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

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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 \( \mu \)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 diguanosine monophosphate] 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 phosphodiesterases. 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, PeiD, L-site effectors, FlhQ, and Cip (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, posttranslational modifications, 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 *silo* 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 xylinum* (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 \( \mu \)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 pilated 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*
indicates that GMP (22), bioinformatics, as well as experimental analysis, multiple enzymes responsible for controlling the levels of c-di-GMP (95). While many species of bacteria were reported to contain c-di-GMP levels, which are controlled by a different species. While many species of bacteria were reported to contain c-di-GMP levels, which are controlled by a 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 ixodex 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/Rp1p) 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 bb002 was used as the wild type throughout the study (a kind gift from R. Rego and P. B. Lussi, Rocky Mountain Laboratories, NIH) (63). This strain is a derivative of A3-68 (63), lacking circular plasmid p (cp9) and linear plasmid pS (lp56), with the bb002 gene inactivated with a pEMB and a cassette to increase transformation frequency (36, 63). B31-A3-68 bb002 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% agarase (52). Swarm plate assays were carried out with 0.35% agarose in plating BSK medium; approximately 1×10⁶ cells in a volume of 5 μl 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-hour 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-BB0363-F (CTTTATTCGAGAAAAATAAAAGAAG) and T-BB0733-R (CTTTGATTAATGGAATATC). The amplified DNA fragment was ligated into pET28a (E. coli expression vector) (Invitrogen, Inc.) by T4 cloning. The vector was then digested and sequenced to confirm its integrity. E. coli cells containing plzA were induced with 0.25 mM isopropyl β-D-thiogalactopyranoside at 30°C for 4 h and N2+ 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 (93, 94). A protein binding assay 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 μM) was injected into one chamber, and 60 μl (10 μM) 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-μm-pore microfilter (Millipore Ultrafuge 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 GraphPad Prism 5 software.

**RT-PCR.** Exponentially growing B. burgdorferi wild-type cells (2×10⁹ 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 RNA-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 μg RNA with SuperScript III reverse transcriptase according to the manufacturer’s protocol (Invitrogen, Inc.). The RT-PCR primer sequences were a1 (TGTAAAATATAGACGAGTTACG), a2 (GGGCGGACATGTATCTTCTTGA), b1 (TTGAGATGAGAATAGGCTCTTATACC), and b2 (GGCGCGGACATGTATCTTCTTGA), c1 (TTGAGTGGTCCTGTTATCTTCTTCT), and c2 (TATGACGAAATCTTACCAAGA). 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 a reference gene (54, 87). The gene-specific primers were RT-enolase-F (TGGAGCGTAGTAAACACAAATT), RT-enolase-R (TGTAAAATATAGACGAGTTACG), BB0733-rt-F (CTTTTATCTAGAAAAATAAAAGAAG), and BB0733-rt-R (TGGAGCGTAGTAAACACAAATT), respectively. The relative level of expression was calculated by the threshold cycle (2⁻ΔΔCt) method (46, 79, 87).

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

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GA) and BB0733-KO-R (ATTCCCAAAAGTGGCCTCTAGT). The amplified DNA was ligated into the pGEM-T Easy vector (Promega, Inc.), yielding pb0733-KO-Easy. Pl-Kan was similarly PCR amplified from pB31-A3-68 with the primers PrLs-Kan AflII-F (GCGCCGACGATCTGAAGAAGAACCTAAGTACGCTGACCCTAA) and PrLs-Kan AflII-R (CCGAAGACACTTAAAC). PCR for the presence of the Pl-Kan cassette with the primers PrLs-Kan AflII-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 bbo733 (plzA) mutant. To complement bbo733:Pl-Kan, the promoter of bbo733 (P_plzA) 400 bp upstream from the ATG start codon of the bbo733:PlzF4 gene and the coding sequence was PCR amplified by PCR with primers bbo733 comp-F-SpeI (GCGCCGACGATCTGAAGAAGAACCTAAGTACGCTGACCCTAA) and bbo733 comp-R-NotI (GCGC GCGCCGACGATCTGAAGAAGAACCTAAGTACGCTGACCCTAA) 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 bbo733:Pl-Kan competent cells. Transformants were selected in plating BSK medium containing 100 μg/ml of kanamycin plus 40 μg/ml of gentamicin, yielding pXLB0733. DNA was ligated into the pGEM-T Easy vector (Promega, Inc.), yielding pBB0733-KO. This deletion-insertion was in frame. Restriction mapping indicated that the direction of transcription of Pl-Kan was the same as that of bbo733. bbo733:Pl-Kan was digested and separated from pb0733:Pl-Kan vector by NotI digestion, precipitated, and electroporated into the wild-type competent cells (92). Mutants were selected on solid growth medium containing 200 μg/ml kanamycin plus 100 μg/ml streptomycin. Resistant colonies were analyzed by PCR for the presence of the Pl-Kan cassette with the primers PrLs-Kan AflII-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).

The AflII-restricted Pl-Kan vector was digested and separated from pb0733:PlzA DNA then inserted into suicide vector pXLFBB0733. Fifty micrograms of the resultant plasmid was electroporated into the bbo733:Pl-Kan competent cells. Transformants were selected in plating BSK medium containing 100 μg/ml kanamycin plus 40 μg/ml gentamicin. Resistant transformants were analyzed by PCR for the presence of kanamycin (Kan) and gentamicin (Gen) genes and an intact bbo733 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 chemiluminescence detection method (GE Healthcare) were carried out as previously reported (23). B. burgdorferi strain B31-A3-68 Δbrain02 (wild-type), plzA, or plzA ΔpilZE 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 polyvinyldene difluoride (PVDF) membrane and Western blotted with monoclonal Flab (A. Barbaro), polyclonal Motb (J. Carroll), FlgI1 and FlgI2 (C. Li), FlmI (D. Blair), Che3Y, and Flil antisera. Specific monoclonal or polyclonal reactivity to B. burgdorferi Flab, Motb, FlgI1, FlgI2, FlimI, Flil, and Che3Y 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 C57/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 guidelines for the care and use of laboratory animals. For infection via needle, 5 × 10^8 to 5 × 10^7 in vitro-grown spirochetes were injected subcutaneously as described previously (16, 35, 64). The number of spirochetes was determined with a Petroff-Hauser chamber, and each clone was verified for its retention of the Ip25, Ip28-1, and Ip36 plasmids. Mice were bled 2 weeks postinfection for immunoblot analysis, and tissues were collected for reisolation of B. burgdorferi. Mice 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 (ID₅₀) 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 (IFA) for the presence of spirochetes (83, 87). A second subset of fed larvae was surface sterilized with 3% H₂O₂ followed by 70% ethanol, washed 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 Actin-F (GATCA TGGTCCGAGACCTTTCA) and Actin-R (CGATACCGGTGTGAAAGA) versus the level of B. burgdorferi flaB by using primers FlaB qRT-F (TGTCGTGATCA GTGCAATATAAACA) and FlaB qRT-R (TGAGACCGCTAAAGTATG 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 naive 6-week-old female C57/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 ID₅₀ 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 ID₅₀ infection experiment and the single-dose infection experiment for each strain were combined for the estimations of the 50% infectious dose. Comparison between strain ID₅₀ 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 concentrated spirochete cultures (5 × 10⁷ cells/ml), which were 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 below.

IFAs. Ticks were dissected in 25 μl PBS-5 mM MgCl₂ 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 MgCl₂ and washed with PBS-5 mM MgCl₂. 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 Axios Imager M1 microscope coupled with an AxioCam MRC digital camera (Carl Zeiss, Inc.).

RESULTS

BB0733 binds c-di-GMP with high affinity. Bioinformatic 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/ZNXXGXG 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 YcgR [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 1RXXXR (number based on amino acid residues of BB0733 protein) (20) is critical for c-di-GMP binding (21). However, a dissociation constant (Kₐ) of PlzA for c-di-GMP was not determined (21). To determine the Kₐ of PlzA, synthetic c-di-GMP (38, 41) and recombinant, purified His₅-PlzA (Fig. 1) were used in equilibrium dialysis, as described previ-
Inactivation and complementation of plzA of unknown function. In Shewanella oneidensis whereas the function of PlzA during the enzootic life cycle of B. burgdorferi and synthetase 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.

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 were 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{\textit{plzA}-plzA}) into the mutant cells. Positive clones were characterized by PCR using kanamycin, gentamicin (Gent) and kanamycin [Gent]) used to confirm the integration of P{\textit{plzA}-plzA} into the mutant. PlzA was reported to be 1.25 ± 0.17 μM, indicating high affinity for c-di-GMP, consistent with the function of PlzA during the enzootic life cycle of B. burgdorferi and the YrdC/YwlC family protein is a putative translation factor involved in translation, as well as ribosomal structure and biosynthesis (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 (Pl-Kan) by allelic exchange, as described previously (87). Electroporation of the plzA::Pl-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 Pl-Kan cassette in all clones, confirming inactivation of plzA (Fig. 3, left panel).
required for infectivity (56, 61) in the \( \text{plzA} \) 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, \( \text{plzA} \) mutant, and complemented \( \text{plzA} / \text{H11001} \) 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 \( \text{plzA} \) altered the \( B. \text{burgdorferi} \) 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 \( \text{plzA} \) mutant cells were indistinguishable from those of the wild-type cells, the swarm diameter of \( \text{plzA} \) 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 \( \text{plzA} \) cells are solely due to the mutation and not a secondary alteration elsewhere. Noticeable growth defects were not observed in \( \text{plzA} \) mutant cells, indicating the decreased-swarming phenotype was not due to a growth defect (not shown). These results indicate that \( \text{plzA} \) is essential for normal \( B. \text{burgdorferi} \) motility and colony morphology.

To determine if the deficient motility exhibited by \( \text{plzA} \) 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 \( \text{plzA} \) mutant cells were not altered compared with those of the wild-type cells. These results indicate that \( \text{plzA} \) is essential for normal \( B. \text{burgdorferi} \) motility and colony morphology.

**plzA mutant cells are significantly attenuated in infecting mice.** In some bacteria, \( \text{pilZ} \) was shown to be required for the
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/H11001 cells to determine the 50% infectious dose (ID$_{50}$). 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 ID$_{50}$ compared to the parental wild-type cells, the ID$_{50}$ 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 ($P_{\text{H}11005} < 0.0031$) (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 $\times$ 10$^6$/mouse), wild-type, or complemented plzA/H11001 strain-infected mice (dose, 5 $\times$ 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 than that of the wild-type or complemented 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 ($P_{\text{H}11005} < 0.001$) between wild-type (WT) and plzA mutant cells.](image-url)

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

<table>
<thead>
<tr>
<th>Type of strain</th>
<th>No. of mice infected/total at dose of:</th>
<th>ID$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 $\times$ 10$^2$</td>
<td>5 $\times$ 10$^3$</td>
</tr>
<tr>
<td>Wild type</td>
<td>0/8</td>
<td>3/8</td>
</tr>
<tr>
<td>plzA mutant</td>
<td>0/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Complemented</td>
<td>1/8</td>
<td>4/8</td>
</tr>
</tbody>
</table>

* plzA$^+$

![FIG. 6. The levels of prominent motility or chemotaxis proteins were not altered in the plzA mutant. Western blots using 5 $\mu$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.](image-url)
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 \( \mu M \), 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-
structured a plzA mutant from the virulent wild-type strain B31-A3-68 Δbbe002 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) phenotype and altered motility (60, 67, 70, 98). We discovered for the first time that the B. burgdorferi plzA mutant exhibited a 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 swimming 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 is 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 yhh gene reduces motility, while a ycgR (encoding a PilZ protein) mutant exhibits normal motility. Furthermore, the motility defect of the yhh mutant was rescued by deleting ycgR (24, 58, 70), indicating that YcgR mediated the reduction in motility due to elevated c-di-GMP (yhh 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 Flil, 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 PilZC and PilZD 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 swimming 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 PilZA or a PilZA-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 swimming motility is attenuated but the swimming pattern was not altered, it appears likely that PilZA 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 parent wild-type cells (47). Therefore, the >1-log-increased ID_{50} 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 were 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 infection from infected mouse to naïve tick to mouse (Table 2).

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In order to determine if the opaque colony morphology of the plzA mutant in the ticks remains, we performed transduction by PilZ domains. EMBO J. 178:263–273.

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REFERENCES


ERRATUM

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

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Page 1822: column 1, lines 15 and 16: “translucent colony morphology” should read “opaque colony morphology.”