Borrelia burgdorferi CheD Promotes Various Functions in Chemotaxis and the Pathogenic Life Cycle of the Spirochete

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Borrelia burgdorferi possesses a sophisticated chemotaxis signaling system; however, the roles of the majority of the chemotaxis proteins in the infectious life cycle have not yet been demonstrated. Specifically, the role of CheD during host colonization has not been demonstrated in any bacterium. Here, we systematically characterized the *B. burgdorferi* CheD homolog using genetics and biochemical and mouse-tick-mouse infection cycle studies. *Bacillus subtilis* CheD plays an important role in chemotaxis by deamidation of methyl-accepting chemotaxis protein receptors (MCPs) and by increasing the receptor kinase activity or enhancing CheC phosphatase activity, thereby regulating the levels of the CheY response regulator. Our biochemical analysis indicates that *B. burgdorferi* CheD significantly enhances CheX phosphatase activity by specifically interacting with the phosphatase. Moreover, CheD specifically binds two of the six MCPs, indicating that CheD may also modulate the receptor proteins. Although the motility of the *cheD* mutant cells was indistinguishable from that of the wild-type cells, the mutant did exhibit reduced chemotaxis. Importantly, the mutant showed significantly reduced infectivity in C3H/HeN mice via needle inoculation. Mouse-tick-mouse infection assays indicated that CheD is dispensable for acquisition or transmission of spirochetes; however, the viability of *cheD* mutants in ticks is marginally reduced compared to that of the wild-type or complemented *cheD* spirochetes. These data suggest that CheD plays an important role in the chemotaxis and pathogenesis of *B. burgdorferi*. We propose potential connections between CheD, CheX, and MCPs and discuss how these interactions play critical roles during the infectious life cycle of the spirochete.

Borrelia burgdorferi, the causative agent of Lyme disease, cycles between *Ixodes* ticks and a mammalian host. Motility and chemotaxis are known to be required for host tissue colonization or disease production by many bacteria, including the spirochetes; however, these processes have not been rigorously characterized in any spirochete (1–13). *B. burgdorferi* is a spirochetal motile bacterium that contains a sophisticated and complicated chemotaxis signal transduction system. This spirochete is a long (10- to 20- μ m) and thin (0.33- μ m) organism with flat-wave morphology, and its motility is provided by flagella that are located within the periplasmic space. There are 7 to 11 periplasmic flagella inserted at each pole of the cell, forming a ribbon-like structure that wraps around the cell cylinder as they extend toward the other pole of the cell (5, 6, 9). When the periplasmic flagellar motors rotate asymmetrically (i.e., flagella at one pole rotate clockwise [CW] and the flagella at other pole rotate counterclockwise [CCW]), the spirochete runs. When the flagellar motors at both poles of the cell rotate in the same direction (both CW or CCW), *B. burgdorferi* cells flex with no net translocation (14). This manner of swimming also produces an unusual “corkscrew-like” locomotion. This distinctive motility pattern is believed to be important for efficient migration within the dense tissues through which these spirochetes preferentially disseminate in both vertebrate and arthropod hosts (5, 15). Interestingly, where most externally flagellated bacteria either slow down or stop within viscous material (16, 17), the velocities of spirochetes actually increase within viscous media (18–20), thus empowering the spirochetes to penetrate into tissues that other bacteria fail to invade.

Chemotaxis is also believed to be very important for the complex vector-host life cycle utilized by *B. burgdorferi*. After transmission from tick to mammalian host, *B. burgdorferi* must quickly perceive its new local environment and make the appropriate ad-

aptations, rapidly disseminating through skin tissues to evade the cellular immune response and migrating to appropriate target tissues, which allows evasion of the expanding adaptive immune response and survival. After residing in the mammalian host for weeks to years, *B. burgdorferi* must then be able to detect that a new tick vector has attached to the host’s skin and then rapidly migrate to the bite site and enter the vector (21).

Chemotaxis allows bacteria to follow gradients of nutrients and other environmental stimuli by governing their motility. The components of the chemotaxis signal transduction systems that mediate these responses are highly conserved among prokaryotes. The best-studied system is found in *Escherichia coli* (or *Salmonella enterica* serovar Typhimurium) (22–24). In this model organism, membrane-spanning methyl-accepting chemotaxis protein receptors (MCPs) detect environmental signals. These MCP proteins are coupled to a histidine kinase (CheA) and a linker protein (CheW). The catalytic activity of CheA is regulated by occupancy of these MCPs with a ligand (e.g., an attractant or repellent). CheA uses ATP to autophosphorylate, and the phosphate group is then

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transferred to the response regulator, CheY. The phosphorylated CheY (CheY-P) then binds to the flagellar motor switch proteins FliM and FliN (25). This binding results in the change of direction of the flagella from the default CCW (running motility) to CW rotation, resulting in tumbling motility. Binding of attractant to the MCPs decreases the kinase activity of CheA, resulting in reduced CheY-P, as well as tumbling frequency. The concentration of CheY-P determines whether a cell runs (which requires low CheY-P) or tumbles (high CheY-P). CheY-P is able to autodephosphorylate; however, the phosphatase CheZ efficiently dephosphorylates the levels of CheY-P in *E. coli* (26). This phosphorylation-dephosphorylation is crucial for the bacterial cells to respond appropriately to environmental stimuli. The chemotaxis system adapts to persistent modification via receptor methylation by the methyltransferase CheR (positive stimuli) and demethylation by the methyl-ersterase CheB (negative stimuli). CheR and CheB are the only known chemoreceptor modification enzymes in this *E. coli* model (23).

Even though the components of the chemotaxis signal transduction system are conserved among prokaryotes, the chemotaxis pathway in *B. burgdorferi* differs from the other well-studied bacterial models and is much more complicated due to the presence of multiple copies of chemotaxis genes, including six *mcp*, two *cheA*, three *cheY*, two *cheB*, two *cheR*, and three *cheW* genes, which may contribute to the asymmetric motility of the spirochete. Moreover, *B. burgdorferi* has a putative gene encoding CheD, which is not found in *E. coli* (5). CheD is relatively well characterized in *Bacillus subtilis* and *Thermotoga maritima* (27–30). In *B. subtilis*, CheD plays an important role in motility and chemotaxis. A *cheD* mutant displays a distinctive motility pattern: wild-type (WT) cells exhibit running and tumbling motility, whereas the Δ *cheD* mutant constantly tumbles. At the molecular level, CheD interacts with the receptor MCPs. MCP proteins contain the well-conserved substrate-binding site of CheD, A/S-X-X-Q/E-Q/E-X-X-A/S (A, alanine; S, serine; Q, glutamine; E, glutamate; X, any amino acid). CheD modifies the MCPs by deamidating glutamine to glutamate residues. The deamidation by CheD is required for the *B. subtilis* chemoreceptors to effectively transduce signals to the CheA kinase (29, 31). Additionally, CheD interacts with the phosphatase CheC, enhancing its enzymatic activity and fine-tuning the intracellular concentration of CheY-P (27, 29). *B. burgdorferi* possesses a phosphatase, CheX, that has been previously reported to possess CheY-P-dephosphorylating activity, and the *cheX* mutant cells constantly flex (32–34). It is noteworthy that the “flex” of *B. burgdorferi* is considered to be equivalent to the tumbling phenotype seen in *E. coli*. Moreover, the MCPs of *B. burgdorferi* contain the well-conserved potential substrate-binding sites of CheD (29, 31, 35). These observations led us to hypothesize that CheD in *B. burgdorferi* plays a role similar to that of its counterpart in *B. subtilis*. Most importantly, even though the role of CheD in motility and chemotaxis has been demonstrated in other bacteria, its role in pathogenesis has not been reported in any organism. To date, only one chemotaxis (*cheA2*) gene has been investigated in the *B. burgdorferi* infectious life cycle (i.e., tick-to-mouse transmission) (7). A rigorous study is therefore warranted in order to understand the functions of other putative chemotaxis genes.

In this study, we determined the requirement for *cheD* in motility and chemotaxis in *B. burgdorferi* by inactivating the *cheD* gene, as well as by demonstrating its enzymatic role in the che-

motaxis signaling system. Moreover, we defined the role of *cheD* in the disease process (mouse-tick-mouse infection cycles) for the first time. We showed that CheD not only is important in chemotaxis, as it enhances the CheX phosphatase activity, but also is essential for the infectious life cycle of *B. burgdorferi*.

MATERIALS AND METHODS

Ethics statement. East Carolina University is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for tick and animal experimentation were approved by the East Carolina University animal care and use committee.

Bacterial strains and growth conditions. The low-passage, virulent *B. burgdorferi* strain B31-A3 was used as a wild-type clone throughout the study (36, 37). The genome of the virulent B31 strain was found to contain a total of 21 plasmids, with 12 linear and 9 circular plasmids, in addition to its 960-kbp linear chromosome (38, 39). Clone B31-A3 lacks circular plasmid 9 (cp9) but remains infectious in tick-mouse cycle studies (37, 40). The construction of *cheD* mutants and complemented strains is described below. *B. burgdorferi* cells were cultured in liquid Barbour-Stoenner-Kelly (BSK-II) medium, and plating BSK was prepared using 0.5% agarose (8, 41, 42). Cells were grown at 35°C in a 2.5% CO₂ humidified incubator as described previously (8, 37, 41). Antibiotics, when required, were included in the *B. burgdorferi* culture medium at the following concentrations: 200 µg/ml kanamycin, 40 µg/ml gentamicin, and 100 µg/ml streptomycin. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) broth or LB agar (43). Antibiotics, when required, were included in the *E. coli* culture medium at the following concentrations: 100 µg/ml ampicillin, 35 µg/ml chloramphenicol, and 100 µg/ml spectinomycin.

Construction and complementation of the *cheD* mutant. Construction of the *cheD* inactivation plasmids, electroporation, and plating conditions were described previously (8, 44). Briefly, the *cheD* gene (locus number *bb0606*) and flanking DNA were first amplified by PCR from the chromosomal DNA of *B. burgdorferi* strain B31-A3 using primers CheD-KO-F (GGAATCAGGCTTAAATCTTG) and CheD-KO-R (AAGCATGGAAAGTTGAAACC). The PCR product was cloned into plasmid pGEM-T Easy (Promega Inc.). The *cheD* gene was inactivated using a kanamycin resistance cassette (*P*_{flgB}-*aph1*) (36), which was inserted at the HindIII sites located within *cheD*. Competent B31-A3 cells were electroporated with *cheD*-*P*_{flgB}-*aph1* DNA that was linearized by NotI restriction digestion to remove the ampicillin restriction marker of the vector, preventing it from being introduced into *B. burgdorferi* (8, 44). The transformants were selected with kanamycin, and the kanamycin-resistant transformants were confirmed by PCR to have *P*_{flgB}-*aph1* integrated within *cheD* (8, 44, 45). Confirmation of *cheD* gene inactivation was achieved by the lack of *cheD* transcripts in the *cheD* mutant (Δ *cheD*) cells by quantitative reverse transcriptase (qRT)-PCR (see below).

The *cheD* mutant was complemented in *cis* by genomic integration using a pXLF14301 suicide vector (35, 46, 47). To complement the mutant, the *cheD* gene and its native promoter (*P*_{*cheD*}) DNA sequences were PCR amplified from genomic DNA of *B. burgdorferi* strain B31-A3 using primers CheDcom.-F (AATTAAATGATTAAACATATTTCCCAATAA CATAGATAC) and CheDcom.-R (GCGGCCGCTTAAAAACCTTTGT TCC) and *P*_{*cheD*}-F (ACTAGTATTGGCCATATCCCCATTAAAGGC) and *P*_{*cheD*}-R (GTATCTATGTTATTGGGAAATATGTTAAATCATTTTAA TT), respectively. The amplified DNA fragments were ligated by overlapping PCR, yielding a *P*_{*cheD*}-*cheD* DNA fragment, and then cloned into plasmid pGEM-T Easy (Promega Inc.). Finally, *P*_{*cheD*}-*cheD* was excised from pGEM-T Easy using SpeI and NotI restriction digestions and then cloned into pXLF14301, yielding plasmid pXLFCheD. The plasmid was then electroporated into the Δ *cheD* mutant cells, followed by selection with gentamicin and kanamycin. The resistant clones were analyzed by PCR for integration of *P*_{*cheD*}-*cheD* within the intergenic region of *bb0445* and *bb0446*. Restoration of *cheD* expression in the

complemented *cheD* (*cheD^{com}*) clones was also verified by qRT-PCR (see below). Retention of *B. burgdorferi* endogenous plasmids in the wild-type, mutant, and complemented strains was verified by PCR as described previously (8, 35, 37, 45).

RT-PCR. Exponentially growing *B. burgdorferi* wild-type cells (2×10^7 cells/ml) were treated with RNase protect, followed by total RNA isolation using 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 (RT)-PCR, cDNA was prepared from 1 µg RNA using the AffinityScript QPCR cDNA synthesis kit (Agilent Technologies Inc.) according to the manufacturer's protocol. The iCycler detection system (Bio-Rad Inc.) was used to measure *cheD* transcript levels according to the manufacturer's instructions. The *B. burgdorferi* enolase gene was used as a reference gene (35, 45, 48, 49). The gene-specific primers (5'-3') were RT-enolase-F (TGGAGCGTACAAAGCCAAACATT), RT-enolase-R (TGAAAAACCTCTGCTGCCATTC), CheD-qRT-F (CCTGGTGAAGCTTTTGTTTC), and CheD-qRT-R (TTGATCAGGAGATATGTCAAGATC). The relative level of expression was calculated using the $2^{-\Delta\Delta CT}$ method (35, 45, 49–51).

Recombinant protein expression in *E. coli*. In order to express *B. burgdorferi* *cheD* in *E. coli*, a DNA fragment harboring the entire *cheD* open reading frame was amplified from chromosomal DNA of B31-A3 using primers R.CheD-F (TTAAATCATTTTAAATTTTAA) and R.CheD-R (TTAA AAAACCTTTGTTCCGT) and cloned into the pASK-IBA7⁺ expression vector (IBA Inc.) to generate Strep-Tactin-tagged CheD (Strep-CheD). *E. coli* BL21(DE3) harboring pASK-IBA7⁺::*cheD* was induced with 200 ng/ml of anhydrotetracycline. Expression and purification of *E. coli* CheA and *B. burgdorferi* His-CheY3 and His-CheX proteins were described previously (33, 52, 53). *B. burgdorferi* *mcp3*, *mcp4*, and *mcp5* open reading frames were cloned separately in the expression vector pMAL c2x (NEB Inc.) to generate MBP-MCP3, MBP-MCP4, and MBP-MCP5 recombinant proteins, respectively. *E. coli* codon-plus cells harboring pMAL c2x::*mcp3*, *mcp4*, or *mcp5* was expressed and purified according to the manufacturer's protocol (NEB Inc.). The purity of the recombinant proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by comparison with standard bovine serum albumin (BSA).

SDS-PAGE and immunoblot analyses. SDS-PAGE and immunoblotting with an enhanced-chemiluminescence detection method (GE Health Inc.) were carried out as reported previously (8, 44). The concentrations of protein in cell lysates were determined with a Bio-Rad protein assay kit using BSA as a standard. Unless otherwise noted, 5 µg of lysate protein was subjected to SDS-PAGE and immunoblotting using *B. burgdorferi*-specific antibodies.

Affinity blotting. Protein-protein interactions were determined using affinity blotting (54). Briefly, 2 µg of purified recombinant Strep-CheD protein was subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PDVF) membranes. The membranes were blocked overnight with shaking at 4°C in blocking solution (5% skim milk, 0.9% NaCl, 10 mM Tris, pH 7.4). After incubation, 10 µg of purified His-CheX, His-CheY3, MBP-MCP3, MBP-MCP4, or MBP-MCP5 was added to the blocking solution for 3 h (54). The membranes were washed 4 times with immunoblotting wash buffer and then probed with the proper antibodies. Detection was performed with the ECL immunoblotting detection kit (GE Healthcare Inc.).

Phosphorylation assays. Phosphorylation of CheA, phosphotransfer from CheA-P to CheY3, and dephosphorylation of CheY3-P by CheX phosphatase have been described previously (32, 33, 53). *B. burgdorferi* has two CheA proteins, CheA1 and CheA2, and both are capable of autophosphorylation by ATP (53). However, the expression level of *B. burgdorferi* CheA1 or CheA2 in *E. coli* is poor, and they are also difficult to purify under native conditions (32). Alternatively, we performed the autophosphorylation assays using *E. coli* CheA. Purified *E. coli* CheA was kindly provided by Ruth Silversmith (University of North Carolina, Cha-

pel Hill, NC) (55). To generate radiolabeled phosphorylated CheA (CheA-³²P), 20 pmol of purified CheA was incubated with 10 µCi of [γ -³²P]ATP and 30 mM nonradiolabeled ATP for 30 min at room temperature. Autophosphorylated CheA was applied to Bio-Spin6 columns according to the manufacturer's instructions to remove unincorporated [γ -³²P]ATP (33, 53). CheA-³²P was then incubated with 160 pmol of purified CheY3 for 5 min, as described previously (32, 53). The CheA and CheY3 reaction mixture was then incubated with 0.15 pmol of CheX or 0.15 pmol of CheX plus 15 pmol of CheD for various times. *In vitro* phosphorylation reactions were conducted in TKM buffer (50 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, pH 8.5), and the reactions were stopped using stop buffer (50 mM Tris-HCl, 100 mM dithiothreitol, 2% [wt/vol] SDS, 0.1% [wt/vol] bromophenol blue, 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, pH 6.8). The reaction mixtures were subjected to SDS-PAGE. The gels were dried, and the CheY3-³²P dephosphorylation levels were analyzed by phosphorimage analysis using a Typhoon 9410 phosphorimager/fluorescence imager (GE Healthcare Inc.) (32, 53). The intensities of phosphorylated proteins (PI) were calculated using Quantity One 1-D analysis software ver. 4.6.9 (Bio-Rad Inc.) and are expressed as the relative "PI volume."

Dark-field microscopy, swarm plate chemotaxis assays, and colony swarm plate assays. Growing *B. burgdorferi* cells were imaged using a Zeiss Imager M1 dark-field microscope connected to a digital camera to determine morphology and motility. Swarm plate assays were performed to determine the spirochete's chemotactic ability as described previously (33, 41). Briefly, approximately 1×10^6 cells in a 5-µl volume were spotted into 0.35% agarose plates containing BSK medium diluted 1:10 in Dulbecco's phosphate-buffered saline. Since *B. burgdorferi* is a slow-growing organism with a 5- to 12-h generation time (56), the swarm plates were incubated for 5 days. Swarming images were documented using a digital camera (8, 45, 49, 57). To determine an individual colony's swarming ability, *B. burgdorferi* clones were plated on separate semisolid BSK plates (20 to 50 colonies per plate). Approximately 1 month after inoculation, the swarming diameter of each individual colony was measured. Each isolate was assayed in at least two independent experiments.

Mouse infection studies. Six- to 7-week-old *Mus musculus* C3H/HeN mice were used for infection studies, as described previously (8, 35, 37, 49, 58). For infection via needle, 5×10^2 to 5×10^6 *in vitro*-grown spirochetes were injected intraperitoneally (8, 37, 59). The number of spirochetes was determined using a Petroff-Hausser chamber, and each clone was verified for its retention of endogenous plasmids before injection. The mice were bled 2 weeks postinoculation for immunoblot analysis with mouse sera against *B. burgdorferi* antigens to determine infectivity, as described previously (35, 45, 59, 60). Reisolation of *B. burgdorferi* from mouse skin, bladder, and joint tissues was performed 3 weeks postinjection to assess the ability of spirochetes to infect mice (35, 37, 45, 58, 61). Tissues of euthanized mice were placed in BSK-II growth medium and incubated for up to 35 days, and the presence of spirochetes was determined by dark-field microscopy. The 50% infectious dose (ID₅₀) was calculated as described below.

Determination of ID₅₀ and statistical analysis. The dose required to infect 50% of the mice was experimentally determined for the wild-type, Δ *cheD* mutant, and *cheD^{com}* strains, as described previously (35, 49). 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 done using a generalized linear model with a probit link function and the log dose. This method is also known as probit regression, and in it, we assume identical slopes in the response-log dose relationship but different intercepts for each strain. Graphically, the assumptions manifest themselves as dose-response curves with lateral shifts corresponding to the changes in the intercept. Additionally, an overdispersion parameter was fitted in order to accommodate greater homogeneity in infection rates than would otherwise be permitted by

the model. All calculations were carried out using JMP V12 software (SAS Institute, Inc., Cary, NC). A *P* value of ≤ 0.05 between samples was considered significant.

Assessment of spirochete acquisition and transmissions using mouse-tick-mouse infection assays. To assess if naive *Ixodes scapularis* ticks are able to acquire *B. burgdorferi* from infected C3H/HeN mice and if the spirochetes can survive in the ticks and then be transmitted from the ticks to naive mice, we performed mouse-tick-mouse cycle experiments (35, 45, 49). Briefly, naive mice were injected with *in vitro*-grown spirochetes to infect 100% of the mice ($100 \times \text{ID}_{50}$). Before performing any infection studies, the spirochetes were verified by PCR to ensure that each clone retained all the endogenous plasmids required for persistent infection in mouse and tick hosts (8, 35, 37, 45, 62). To verify the infection status, the mice were bled 2 weeks postinoculation and the sera were tested by immunoblotting using *B. burgdorferi* cell lysates. Three weeks postinjection, naive larval ticks were fed to repletion on separate spirochete-infected mice (3 mice per bacterial strain; ~ 200 larvae/mouse) for 5 to 7 days and collected once they dropped off the mice to determine acquisition of spirochetes from the mice by the ticks. To determine the percentage of spirochete-positive ticks for each *B. burgdorferi* clone, fed ticks were squashed individually to isolate genomic DNA, followed by *B. burgdorferi* *flaB* gene-specific PCR, as described previously (35, 46, 49, 63). Seven days after drop-off, the fed larvae were surface sterilized using 3% H_2O_2 followed by 70% ethanol, crushed individually in BSK-II medium, and plated in semisolid BSK to determine the number of viable spirochetes per tick (i.e., acquisition of spirochetes from infected mice by larval ticks) (8, 37, 49, 58, 61). In determining the total number of spirochetes per fed tick, 10 ticks were analyzed for each strain, and the results were expressed as a scatter plot with the mean. Statistical analysis was performed as described below. A second subset of fed larvae was allowed to molt. Two to 3 weeks after the molt, the nymphal ticks were allowed to feed on naive mice to determine transmission of spirochetes from infected nymphs to mice (8, 9). Naive C3H/HeN mice were anesthetized, and groups of nymphs were confined to capsules affixed to the shaved backs of the mice (3 mice per strain; 15 nymphs/mouse) (8, 64, 65). The ticks were allowed to feed until repletion (3 to 5 days) and then collected from the capsules. At 7 days postrepletion, the ticks were squashed individually, and spirochete burdens were determined as described above. Statistical analysis was performed as described below. The mice were euthanized 3 weeks postrepletion, and the transmission of the spirochetes was evaluated by reisolation.

Statistical analysis. The significance of the difference between the mean values of the groups was analyzed as follows. Unless otherwise stated, for all data, normality was checked using a Shapiro-Wilk normality test. If the data passed normality, a multiple-comparison analysis was performed by using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. If the data did not pass normality, a multiple-comparison analysis was performed by using a Kruskal-Wallis test, followed by a Dunn test. A *P* value of ≤ 0.05 between samples was considered significant.

RESULTS

Phosphatase activity of CheX is enhanced by CheD. Amino acid sequence analysis of *B. burgdorferi* CheD indicated that it shares approximately 30% identity with *B. subtilis* and *T. maritima* CheD proteins. Most importantly, the essential residues of *B. subtilis* and *T. maritima* CheD are also conserved in *B. burgdorferi* (data not shown) (29). In *B. subtilis*, CheD plays an important role in chemotaxis by modification of MCPs by deamidation of glutamine to glutamate residues or by enhancing CheC phosphatase chemotaxis activity to efficiently dephosphorylate the response regulator CheY, thereby regulating the levels of CheY-P (27–30). Because of its possession of the conserved residues, we predicted that

FIG 1 Affinity blot analysis indicates that CheD specifically interacts with CheX. (A) Purified Strep-CheD was incubated with (right) or without (left) His-CheX, followed by immunoblotting with polyclonal anti-CheX. The band at an apparent molecular mass of 19 kDa (Strep-CheD) was observed in blots probed with His-CheX. (B) Purified Strep-CheD was probed with (right) or without (left) His-CheY3, followed by Western blotting with polyclonal anti-CheY3. The bands at an apparent molecular mass of 19 kDa (Strep-CheD) were not detected with the His-CheY3 probe.

B. burgdorferi CheD may exhibit functions similar to those seen in other bacteria (27, 29, 31). The *B. burgdorferi* genome lacks a homolog of the CheC gene but encodes a functional phosphatase, CheX, that efficiently dephosphorylates CheY3-P (33, 34). In order to test if *B. burgdorferi* CheD is able to stimulate the phosphatase activity, we first performed protein-protein interaction assays using affinity blotting, as we supposed that CheD should bind to CheX in order to enhance the phosphatase activity. The affinity-blotting data indicated that CheD interacts with CheX but not with the chemotaxis response regulator CheY3 (Fig. 1). These observations suggest that CheD may be able to stimulate the phosphatase activity.

To determine if CheD is able to enhance phosphatase activity, we performed phosphorylation-dephosphorylation assays using recombinant purified CheA, CheY3, and CheX. When CheY3 was incubated with CheA- ^{32}P for 5 min, CheY3 was phosphorylated, as we have reported previously (Fig. 2A, lane 2) (32, 33, 53). Moreover, incubation of CheY3- ^{32}P with CheX for 2.5 min resulted in dephosphorylation of CheY3- ^{32}P by CheX (Fig. 2A, lane 3) (33). Importantly, when CheD was added in the CheA-CheY3-CheX reaction mixture for 2.5 min, CheY3- ^{32}P dephosphorylation was enhanced (Fig. 2A, lane 4) and the level of CheY3- ^{32}P was completely diminished within 5 min (Fig. 2A, lane 8). CheD itself did not affect CheY3- ^{32}P dephosphorylation (Fig. 2A, lanes 5 and 9). Moreover, the enhancement of CheX phosphatase activity was dependent on the CheD concentration (Fig. 2B, lanes 4 to 8), indicating that CheD efficiently enhances CheX phosphatase activity.

FIG 2 CheD enhances phosphatase activity of CheX. Shown are phosphorylation-dephosphorylation assays using purified recombinant CheA (A), CheY3 (Y), CheX (X), and CheD (D) proteins. (A) Twenty picomoles of CheA was autophosphorylated using 10 μ Ci of [γ - 32 P]ATP (lane 1). CheA- 32 P was then incubated separately with 160 pmol of CheY3 for 5 min (lanes 2 and 6). Radiolabeled CheY3- 32 P was incubated with 0.15 pmol of CheX for 2.5 min or 5 min (lanes 3 and 7, respectively). The CheY3- 32 P and CheX reaction mixture was incubated with (lanes 4 and 8) or without (lanes 5 and 9) 15 pmol of CheD. The arrowheads indicate the positions of CheA- 32 P and CheY3- 32 P. (B) CheX phosphatase activity was efficiently enhanced by CheD. CheA was autophosphorylated as described above (lane 1). CheA- 32 P was then incubated with 160 pmol of CheY3 for 10 min (lane 2). The radiolabeled CheY3- 32 P was mixed with 0.15 pmol of CheX for 5 min without CheD (lane 3) or with 0.2 pmol (lane 4), 2 pmol (lane 5), 5 pmol (lane 6), 10 pmol (lane 7), or 20 pmol (lane 8) of CheD. CheY3- 32 P was also incubated with 20 pmol of CheD to confirm that CheD did not interfere with CheY3-P dephosphorylation (lane 9). The arrowheads indicate the positions of CheA- 32 P, CheY3- 32 P, and CheD. The relative intensities ("PI volumes") of CheY3- 32 P are shown on the right with the corresponding phosphorimage lane numbers above.

CheD interacts with MCP3 and MCP4. To determine if CheD is able to modify a *B. burgdorferi* chemoreceptor MCP protein, we performed deamidation assays using recombinant purified proteins, as described previously (29, 31). Even though we were able to detect deamidation with the positive-control (*B. subtilis* chemoreceptor) MCP-A, our multiple attempts under various assay conditions failed to deaminate any *B. burgdorferi* MCP. Since we were unable to detect deamidation of an MCP by CheD, we performed CheD-MCP protein-protein interaction assays alternatively, reasoning that CheD must first bind to an MCP in order to deaminate it. Using recombinant purified MCP and CheD proteins in affinity blotting, we were able to determine CheD-MCP

interactions. Specifically, we found that CheD interacts with MCP3 and MCP4, but not MCP5 (Fig. 3).

Construction of *cheD* mutant and complemented strains. In order to determine the role of CheD in motility, chemotaxis, or the infectious cycle of *B. burgdorferi*, we inactivated the gene using a kanamycin resistance cassette (P_{flgB} -*aph1*) in the low-passage-number virulent wild-type clone B31-A3 (see Fig. S1 in the supplemental material) (35, 45, 49, 57). PCR analysis of the kanamycin-resistant clones indicated that the P_{flgB} -*aph1* cassette was inserted in the *cheD* gene (not shown), resulting in successful construction of the mutant. Furthermore, we confirmed by PCR that the *cheD* mutant retained all the linear and circular endogenous plasmids seen in the parental wild-type cells (data not shown).

The *cheD* mutant (Δ *cheD*) was complemented in *cis* by genomic integration to ensure that the phenotype of the mutant was solely attributed to the mutation and not due to a secondary mutation elsewhere in the genome (see Fig. S1 in the supplemental material) (35, 57). Accordingly, the *cheD* gene and its native promoter (P_{cheD}) were fused together and inserted within *bb0445* and *bb0446* in the suicide vector pXLFcheD (46, 47). The insertion of P_{cheD} -*cheD* was downstream of both *bb0445* and *bb0446* and less likely to affect their expression, as reported previously (35, 46, 47). To detect the expression of *cheD* in the wild-type, Δ *cheD* mutant, and complemented *cheD* (*cheD*^{com}) cells, we performed qRT-PCR using gene-specific primers. Real-time PCR detected the *cheD* transcripts in wild-type and *cheD*^{com} cells, but not in the *cheD* mutant cells (see Fig. S2 in the supplemental material). Moreover, the level of *cheD* transcripts synthesized in the *cheD*^{com} cells was approximately 84% of that in wild-type cells, indicating the resto-

FIG 3 CheD specifically interacts with MCP3 and MCP4. Affinity blotting was performed as described in the text. Purified Strep-CheD was probed with MBP-MCP3, MBP-MCP4, or MBP-MCP5, followed by immunoblotting with monoclonal MBP antibodies (Thermo Scientific). The bands at an apparent molecular mass of 19 kDa (Strep-CheD; arrows) were always observed in blots probed with MCP3 and MCP4, but not MCP5.

FIG 4 $\Delta cheD$ mutant cells are deficient in chemotaxis. (A) Swarm plate assays indicated a defect in chemotaxis exhibited by $\Delta cheD$ cells. Approximately 1×10^6 *B. burgdorferi* cells from the indicated strains were spotted into 0.35% soft agarose plates, which were then incubated for 5 days. The swarm diameter of each clone was measured in millimeters. A nonchemotactic $cheY3$ mutant was used as a control (53). The bars represent the means $\pm$ standard deviations of the mean for each clone (with data from at least 3 plates per clone). Statistical analysis was performed by using ANOVA, as described in Materials and Methods. *P* values between samples are shown at the top. (B) $\Delta cheD$ mutants display reduced colony size compared to the wild-type or complemented strain. *B. burgdorferi* cells from the indicated strains were plated on 0.4% soft agarose plates. An individual colony's swarming ability was determined 4 weeks after inoculation. The colony size of each clone was measured in millimeters. *P* values were determined using ANOVA. The values are the means $\pm$ standard deviations of the averages of at least 3 plates (20 individual colonies) per strain. *P* values between samples are shown at the top; a *P* value of ≤ 0.05 was considered significant.

ration of *cheD* synthesis in the $cheD^{com}$ cells (see Fig. S2 in the supplemental material).

In vitro motility and chemotaxis phenotypes of the $\Delta cheD$ mutant cells. We analyzed the $\Delta cheD$ mutant to determine any alteration of motility or chemotaxis behavior and compared the results with the wild-type or $cheD^{com}$ cells. Since we showed that CheD enhanced the phosphatase activity of CheX, we postulated that the *cheD* mutant cells might display an altered motility or chemotaxis phenotype. Dark-field microscopic analysis indicated that the motility patterns of the *cheD* mutant (run-flex/pause-reversal) and its flexing frequency during swimming were not noticeably different from those of the wild-type cells (9 to 12 flexes per minute [data not shown]). However, the chemotaxis was found to be significantly reduced compared to the wild-type ($P = 0.002$) or the $cheD^{com}$ ($P = 0.007$) cells (Fig. 4A). In swarm plate assays, the average swarm diameters of the $\Delta cheD$ mutant were approximately 18% smaller than those of the wild-type or $cheD^{com}$ cells (Fig. 4A). The swarm plate assay is a group event where millions of bacteria attempt to migrate out from the initial site of inoculation (in a semisolid plate) as they chemotax and metabo-

lize neighboring nutrients, resulting in a swarm ring. However, the results obtained from such an assay may not accurately determine the chemotactic ability of an individual spirochete. Accordingly, we plated 20 to 50 *B. burgdorferi* cells in a semisolid plate (the same plates used for the swarm plate assay) to determine the chemotactic ability of individual cells by measuring their colony (swarm) size. Prolonged incubation of the plates produced colony sizes that were significantly smaller than those of the wild-type ($P = 0.0001$) or $cheD^{com}$ ($P = 0.014$) cells. On average, the colony size of $\Delta cheD$ mutants was approximately 13% smaller than that of wild-type or $cheD^{com}$ cells (Fig. 4B). Together, our results indicate that *cheD* plays an important role in *B. burgdorferi* chemotaxis.

***B. burgdorferi* $\Delta cheD$ mutant cells can establish infection in C3H/HeN mice by needle inoculation but display reduced infectivity.** While the role of CheD in motility and chemotaxis has been demonstrated in other bacteria, its role in pathogenesis has not been determined in any bacterium. In order to evaluate the infection potential of the mutant, groups of C3H/HeN mice were challenged with 10-fold-increasing doses of wild-type, $\Delta cheD$, or $cheD^{com}$ cells to determine the ID_{50} . Immediately before the injections, we confirmed by PCR that these strains retained their endogenous plasmids (data not shown) (8, 35, 37, 45). 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 (59, 60). Furthermore, to confirm the serology results, mice were euthanized 3 weeks postinoculation and spirochetes were reisolated from ear, bladder, and joint tissues. The serology results indicated that *cheD* mutant cells were attenuated in establishing an infection in mice (not shown) and correlated well with reisolation of spirochetes from the tissues examined (Table 1). Whereas the $cheD^{com}$ cells did not show a significant increase in the ID_{50} compared to the parental wild-type cells ($P = 0.3$), the ID_{50} for the *cheD* mutant was approximately 1

TABLE 1 $\Delta cheD$ mutant cells are able to establish infection in C3H/HeN mice, but infectivity is considerably reduced^a

Strain	Dose (CFU)	No. of mice infected/no. tested	ID_{50}
WT	7.5×10^2	0/7	1.8080×10^4
	7.5×10^3	1/7	
	7.5×10^4	5/7	
	9.3×10^5	7/7	
$\Delta cheD$	6.2×10^2	0/7	1.5694×10^5
	6.2×10^3	1/7	
	6.2×10^4	1/7	
	1.00×10^6	6/7	
	1.00×10^7	7/7	
$cheD^{com}$	5.6×10^2	0/7	1.9483×10^4
	5.6×10^3	2/7	
	5.6×10^4	5/7	
	8.2×10^5	7/7	

^a Mice were injected with the indicated *B. burgdorferi* clones and doses. Infectivity was determined by reisolation of *B. burgdorferi* from the tissues of euthanized mice (ear skin, ankle joint, and urinary bladder). ID_{50} and statistical analyses were performed using probit regression, as described in Materials and Methods. The $\Delta cheD$ mutant spirochetes were significantly attenuated compared to the ID_{50} of the wild type ($P = 0.0125$) or the complemented *B. burgdorferi* ($P = 0.0005$) (significance was defined as a *P* value of ≤ 0.05).

FIG 5 Naive ticks are able to acquire and transmit $\Delta cheD$ mutant spirochetes during mouse-tick-mouse infection studies. (A) Naive ticks were allowed to feed on infected mice to determine acquisition of spirochetes. Ten larvae fed upon the wild-type, $\Delta cheD$, or complemented-spirochete-infected mice were squashed individually to isolate DNA. The DNA samples were then used to detect *B. burgdorferi* *flaB* by PCR. The PCR data indicated that 100% of the larval ticks were able to acquire the spirochetes from *B. burgdorferi*-infected mice (all groups). Tick DNA that we had previously confirmed to be spirochete positive was used as a positive control (+), and double-distilled water (ddH₂O) was used as a negative control (−). (B) $\Delta cheD$ mutant spirochetes are able to survive in fed larvae. Viable $\Delta cheD$ spirochetes in fed larvae are reduced compared to the wild-type or complemented cells. The *P* values shown were determined by using ANOVA, as described in Materials and Methods. A *P* value of ≤ 0.05 between samples was considered significant. (C) Mutant spirochetes are able to survive in fed nymphs, but the number of viable $\Delta cheD$ cells in fed nymphs is considerably less than that of the wild type ($P = 0.013$) but not statistically different from that of the $cheD^{com}$ cells ($P = 0.452$). The *P* values were determined as for panel B, and significance was defined as a *P* value of ≤ 0.05 . A representative result from two independent studies with different batches of ticks is shown. The bars represent the numbers of mean viable spirochetes per tick $\pm$ standard errors of the mean (SEM).

logarithm higher than that of wild-type or $cheD^{com}$ cells, indicating significant attenuation in virulence ($P = 0.012$ and $P = 0.0005$, respectively) (Table 1).

***I. scapularis* ticks are able to acquire and transmit mutant *B. burgdorferi*.** Because infection of and survival within the mammalian host represents only one aspect of the *B. burgdorferi* enzootic life cycle, a more comprehensive evaluation of the activities of these mutants in the tick vector was justified. Naive *I. scapularis* larvae were allowed to feed on infected mice in order to determine acquisition, as well viability, of *B. burgdorferi* in larval ticks. Seven days after feeding on infected mice, a subset of larvae were

squashed individually, and genomic DNA was purified in order to determine spirochete-positive ticks by PCR using *B. burgdorferi* *flaB* gene-specific primers. Another set of fed larvae were squashed individually for plating in semisolid medium in order to determine the number of viable spirochetes per tick. The PCR data shown in Fig. 5A indicate that 100% of the larvae that fed on wild-type-, $\Delta cheD$ -, or $cheD^{com}$ -infected mice were able to acquire the spirochetes (i.e., were spirochete positive). The burden of the mutant spirochetes per tick was not significantly lower than that of the wild type ($P = 0.129$) (Fig. 5B). However, the burden of mutant *B. burgdorferi* spirochetes was statistically less than the burden seen in larval ticks infected with the complemented $cheD^{com}$ ($P = 0.007$).

In order to determine if the infected ticks were able to survive the molting and to transmit the mutant *B. burgdorferi* in naive mice, a subset of fed larvae were allowed to molt. Two to 3 weeks after molting, the infected nymphs were allowed to feed on naive C3H/HeN mice (3 mice per strain; 15 nymphs per mouse). Seven days postrepletion, the nymphs were analyzed individually by plating on semisolid plates as described above. The plating results indicated that the mutant spirochete burden in fed nymphs was considerably reduced compared to that of the wild type ($P = 0.013$); however, the $\Delta cheD$ mutant load was not statistically different from that of the $cheD^{com}$ -infected nymphs ($P = 0.452$) (Fig. 5C) despite the fact that 100% of the nymphs were spirochete positive by all clones (data not shown).

To determine the transmission of *B. burgdorferi* from infected nymphs to mice, the tick-fed animals were bled at 2 weeks post-feeding to perform serology, followed by euthanization of the mice to reisolate *B. burgdorferi* from ear, ankle joint, and urinary bladder tissues at 3 weeks postrepletion. Serology, as well as bacterial outgrowth analyses, indicated that the $\Delta cheD$ mutants were able to transmit and establish infection in all the mice with no detectable deficiency (Table 2; see Fig. S3 in the supplemental material) even though we performed these *in vivo* studies twice using separate batches of ticks and mice.

DISCUSSION

In this study, we systematically demonstrated that CheD specifically interacts with CheX and enhances phosphatase activity (Fig. 1 and 2). It is notable that CheC-type phosphatases are monomers that possess two CheY-P dephosphorylation sites whereas the CheX-type phosphatases are dimers containing only one dephosphorylation site (29, 33, 34, 66). Considering this distinction, this is the first report demonstrating the stimulation of CheX-type phosphatases by CheD. CheD was reported to possess various functions in *B. subtilis* (27, 31, 67). While we were able to deter-

TABLE 2 $\Delta cheD$ spirochete-infected nymphal ticks are able to transmit the organisms into naive C3H/HeN mice^a

Strain	No. of tissues positive/no. examined	No. of mice infected/no. tested
Wild type	8/9	3/3
$\Delta cheD$	9/9	3/3
$cheD^{com}$	8/9	3/3

^a Fifteen infected nymphs molted from larvae (from an acquisition assay) were allowed to feed on each C3H/HeN mouse. The mice were euthanized 3 weeks postrepletion. Infectivity was determined by reisolation of *B. burgdorferi* from the tissues of the euthanized mice. A representative result from two independent studies is shown.

mine the CheD-CheX relationship, our multiple attempts to deamidate *B. burgdorferi* MCPs using CheD were unsuccessful despite the fact that our positive-control *B. subtilis* MCP deamidation was successful (27, 29), and we performed the assays under various conditions (data not shown). However, we were able to show that CheD specifically interacts with MCP3 and MCP4, but not MCP5 (Fig. 3). It is possible that CheD binds to other MCPs, but we chose to use these three MCPs because MCP3 and MCP5 are homologs of the major chemoreceptors Tar and Tsp of *E. coli*, respectively, and MCP4 possesses three CheD binding sites (see Table S1 in the supplemental material). CheD binding to MCP3 and MCP4 suggests that either our deamidation assay conditions were not optimized or the CheD/MCP proteins purified from *E. coli* cells were nonfunctional/inactive.

Because we found that CheD enhances CheX phosphatase activity and the *cheX* mutant constantly flexes (33), we supposed that the *cheD* mutant would also exhibit a motility phenotype. However, we failed to detect any noticeable difference in motility behavior exhibited by the $\Delta cheD$ cells. Even though the motility phenotype of the *cheD* mutant was indistinguishable from that of the wild-type cells, we demonstrated that CheD is important for chemotaxis (Fig. 4). The chemotaxis effect is unrelated to the mutant's growth rate *in vitro*, since the cells grow normally in the BSK-II growth medium (not shown). Based on these findings, we propose that the enhancer function associated with CheD is not likely to have a noticeable effect on CheX function in motility *in vitro*.

Our mouse infection studies demonstrated that the mutant cells are able to establish infection in C3H/HeN mice; however, the mutant was significantly attenuated (Table 1). These results were not completely surprising given the fact that the nonchemotactic *cheX* mutant cells are noninfectious in mice (M. A. Motaleb, unpublished data). In $\Delta cheD$ mutant cells, CheX, the only phosphatase in *B. burgdorferi*, is intact. Deletion of CheD likely had a minor effect on the CheX protein's ability to dephosphorylate CheY-P. Thus, the attenuation in mice that we observed with the *cheD* mutant spirochetes is reasonable, as the mutant did exhibit a reduced chemotaxis phenotype. It is also plausible that the attenuation we found with the mutant spirochetes could be a result of the chemoreceptors not being modified/deamidated normally due to the deletion of *cheD*. Moreover, we found that CheD is dispensable for acquisition or transmission of *B. burgdorferi* (Table 2; see Fig. S3 in the supplemental material) despite the fact that the viability of mutant spirochetes was reduced in ticks (both larvae and nymphs), and this reduced burden was detected consistently even though the *P* values of the wild type versus $\Delta cheD$ or *cheD*^{com} versus $\Delta cheD$ were not always statistically significant (Fig. 5).

Chemotaxis has been found to be important for the disease processes of many bacteria, including the spirochetes (1, 2, 4, 5, 7, 68); however, our findings with the $\Delta cheD$ mutant suggest that either the *in vitro* assay conditions do not reflect the *in vivo* host conditions (*in vivo*, the *cheD* mutant may be normal with respect to its chemotactic ability) or the chemotaxis defect we observed with the $\Delta cheD$ mutant *in vitro* was not reduced enough to affect the acquisition or transmission of spirochetes. However, our coherent observation of marginally reduced burdens of *cheD* mutant spirochetes in ticks could be related to its activity in CheX, as the *cheX* mutant spirochete burdens in ticks are also marginally reduced compared to those of the wild-type (Motaleb, unpub-

lished). Moreover, we postulate that this phenotype is linked to fewer mutant spirochetes having been acquired by the ticks while feeding or that the viability defect is connected to the receptor MCP modification function. Further evaluation of the roles of MCP proteins in *B. burgdorferi* or how CheD affects the receptors and their subsequent effect on virulence needs to be done.

The Lyme disease spirochete, *B. burgdorferi*, exists primarily in a zoonotic cycle involving *Ixodes* ticks and mammalian hosts. Effective chemotaxis in these disparate habitats potentially explains the need for multiple chemotaxis systems in the organism. We propose that different adaptation systems (CheD or CheR1/CheR2 mediated) are likely employed to sense gradients of different scales and contours in these diverse milieus. However, exactly how these chemotaxis systems contribute to the fitness of the Lyme disease spirochete is an open question.

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