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# Identification of Specific Chemoattractants and Genetic Complementation of a *Borrelia burgdorferi* Chemotaxis Mutant: Flow Cytometry-Based Capillary Tube Chemotaxis Assay▽

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**Measuring the chemotactic response of *Borrelia burgdorferi*, the bacterial species that causes Lyme disease, is relatively more difficult than measuring that of other bacteria. Because these spirochetes have long generation times, enumerating cells that swim up a capillary tube containing an attractant by using colony counts is impractical. Furthermore, direct counts with a Petroff-Hausser chamber is problematic, as this method has a low throughput and necessitates a high cell density; the latter can lead to misinterpretation of results when assaying for specific attractants. Only rabbit serum and tick saliva have been reported to be chemoattractants for *B. burgdorferi*. These complex biological mixtures are limited in their utility for studying chemotaxis on a molecular level. Here we present a modified capillary tube chemotaxis assay for *B. burgdorferi* that enumerates cells by flow cytometry. Initial studies identified *N*-acetylglucosamine as a chemoattractant. The assay was then optimized with respect to cell concentration, incubation time, motility buffer composition, and growth phase. Besides *N*-acetylglucosamine, glucosamine, glucosamine dimers (chitosan), glutamate, and glucose also elicited significant chemoattractant responses, although the response obtained with glucose was weak and variable. Serine and glycine were nonchemotactic. To further validate and to exploit the use of this assay, a previously described nonchemotactic *cheA2* mutant was shown to be nonchemotactic by this assay; it also regained the wild-type phenotype when complemented in *trans*. This is the first report that identifies specific chemical attractants for *B. burgdorferi* and the use of flow cytometry for spirochete enumeration. The method should also be useful for assaying chemotaxis for other slow-growing prokaryotic species and in specific environments in nature.**

Lyme disease is caused by the motile spirochete *Borrelia burgdorferi*. This disease is the most prevalent arthropod-borne infection in the United States, with 19,804 cases reported in 2004 (11). The clinical course of *B. burgdorferi* infections includes symmetrical spread of the spirochetes through the dermis resulting in a rash referred to as erythema migrans and invasion of the blood and deep organs (6). Disease manifestations also include arthritis, cardiac abnormalities, and neuropathies. The life cycle of *B. burgdorferi* involves transmission from a tick vector to mammals or birds and back to the tick over the course of several seasons (54).

A robust motility-and-chemotaxis system is likely to be vital for *B. burgdorferi* in its overall life cycle. Many of the motility and chemotaxis genes are expressed in both the tick and the mammalian host, and several are upregulated in the laboratory that mimic in vivo conditions (8, 16, 21, 45, 48, 57). Furthermore, approximately 6% of its chromosomal genome encodes putative chemotaxis and motility genes (18) and between 10 and 14% of the total cellular protein is composed of flagellar filament proteins (42). Thus, this system is both evidently important but energetically expensive to the spirochete. Motility and chemotaxis have been postulated as being important for the spirochetes to migrate from the tick gut to salivary glands for deposition into the new host upon infection (12, 16). In the mammalian host, motility may be important for the spirochetes to penetrate the bloodstream after being deposited in the skin by the tick bite and also for specific tissue and organ localization (32, 55). For the cycle to be completed, salivary proteins and other compounds could conceivably serve as attractants during tick feeding (12, 32, 53). This chemotactic signaling would result in the spirochetes concentrating at the site of the tick bite for cycle continuation. Chemotaxis and motility have been extensively studied in several species of bacteria and are best understood in the enteric species *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (3, 33, 47, 59). Bacterial chemotaxis involves a sensory transduction system that enables cells to swim toward a favorable environment or away from one that is toxic.

We have begun to understand the basic movements of *B. burgdorferi* motility (12). These spirochetes have two bundles of periplasmic flagella (PFs) located between the cell cylinder and outer membrane sheath. Each bundle consists of 7 to 11 PFs that overlap in the center of the cell. As with other spirochete species, the PFs are attached to basal bodies similar to those of other bacteria (44); these basal bodies are subterminally positioned at each pole of the cell cylinder. A model of *B. burgdorferi* motility states that during a run there is a coordinated rotation of the two PF bundles that results in backward-

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TABLE 1. Primers used to amplify genetic elements

| Fragment | Primer name | Primer sequence[a] |
|---|---|---|
| *flgB* promoter | pflgB1-forward | 5′-CTGCAG TAATACCCGAGCTTCAAG-3′ |
| | pflgB2-reverse | 5′-CATATGACCTCCCTCATTTAAAATTGC-3′ |
| *cheA2* | FPA2-forward | 5′-CATATGATTAAAGAGGAGAAATTAACTATG-3′ |
| | RPA2-reverse | 5′-GCATGCTCAGCTAATTAAGCTTGGCTG-3′ |
| T7 terminator | $pFT_7$-forward | 5′-GCATGCGCTAACAAAGCCCGAAAGGAAGCT-3′ |
| | $pRT_7$-reverse | 5′-AAGCTTTGCAGATCCGGATATAGTTCCTCC-3′ |
| *cheA2* probe | DFA2-forward | 5′-AAAAGACAATCCTATGGCTAC-3′ |
| | DFA2-reverse | 5′-TAACTCTCTCTTCAACTGTTTC-3′ |

[a] Engineered restriction sites are underlined.

moving flat waves (12, 22, 23, 31). These waves propel the spirochete in a given direction. To balance the rotation of the PFs, the cell body rolls around its axis in the opposite direction. During the run, the two bundles rotate in opposite directions, i.e., asymmetrically, one going clockwise (the frame of reference is viewing the PF from its distal end to its insertion into the cell) and the other counterclockwise. Cells reverse directions; they also have a stop mode referred to as a flex when the bundles are believed to rotate in the same direction (clockwise or counterclockwise).

The subpolar localization of the bundles of PFs with their consequential asymmetrical rotation during runs adds a level of complexity in deciphering *B. burgdoferi*'s chemotactic response (12). Nonchemotactic *cheA* mutants of *B. burgdorferi* constantly run (30), as do *cheA* mutants of the spirochete *Treponema denticola* (34) and also *E. coli* and *S. enterica* serovar Typhimurium (46). In addition, inactivation of *B. burgdorferi cheX*, which encodes a specific *cheY* phosphatase, was recently found to result in constant flexing (41). However, a chemosensory model that integrates the phenotypes of these *cheA2* and *cheX* chemotaxis mutants has yet to be formulated.

Identifying compounds that are chemoattractants for *B. burgdorferi* will not only permit a further understanding of its motility and chemotaxis on a molecular level but should also facilitate better understanding of how these organisms shuttle between their mammalian or avian and arthropod hosts. The capillary tube assay is a well-documented procedure for quantitatively assessing bacterial chemotaxis and identifying attractants (1). In this assay, bacteria that have entered capillary tubes containing attractants are usually counted by colony formation. However, measuring *B. burgdorferi* chemotaxis with the capillary tube assay has been difficult. *B. burgdorferi* bacteria are fastidious and slow growing, with approximately 2 to 3 weeks required for colony formation. While direct counting of *B. burgdorferi* bacteria in capillary tubes with Petroff-Hausser chambers has been used to demonstrate that rabbit serum and tick saliva are attractants (52, 53), this approach is highly labor-intensive and has a low throughput. In addition, it requires a high density of cells (at least $1 \times 10^8$/ml in the motility buffer medium) (30, 52). This high cell density can lead to misinterpretation of results. Specifically, cell metabolism could conceivably lead to an alteration in the gradient such that attractant depletion (1) or the generation of a secondary metabolite could interfere with or even promote chemotaxis. To overcome these limitations, a flow cytometry approach was developed to accurately and rapidly enumerate *B. burgdorferi* bacteria at a relatively low cell concentration. This method was optimized and used to identify specific chemicals that elicit a chemotactic response in *B. burgdorferi*. In addition, it was also exploited to further characterize the chemotaxis *cheA2* mutant by complementation (30). A brief description of the use of this method to analyze a *B. burgdorferi cheX* mutant has recently been published (41).

## MATERIALS AND METHODS

**Bacterial strains and culture.** A single clone of high-passage *B. burgdorferi* B31 (B31A) and its *cheA2*::*kan* deletion mutant (LC-A2, referred to as the *cheA2* mutant) were grown at 33 to 34°C in liquid BSK-II medium (7, 30). Kanamycin (350 μg/ml) and streptomycin (80 μg/ml) were used as needed. Swarm plate chemotaxis assays were carried out as previously described (30, 40).

**DNA manipulation, primers, and *cheA2* complementation.** Restriction mapping, enzyme modification, and transformation were carried out by standard procedures. Based on the published *B. burgdorferi* genome sequence (18), DNA amplifications with the PCR primers listed in Table 1 were carried out with *Taq* polymerase. *cheA2* mutant cells were transformed with the intact *cheA2* gene for construction of the complemented strain, referred to as *cheA2*+, as follows. A 409-bp *B. burgdorferi flgB* promoter fragment with PstI and NdeI sites at the 5′ and 3′ ends, respectively, was PCR amplified and cloned into pGEM-T Easy (Promega Co., Madison, WI). The entire *cheA2* gene was PCR amplified with primers engineered with NdeI and SphI sites at the 5′ and 3′ ends, respectively, and ligated into pGEM-T Easy. This *cheA2* fragment was digested from the pGEM-T easy vector and fused to the 3′ end of the *flgB* promoter at the NdeI site. The resultant *flgB-cheA2* fusion fragment was excised with PstI/SphI and subcloned into *B. burgdorferi* shuttle vector pKFSS1 (17) that was modified by the insertion of a T7 terminator at SphI/HindIII, generating the complementation plasmid pFlgBA2com. DNA sequencing confirmed that the insert was present in the proper orientation. The preparation of *B. burgdorferi* competent cells and electrotransformation were performed as previously described (40, 49). Approximately 10 μg of the pFlgBA2com plasmid was transformed into *B. burgdorferi cheA2* mutant cells by electroporation. After 2 to 3 weeks of incubation in BSK-II agar plates containing 80 μg/ml streptomycin, antibiotic-resistant colonies were picked, transferred to BSK-II medium, and further analyzed.

**Western and Southern blotting.** Western blotting was carried out as previously described, by the ECL detection method (Amersham Pharmacia, Piscataway, NJ) (19). P. Matsumura (University of Illinois, Chicago) and J. Benach (State University of New York at Stony Brook) generously provided rabbit antiserum to *E. coli* CheA (used at a 1:1,500 dilution) and monoclonal antibodies to *B. burgdorferi* DnaK (used at a 1:3,000 dilution), respectively. Southern blot analysis was used to determine whether the intact *cheA2* gene in complemented cells resided on the chromosome or a plasmid (50). Chromosomal DNA was isolated by phenol-chloroform extraction; plasmids were isolated with a Wizard plus SV Minipreps Kit (Promega Co., Madison, WI) and a HiSpeed Plasmid Midi Kit (QIAGEN Co., Hilden, Germany). A 906-bp fragment within *cheA2* was PCR

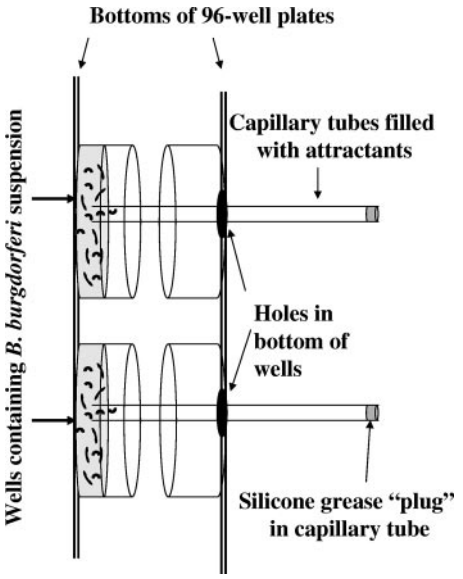


FIG. 1. Schematic of chemotaxis assay using two 96-well plates. *B. burgdorferi* cells in motility buffer plus methylcellulose were placed in wells of a 96-well plate. Holes were formed in the corresponding bottoms of wells of another 96-well plate with a heated metal cylinder. These 96-well plates were taped together face to face, with the plate containing the cells lying face up. Capillary tubes filled with attractant in motility buffer plus methylcellulose were plugged at one end with silicone grease and placed through the holes in the top 96-well plate. The ends of the capillary tubes dipped into the cell suspension in the corresponding wells of the other 96-well plate. The sandwiched plates were incubated such that the attractant-filled capillary tubes were horizontal but still remained inserted in the cell suspension.

amplified (Table 1), labeled with digoxigenin (DIG DNA Labeling and Detection Kit; Boehringer, Mannheim, Germany), and used as a probe.

**Flow cytometry enumeration of *B. burgdorferi*.** All culture media and solutions, except methylcellulose, were filtered (0.1 μm) before flow cytometry. *B. burgdorferi* cells were diluted in counting solution (0.01 M HEPES, 0.15 M NaCl, pH 7.4, 10 nM fresh Syto61 [Molecular Probes Inc., Eugene, OR]), with or without 6-μm-diameter polystyrene beads (final concentration, $3 \times 10^3$ to $5 \times 10^3$/ml; Duke Scientific Co., Palo Alto, CA). Syto61 is a membrane-permeating, nucleic acid-binding, fluorescent dye excitable at 635 nm with emission at 647 nm. The beads were used in the initial experiments as an internal standard to ensure consistent volumes. Samples were analyzed in a Becton-Dickinson FACScalibur with 15-mW air-cooled argon and red diode lasers operating at 488 and 635 nm, respectively, at a rate of 12 or 16 μl/min for 60 or 120 s to ensure at least 100 events in the gate where cells were observed. Compensation was unnecessary, as there was no spectral overlap between detectors. All data acquisition and analysis was performed with CellQuestPro (Becton-Dickinson, San Jose, CA) and Excel (Microsoft Co., Redman, WA).

**Modified capillary tube chemotaxis assay.** The capillary tube assay developed by Pfeffer and described by Adler was optimized to measure *B. burgdorferi* chemotaxis (1). *B. burgdorferi* cells were grown to late logarithmic phase ($\sim 7.5 \times 10^7$ cells/ml) from an initial concentration of $2 \times 10^5$ cells/ml and centrifuged at 23°C for 8 min at $1,800 \times g$ and gently resuspended in a motility buffer consisting of 136.9 mM NaCl, 8.10 mM $Na_2HPO_4$, 2.7 mM KCl, 1.47 mM $KH_2PO_4$, 2% recrystallized bovine serum albumin (BSA; Sigma-Aldrich Co., St. Louis, MO), and 0.1 mM EDTA and adjusted to pH 7.4. Cells were first enumerated by flow cytometry and then diluted to $1 \times 10^7$/ml in motility buffer containing 1.0% methylcellulose (400 mesh; Sigma-Aldrich Co., St. Louis, MO), yielding a final viscosity of 224 cP (33°C, Cannon-Fenske viscometer). Attractants were dissolved in motility buffer–1.0% methylcellulose and readjusted to pH 7.4. Chemotaxis chambers consisted of either 2.0-ml Microfuge tubes with a Parafilm sheet closed under a perforated cap or two 96-well plates sandwiched face to face with tape and with holes on one side for inserting capillary tubes in well bottoms (Fig. 1). Cells were transferred to Microfuge tubes (0.3 ml) or 96-well plates (0.15 to 0.2 ml), and attractant-filled 70-μl capillary tubes (catalog no. 22-362-574; Fisher Scientific, Pittsburgh, PA) plugged with silicone grease were inserted into the chambers. After the chambers were incubated at 33°C in 3% $CO_2$ for 120 min, the capillary tubes were removed, the outsides were carefully wiped with a paper towel, and the contents were expelled by centrifugation at $1,000 \times g$ for 3 to 4 s. Approximately 0.01-ml volumes of expelled solutions were mixed with 0.49 ml of counting solution, and bacteria were enumerated by flow cytometry. The mean of four to five replicas was determined, and the results are expressed as the mean relative increase over a buffer control containing no attractant (referred to as the relative chemotactic response) or as the number of cells that migrated into the capillary tube. At least three independent experiments were done for each test compound, and the results are expressed as the mean ± the standard deviation (SD) for individual experiments or the standard error of the mean for multiple experiments. An increase in the number of spirochetes equal to or greater than twice that of the buffer control was considered significant (26, 27, 35, 43). All chemicals were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, with the exception of chitosan dimers [β-(1-4)-linked D-glucosamine; U.S. Biological, Swampscott, MA] and *N-N*-diacetyl-chitobiose [β-(1-4)-linked *N*-acetyl D-glucosamine; Associates of Cape Cod, Inc., East Falmouth, MA].

**Motion analysis.** To track cells, the spirochetes were prepared as described for the chemotaxis assay and then visualized at ×200 magnification with a Zeiss Axioskop 2 under dark-field illumination (Carl Zeiss Inc., Oberkochen, Germany) equipped with a 35°C heated stage (Physitemp Inc., Clifton, NJ). The Hobson BacTracker was used in the initial tracking experiments (30), and the software package Volocity (Improvision Inc., Coventry, United Kingdom) was used in later analyses. With Volocity, video sequences of swimming cells were captured with iMovie on a PowerMac G4 (Apple Computer Inc., Cupertino, CA) with a Scion LG-5 (Scion Inc., Fredrick, MD) frame grabber card and a Dage MTI (Dage-MTI Inc., Michigan City, IN) black-and-white video camera. Videos were exported as QuickTime (Apple Computer Inc., Cupertino, CA) movies and imported into OpenLab (Improvision Inc., Coventry, United Kingdom) where the frames were cropped, calibrated with a stage micrometer, and saved as LIFF files. Volocity was then used to track the LIFF files.

## RESULTS

**Flow cytometry enumeration of *B. burgdorferi* bacteria.** Although flow cytometry has been previously used to assay for gene expression of *B. burgdorferi* (2, 9, 10, 13–15), it has not been used to enumerate cells. We developed a flow cytometry method to quantitate *B. burgdorferi*. In the absence of fluorescent dyes in our chemotaxis assays, flow cytometric analysis by side versus forward scatter indicated a distinct cell population but with considerable background (Fig. 2A). This background had several causes, including electronic noise, residual particulate material in the BSK-II medium, and *B. burgdorferi*'s flat-wave morphology and variable length. To separate *B. burgdorferi* from this background, the fluorescent, nucleic acid-staining dye Syto61 was tested. We first determined that neither the debris nor *B. burgdorferi* cells autofluoresced with the FLH-4 fluorescence detector. In addition, the debris did not take up the dye. Staining *B. burgdorferi* cells yielded a highly uniform population (Fig. 2B) well resolved from the latex bead control (rectangle) and the background. These beads were initially incorporated into the counting solution as an internal control for sample flow rates through the cytometer, but because of high reproducibility, the beads were eliminated in later studies. To test the validity of this assay for enumerating *B. burgdorferi* bacteria, several different concentrations of cells were counted both with the Petroff-Hausser counting chamber and by flow cytometry. As shown in Fig. 3, the correlation between the two methods was excellent, with a correlation coefficient greater than 0.98.

**Optimization of the capillary tube chemotaxis assay for *B. burgdorferi*.** The capillary tube chemotaxis assay using flow cytometry was optimized for *B. burgdorferi*. We found that 100 mM *N*-acetylglucosamine served as a relatively strong and con-

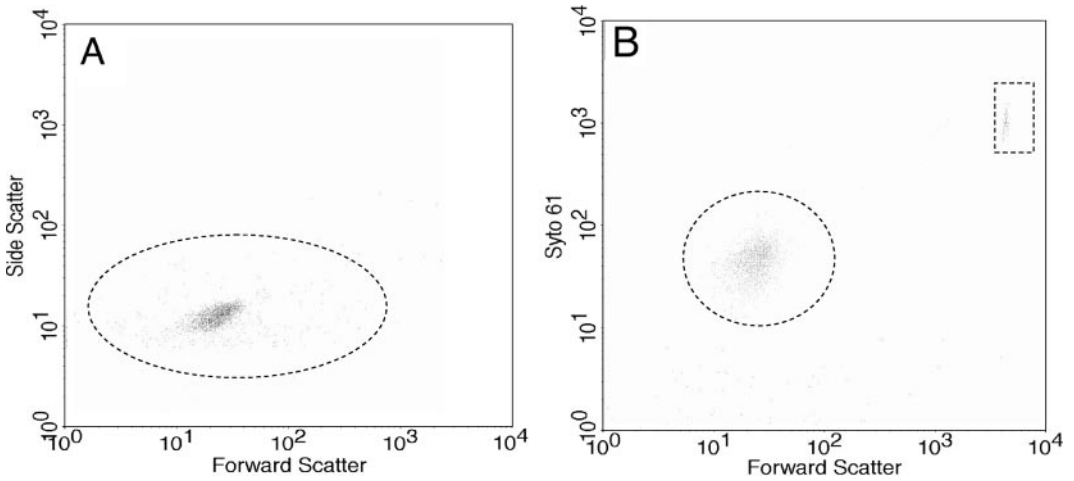


FIG. 2. Flow cytometric analysis of *B. burgdorferi*. Wild-type cells were analyzed by flow cytometry in the absence (A) or presence (B) of Syto61. The *y* axis represents side scatter in panel A and intensity of Syto61 staining in panel B. The dotted circle in panel A indicates that *B. burgdorferi* cells are detected by side scatter, and that in panel B indicates detection of cells stained with Syto61. Polystyrene beads formed a distinct fluorescent population (dotted box) in the presence of Syto61 (B). Results identical to those in panel B were also obtained with *cheA2* mutant cells (not shown).

sistent attractant. Accordingly, the parameters for the chemotaxis assay were established with this compound. A concentration of $1 \times 10^7$ cells/ml in the buffer chamber was found to be optimal. If we used a lower concentration ($1 \times 10^5$ to $1 \times 10^6$ cells/ml), the results were variable due to the lower number of cells that entered the capillary tubes. Assays using $1 \times 10^7$ cells/ml in the motility buffer provided sufficient cell numbers for reproducible counts, and this low number limited the possibility that metabolism of the test compound could influence the chemotaxis assay. This cell concentration is in the range ($6 \times 10^7$ cells/ml) of the standard chemotaxis assay used for *E. coli* (1). The number of *B. burgdorferi* bacteria entering capillary tubes containing motility buffer alone (control) or motility buffer with *N*-acetylglucosamine (100 mM) steadily increased as a function of time up to 2 h. The relative chemotactic response to *N*-acetylglucosamine was greatest at 2 h, which was adopted as the standard incubation time; Shi et al. (52) also used 2 h in their *B. burgdorferi* chemotaxis assay.

Several other parameters were examined to optimize the chemotaxis assay. Gentle resuspension of the cells in the motility buffer after centrifugation was essential for the cells to maintain motility. At least 90% of the resuspended cells were motile at the beginning of the assay, and at least 50% remained motile when the assay was stopped. A second wash and recentrifugation markedly inhibited motility in the buffer solution. Furthermore, preincubating or starving the centrifuged cells in motility buffer longer than the 45 min required to set up the assay resulted in a diminished chemotactic response. Initial experiments indicated that the composition of the buffer was critical for the cells to maintain motility throughout the 2-h incubation period. Specifically, suspending the cells in buffer containing recrystallized rather than Cohn fraction V BSA, or fatty acid-free BSA, was essential. Because *B. burgdorferi* and other spirochetes increase their velocity in a gel-like medium (23, 29), we tested whether methylcellulose added to the motility buffer and attractant solutions influenced the chemotaxis assay. We found that the addition of 1% methylcellulose resulted in more reproducible results from one experiment to another. Thus, methylcellulose was incorporated in all assays. We also determined whether the age of the cells influenced the chemotactic response. Adler found early logarithmic-phase cells of *E. coli* to be optimal (1). In contrast, we found that cells taken from the late logarithmic phase of growth responded best to the attractant (Fig. 4). In addition, these cells were of more uniform size than stationary-phase cells and were thus easily counted with the gating parameters chosen. While conventional bacterial capillary tube chemotaxis assays use small (usually 1-μl) capillary tubes with heat sealing of one end (1), we used 70-μl microhematocrit capillary tubes (30, 41). These tubes provided the volume and numbers of cells necessary for flow cytometric analysis. In addition, these tubes can be more readily filled and plugged at one end with silicone than the 1-μl capillary tubes. Because the filling solution contains BSA, heat sealing of the tubes was not a reasonable option. In our initial chemotaxis assays, we used the standard U-shaped tube sandwiched between a cover glass, a capillary tube, and a slide (1). However, Eppendorf tubes, as well as sandwiched 96-well

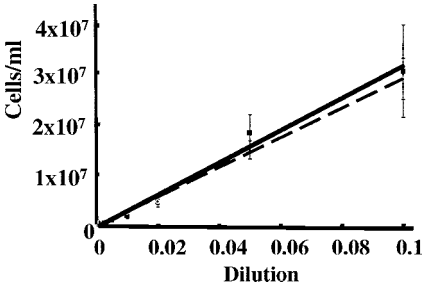


FIG. 3. Comparison of cell enumeration by flow cytometry and the Petroff-Hausser counting chamber. *B. burgdorferi* bacteria were collected by centrifugation, suspended in motility buffer, and serially diluted. Cell concentrations were determined manually with a Petroff-Hausser counting chamber (squares, solid line) or by flow cytometric analysis (circles, dashed line). The coefficient of correlation between the two methods was >0.98.

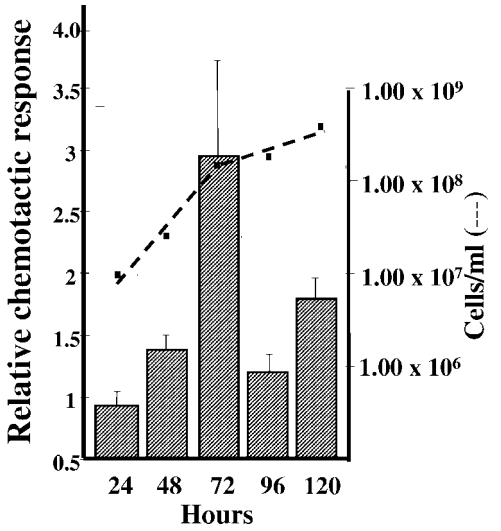


FIG. 4. Growth curve dependence of chemotaxis. *B. burgdorferi* cells were assayed for chemotaxis during different phases of growth. The chemotactic response is represented by the bars, and cell numbers per milliliter are represented by the dashed line. The cell division time during logarithmic growth was 9.33 h. Error lines represent SDs of quadruplicate samples.

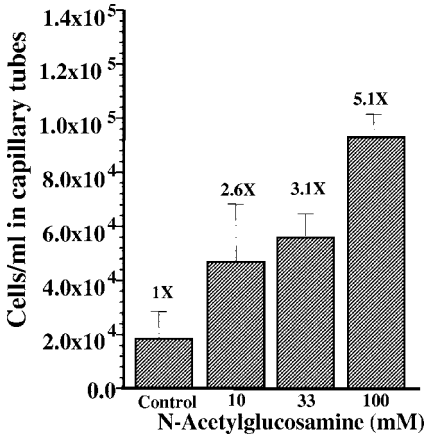


FIG. 5. Dependence of chemotaxis on *N*-acetylglucosamine concentration. Capillary tubes filled with motility buffer alone (control) or the indicated concentrations of *N*-acetylglucosamine were incubated with *B. burgdorferi* cells and assayed for chemotaxis. The results of a representative experiment are depicted, showing the mean and SD of quadruplicate samples. The relative chemotactic response is indicated as a times value at the top of each bar.

plates with submerged capillary tubes, containing attractant yielded results comparable to those of the standard assay. The latter assays were adopted, as the materials were easier to manipulate and consequently were less likely to lead to a laboratory-acquired infection.

**Defined attractants for *B. burgdorferi*.** In many bacterial species, compounds that are transported and are also metabolized by cells are often chemoattractants. Genomic analysis indicated that a putative *N*-acetylglucosamine transporter is encoded in the *B. burgdorferi* genome (18). In addition, either *N*-acetylglucosamine or the *N*-acetylglucosamine dimer chitobiose is an essential nutrient for *B. burgdorferi* (56). Accordingly, *N*-acetylglucosamine was tested as a chemoattractant. *N*-Acetylglucosamine induced a concentration-dependent increase in the number of cells that migrated into the capillary tube compared to the buffer control (Fig. 5). The relative chemotactic response to 200 mM *N*-acetylglucosamine was not significantly greater than that to 100 mM *N*-acetylglucosamine (data not shown), suggesting that the chemotactic response may have been saturated at 100 mM *N*-acetylglucosamine. Although these results suggested that *N*-acetylglucosamine was a chemoattractant, other explanations are possible. One possibility is that *N*-acetylglucosamine increased the velocity of the cells, and this increase resulted in more cells migrating into the capillary tube. We found that the velocity of the cells with different concentrations of *N*-acetylglucosamine were not different than the buffer control (3 to 5 μm/s). We also tested if a gradient of attractant was necessary for the cells to migrate into the capillary tube (37). When cells were suspended in the buffer containing the same concentration of *N*-acetylglucosamine as found in the capillary tube, there was no increase in cells in the capillary tube compared to the control (Fig. 6, no-gradient control). Finally, we tested nonchemotactic *cheA2* mutant cells in the capillary tube assay. This mutant has been previously shown to be nonchemotactic to rabbit serum, and it had a constantly running phenotype (30). We found that this mutant failed to show an increase in cell numbers in the capillary tube compared to the buffer control (Fig. 6). Taken together, the results indicate that *N*-acetylglucosamine is a chemoattractant for *B. burgdorferi*.

Based on known *B. burgdorferi* nutritional requirements and/or the presence of putative transporters in the genome (5, 18), other compounds were assessed for a chemotactic response. In each experiment, a nongradient control and *cheA2*

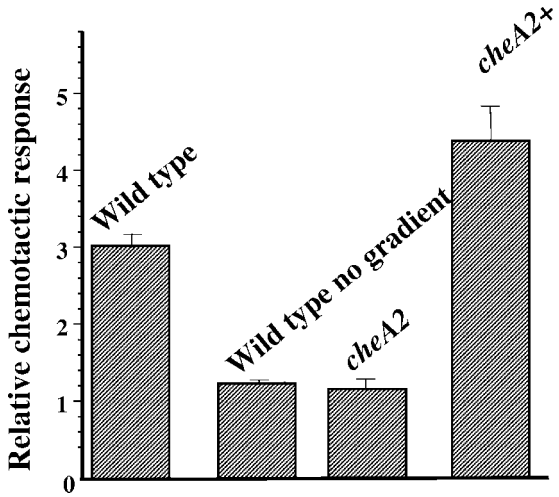


FIG. 6. CheA2 and attractant gradient dependence of *B. burgdorferi* on chemotaxis to *N*-acetylglucosamine. Capillary tubes filled with motility buffer containing 100 mM *N*-acetylglucosamine were assayed for chemotaxis with wild-type *B. burgdorferi*, the *cheA2* mutant, or complemented strain *cheA2*+. The relative chemotactic response compared to the buffer control was determined. Results are expressed as the mean of four experiments ± the standard error. "No gradient" indicates a control in which both the capillary tube and the suspending motility buffer contained equal concentrations of *N*-acetylglucosamine.

TABLE 2. Chemotactic responses of *B. burgdorferi* to specific compounds[a]

| Compound | Relative chemotactic response ± SEM |
|---|---|
| Chitosan dimer [β-(1-4)-linked D-glucosamine] | 4.5 ± 1.4 |
| Glucosamine | 3.5 ± 0.3 |
| *N*-Acetylglucosamine | 2.7 ± 0.3 |
| Glutamate | 2.2 ± 0.1 |
| Glucose | 2.0 ± 0.5 |
| β-Alanine | 1.4 ± 0.1 |
| Glycine | 1.2 ± 0.2 |

[a] The relative chemotactic response is the mean response to the test compound relative the buffer control for at least three experiments. All test compounds were at 100 mM, except the chitosan dimer, which was at 10 mM. Lower concentrations of the above compounds did not elicit a stronger response (not shown except in Fig. 5). All of the compounds shown except β-alanine and glycine elicited relative chemotactic responses of ≥2 and were considered chemoattractants.

mutant cells were used to verify the chemotactic response. The putative *N*-acetylglucosamine transporter is postulated to also be involved in the transport of glucosamine (18). However, glucosamine is not an essential nutrient as is *N*-acetylglucosamine. We found that glucosamine elicited a concentration-dependent response, similar to *N*-acetylglucosamine. Besides *N*-acetylglucosamine and glucosamine, chitosan dimers, glucose, and L-glutamate were found to be chemoattractants for *B. burgdorferi* (Table 2). These compounds induced a relative chemotactic response equal to or greater than twice the buffer control in a concentration-dependent manner. Both glutamate and glucose were weak attractants, as their response approximated twice the buffer control. Furthermore, the response to glucose was variable whereas that for glutamate was consistent. Several of the compounds tested showed no chemotactic response, and these included glycine and β-alanine.

**Complementation of *cheA2*.** We used the flow cytometry chemotaxis assay to test for complementation of *cheA2* mutant cells. We previously reported that inactivation of *cheA2* by targeted mutagenesis with either a *kan* or an *erm* cassette inhibited CheA2 synthesis and chemotaxis toward rabbit serum (30). These mutants failed to reverse and constantly ran. The *kan* cassette inserted into *cheA2* did not affect expression of the downstream gene *cheY3*, but insertion of the *erm* cassette resulted in a decrease of CheY3 accumulation by one-third (30). Although these results pointed toward *cheA2* being involved in chemotaxis, it was not rigorously determined whether the resulting phenotype in the *cheA2* mutant cells was due to inactivation of *cheA2* or to a secondary mutation. Accordingly, we complemented this strain with shuttle plasmid pFlgBA2com, a derivative of pKFSS1 (17), and analyzed the resulting genotype and phenotype. The strong *flgB* promoter (20) was used to drive transcription of *cheA2* in the complemented strain (referred to as *cheA2*+). Western blot analysis indicated that CheA2 was produced in the *cheA2*+ strain but not in *cheA2* mutant cells (Fig. 7a). Using reactivity to DnaK to normalize the amount of protein loaded in the gels, we found that the level of CheA2 produced in *cheA2*+ cells was greater than that produced in wild-type cells. These results are consistent with reports that the *flgB* promoter is quite strong (20).

We also determined if pFlgBA2com is extrachromosomal in *cheA2*+ or if it is integrated into the chromosome. Southern blot analysis was used to localize intact *cheA2*. Chromosomal and plasmid DNAs from wild-type, *cheA2* mutant, and *cheA2*+ cells were separated from one another. With a probe complementary to the deleted fragment in the *cheA2* mutant (Table 1), positive reactions were detected with chromosomal DNA isolated from wild-type cells (Fig. 7b, lane 4) and plasmid DNA from *cheA2*+ cells (lane 1). No reaction was detected with plasmid DNA isolated from cells of the wild type (lane 2) or with chromosomal DNA isolated from *cheA2*+ (not shown) or *cheA2* mutant (lane 3) cells. These results indicate that pFlgBA2com replicated as a plasmid in *cheA2*+ cells.

We determined if pFlgBA2com restored the wild-type phenotype in the *cheA2* mutant. With the flow cytometry chemotaxis assay, we found that *cheA2*+ cells had a strong chemotactic response to *N*-acetylglucosamine (Fig. 6). Restoration of the chemotactic response in *cheA2*+ cells was corroborated by the swarm plate chemotaxis assay (30); wild-type and *cheA2*+ cells both formed 17- to 18-mm-diameter swarm rings in diluted growth medium at 3 days of incubation. These swarm ring diameters were considerably larger than that of the *cheA2* mutant, which was 3 to 5 mm (not shown).

We also tracked the swimming behavior of *cheA2*+ cells (30) and compared it to that of the wild type. Whereas *cheA2* mutant cells failed to reverse and continuously translated in one direction, *cheA2*+ cells readily reversed. The reversal frequency of *cheA2*+ cells (22 ± 1.0 reversals/min) was the same that of wild-type cells (21 ± 1.5 reversals/min). These results indicate that pFlgBA2com restored the wild-type phenotype in the *cheA2* mutant cells and that a secondary mutation was not responsible for its altered phenotype. In addition, these results also illustrate that the flow cytometry chemotaxis

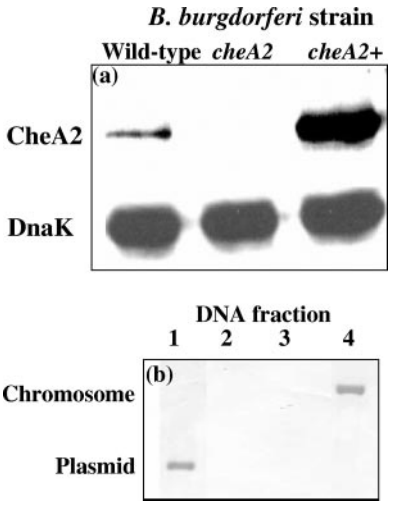


FIG. 7. *cheA2* inactivation and complementation. (a) Ten micrograms of protein from lysates of wild-type, *cheA2*, and *cheA2*+ cells were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, blotted onto nitrocellulose, and reacted with *E. coli* CheA and *B. burgdorferi* DnaK antibodies. (b) Southern blot analysis of chromosomal or plasmid DNA from *cheA2*+, wild-type, and *cheA2* mutant cells (see the text). Southern blot analysis was carried out with a digoxigenin-labeled DNA probe complementary to a fragment of *cheA2* deleted in the mutant (Table 1). The chromosomal and plasmid DNAs migrated at rates represented by the vertical axis.

assay is applicable in analyzing the chemotactic response of *B. burgdorferi*.

## DISCUSSION

The routinely used capillary tube chemotaxis assay has obvious limitations when adopted for use with slow-growing bacteria. To facilitate identification of defined chemoattractants and to begin to define the complex molecular events involved in *B. burgdorferi* chemotaxis, we developed a rapid capillary tube chemotaxis assay in combination with flow cytometry. The enumeration of Sty061-stained cells by flow cytometry produced results identical to those obtained with a Petroff-Hausser chamber. The main advantages of flow cytometry over manual counting are that it enables the use of a lower concentration of cells and it has a much higher throughput.

Results of capillary tube assays are often reported as the "relative chemotactic response" (26, 27, 35, 43). This response is defined as the ratio of cells that migrate into the attractant versus buffer-filled capillary tubes, and it is used to normalize day-to-day variability. A relative chemotactic response of $\geq 2$ is frequently used to indicate a significant chemotactic response (26, 27, 35, 43). We identified a number of compounds as being chemoattractants by this criterion (Table 2). Furthermore, other results also indicate that these compounds are chemoattractants. Specifically, no response occurred with these compounds in the absence of a concentration gradient. In addition, *cheA2* mutant cells failed to show a positive response to these compounds. We show here that *trans* complementation of this mutant resulted in cells regaining the chemotaxis response. Finally, two of these compounds, *N*-acetylglucosamine and glucosamine, were tested for the ability to increase cell speed and no increase was detected. Thus, the positive results found with these two test compounds were not a consequence of a general physiological effect that resulted in an increase in cell velocity.

The compounds identified to date as *B. burgdorferi* chemoattractants elicited relative chemotactic responses ranging from ~2 to 5 (Table 2), which is at the lower end of the range relative to other bacteria. For example, the relative chemotactic responses of several bacterial species are approximately the following (calculated): *E. coli*, 10 to 72 (1, 38); *Spirochaeta aurantia*, 4 to 155 (24); *Pseudomonas aeruginosa*, 2.5 to 25 (26, 43); *Brachyspira hyodysenteriae*, 2 to 16 (27). The apparent lower relative chemotactic responses found with *B. burgdorferi* may be attributed, in part, to shallower attractant gradients with the larger capillary tubes in the modified chemotaxis assay; a decrease in the number of bacteria entering larger capillary tubes in chemotaxis assays occurs for *E. coli* (1). The assay described herein uses 70-μl capillary tubes, whereas 1-μl capillary tubes are used in the standard assay (1). Seventy-microliter capillary tubes have an aperture ~7.6 times the area of 1-μl capillary tubes. Calculations of diffusion gradients indicate a gradient that is shallower with 70-μl capillary tubes than with 1-μl capillary tubes but is still present at 2 h of incubation (4). With a different chemotaxis assay, in which *B. burgdorferi* bacteria were inoculated into one side of a U-shaped tube partially filled with semisolid medium and attractants were placed at the other end, somewhat higher relative chemotactic responses (~10 to 25) were reported for salivary gland extracts prepared from ticks that fed on hosts over a 12- to 48-h assay period (53). This higher response may be attributed to the different type of assay or to more potent chemoattractants in the salivary gland extracts.

The use of capillary tubes with large apertures, producing shallow gradients, may also have required higher concentrations of most attractants (100 mM) to elicit significant chemotactic responses in *B. burgdorferi* than would be required for smaller capillary tubes. However, similar concentrations of attractants (~100 mM) elicited the maximal chemotactic response for *E. coli* and *S. enterica* serovar Typhimurium to alanine, asparagine, glycine, and several other amino acids (25, 36, 37). Furthermore, maximal chemotactic responses were observed with 100 mM chemoattractants for *S. aurantia* (24) and for *B. hyodysenteriae* (27) to 12 of 21 and to 3 of 9 defined chemoattractants, respectively.

Both rabbit serum and tick saliva have been identified as *B. burgdorferi* chemoattractants, but these attractants are complex and the specific attractants in these mixtures have not been identified (52, 53). Here we identified the first specific chemoattractants of *B. burgdorferi*: *N*-acetylglucosamine, glucosamine, chitosan dimers [β-(1-4)-linked D-glucosamine], and glutamate; the response to glucose was variable and weak. Both genetic and physiological analyses indicate that *N*-acetylglucosamine, glucose, and glutamate are metabolized by *B. burgdorferi* (5, 18, 58). *N*-Acetylglucosamine dimer (chitobiose) was reported to support *B. burgdorferi* growth (56), but we found no evidence that it is an attractant at 10 mM (data not shown), a concentration approximately 50-fold greater than that reported to support *B. burgdorferi* growth (56). Glucose, *N*-acetylglucosamine, and *N*-acetylglucosamine dimers were reported to be nutrients utilized by *B. burgdorferi*, while glucosamine was not (58); however, glucosamine was an attractant. We found that glutamate was a weak attractant, but Shi et al. found that it was not (52). Our positive response was at 100 mM, whereas Shi et al. tested glutamate at 10 mM. When we tested lower concentrations of glutamate (25 mM), we also obtained a negative response $1.02 \pm 0.19$ (not shown), suggesting that the threshold concentration of glutamate was higher than that of most of the sugars that were identified as chemoattractants (Table 2). It is not clear if any of the *B. burgdorferi* attractants identified to date play a role in the life cycle of the spirochete as it shuttles between tick and mammalian hosts. However, some of the pathologies associated with Lyme disease are attributed to *B. burgdorferi* colonization of the nervous system and joints. It is tempting to speculate that glutamate, a major central nervous system neurotransmitter, and *N*-acetylglucosamine and glucosamine, major components of connective tissue in joints, may play a role in the migration of *B. burgdorferi* to these tissues.

The development of this assay is timely. Recent microarray results indicate that several motility and chemotaxis genes are markedly up-regulated in mammalian infection or under in vitro conditions that mimic infection (8, 16, 48, 57). Our results set the stage for the detailed phenotypic analysis of targeted mutations of these genes and other chemotaxis genes and their roles in chemotaxis and virulence (41). Defined chemoattractants will also be important in sorting out how *B. burgdorferi* coordinates rotation of the two PF bundles. For example, the response of individual cells to an immediate release of an attractant (28) can be determined by using glutamate as a

caged compound, and pulsed release of an attractant at one cell pole will facilitate determination of the response speed to the other cell pole (51). The modified capillary tube flow cytometry chemotaxis assay is likely to have important uses of a general nature: First, it should be quite useful in enumerating spirochetes and in measuring the chemotaxis of other slow-growing or difficult-to-manipulate bacteria such as anaerobes. Second, the assay should also be helpful in measuring chemotaxis of bacteria in natural settings where motile bacteria are present (39). Specifically, capillary tubes containing various putative attractants could be inserted and incubated in specific environments in nature. Flow cytometry could then be used to rapidly enumerate, and even identify, the organisms responding to the attractants.

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