino acid residues K53 to T64 of MotB act as a plug to prevent proton flow before the MotA-MotB complex associates with the flagellar structure (31, 32). Because in *plzA* cells the swarming motility is attenuated but the swimming pattern was not altered, it appears likely that PlzA acts as a positive regulator in *B. burgdorferi* motility or chemotaxis.

Several studies have shown that a mutation in the gene encoding PilZ protein affects virulence of some bacteria. For example, a mutation in the *plzD* gene of *V. cholerae* results in cells that are 10-fold less infectious than the parental cells in a murine model (60). A mutation in the plant pathogen *Xanthomonas campestris* pv. *campestris pilZ* (*xc3221*) gene also resulted in cells that are significantly less virulent in the Chi-

nese radish than their parental wild-type cells (47). Therefore, the >1-log-increased ID₅₀ observed with the *B. burgdorferi* *plzA* mutant cells during mammalian infections is consistent with these other reports (Table 1). Although why *plzA* mutant cells are significantly ($P = 0.0031$) less infectious than the wild-type cells is unknown, we propose that motility is likely to be a factor and that wild-type motility is critical for *B. burgdorferi* infectivity (9, 43, 87; our unpublished observation). Future studies will test this hypothesis. However, we were surprised that the *plzA* cells are defective in transmitting the infection from infected mouse to naïve tick to mouse (Table 2). The defect in the transmission between tick and mammalian hosts was not due to the number of ticks allowed to feed on naïve mice (30 *plzA* mutant ticks versus 10 ticks per mouse for wild-type or *plzA*⁺ cells). Using mouse-tick-mouse infection cycle and tick-immersion studies (Tables 2 and 3), we determined that *plzA* cells were defective in surviving in fed ticks, although the mechanism is currently unknown (see below). Reduced motility is likely not responsible for the reduced survival of spirochetes in ticks since motility- or chemotaxis-defective mutants are able to survive and multiply before or after a blood meal from mice (87; our unpublished observation). We speculate that PilZ itself is required for survivability within ticks or PilZ is a regulator of a virulence determinant(s) impacting survivability in ticks and/or attenuation in mice. It is also possible that the opaque colony morphology of the *plzA* mutant cells may reflect an altered cell surface membrane structure, which may be related to the impaired ability of *plzA* cells to infect mice and/or decreased survivability within ticks. Tick saliva contains bioactive molecules and a plethora of proteolytic enzymes (19, 75). Whether tick-derived elements, host-derived products acquired from the tick's blood meal, or salivary gland factors (18, 19, 34, 75, 81, 82) were responsible for the reduced survival of the *plzA* mutant in the ticks remains to be determined. We and others have shown that *B. burgdorferi* uses different mechanisms to survive in different hosts (ticks/mammalian). Future studies are warranted to decipher the wide spectrum of PilZ effects on *B. burgdorferi*.

ACKNOWLEDGMENTS

We thank Rita Tamayo and Philip Stewart for comments on the manuscript. We thank R. Rego, P. Rosa, C. Li, J. Benach, A. Barbour, J. Carroll, M. Gomelsky, D. Blair, U. Pal, E. Fikrig, and S. Samuels for providing reagents. We appreciate technical discussions from P. Policastro and A. Bestor, RML, NIH.

The research was sponsored by a National Institute of Arthritis, Musculoskeletal, and Skin Diseases, NIH, grant (R03AR054582) and an award of an East Carolina University Research and Development "start-up" fund to M.A.M.

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Editor: A. Camilli