UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

UPREGULATION OF OUTER SURFACE PROTEIN C EXPRESSION BY LYME DISEASE SPIROCHETES IN LABORATORY MEDIUM

A Manuscript Style Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of Master of Science in Biology

Macy Olson

College of Science and Health
Clinical Microbiology

December, 2015

UPREGULATION OF OUTER SURFACE PROTEIN C EXPRESSION BY LYME DISEASE SPIROCHETES IN LABORATORY MEDIUM

By Macy Olson

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science Biology - Clinical Microbiology

The candidate has completed the oral defense of the thesis.

Steve Callister, Ph.D. — 9-28-15
Thesis Committee Chairperson

Dean Jobe, M.S. — 9-28-15
Thesis Committee Member

Bernadette Taylor, Ph.D. — 10/13/15
Thesis Committee Member

Peter Wilker, Ph.D. — 9-28-15
Thesis Committee Member

Thesis accepted

Steven Simpson, Ph.D. — 10/13/15
Graduate Studies Director

ABSTRACT

Olson, M.G. Upregulation of outer surface protein c expression by Lyme disease spirochetes in laboratory medium. MS in Clinical Microbiology, December 2015, 56pp. (S. Callister)

Lyme disease is the most common vector borne illness in the United States and there is considerable interest in the ability of vaccination with OspC to provide protection. However, studies to evaluate the efficacy of this approach have been hampered by the lack of OspC expression by spirochetes that have been cultured in traditional Barbour-Stoenner-Kelly (BSK) laboratory growth medium. In this study, I show that maintaining an incubation temperature of 33 °C, limiting the number of passages to fresh BSK medium, and adjusting the pH of the culture medium to 6.6, induced the *B. burgdorferi* ss 297 spirochetes to produce significantly higher levels of OspC. In addition, I developed and used a flow cytometric procedure to confirm that OspC was expressed on the surface of some of the spirochetes. Therefore, additional studies to refine the expression of OspC, so that the Osp is expressed on the surface of each spirochete, remain necessary. However, the findings should help facilitate future efforts to examine the feasibility of OspC-based vaccines.

ACKNOWLEDGEMENTS

I would like to thank the members of my thesis committee, Dr. Steve Callister, Dean Jobe, Dr. Bernadette Taylor and Dr. Peter Wilker for their time and advice. I would like to express extra gratitude to Dr. Steve Callister for his support throughout the research and scientific writing process. This thesis would not have been possible without his advice and support. I am grateful to Dean Jobe for his expert technical training, assistance and encouragement, and I would also like to thank Dr. Steve Lovrich for his guidance during the research process. Finally, I'd like to thank my family and friends for their unwavering support and acknowledge the Gundersen Medical Foundation, whose financial support made this project possible.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES……...vii

LIST OF TABLES……..……....ix

INTRODUCTION……...1

Discovery…….....1

Tick vector.....…….….1

Classification of Lyme disease spirochetes…….…2

Morphology of *B. burgdorferi* ss…….2

Expression of outer surface proteins……....3

Clinical signs and symptoms……...3

Humoral immune response after infection……...……4

Laboratory confirmation of infection……...4

Prevention and treatment…….....5

STUDY RATIONALE AND OBJECTIVES…….……7

METHODS AND MATERIALS……...10

Infectious *B. burgdorferi*……10

Control *B. burgdorferi* 50772……10

Early Lyme disease sera…...……..10

Monoclonal anti-OspA antibodies ……...……...……..10

Recombinant (r)OspC..……..11

OspC7 peptide……....11

Mice……..…….12

Vaccination and collection of mouse immune sera……..……...……..12

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis…….……...12

Western blotting…..……....12

rOspC7 ELISA…….…...……...13

Removal of anti-OspC7 antibodies……...…….……...…....14

rOspC ELISA…….14

B. burgdorferi 50772 or 297 ELISAs…….…...15

Flow cytometric detection of OspC expression……...…….....15

Detection of borreliacidal antibodies.....16

RESULTS…….....18

Antibody "marker" for confirming surface expression of OspC……..…..……18

Detection of surface expression of OspC ……..……...19

OspC expression by *B. burgdorferi* 297 after growth in traditional BSK medium……..……19

Effect of incubation temperature/passage on OspC expression…….....28

Effect of pH on OspC expression……..31

Quantitation of OspC expression…..…….31

Surface accessibility of *B. burgdorferi* 297 OspC after increased expression.....32

DISCUSSION……37

REFERENCES……...……...41

LIST OF FIGURES

FIGURE PAGE

1. Structure of *B. burgdorferi* OspC with surface expressed C-terminus highlighted……....21

2. Western blots of antibodies in normal serum (lane 1) or Lyme disease serum number #4 (lane 2) reactive against *B. burgdorferi* 297. Panels A and B are IgM and IgG Western blots, respectively.…….23

3. Fluorescence intensity of *B. burgdorferi* 50772 and normal serum (panel A), *B. burgdorferi* 50772 and early Lyme disease serum #4 (panel B) or *B. burgdorferi* 50772 and early Lyme disease serum #4 after removal of anti-OspC7 antibodies (panel C) after the addition of FITC-labelled anti-human IgM antibodies. Panel 1 shows dot plots where the side scatter and fluorescence intensity of each organism are depicted. Panel 2 shows histograms where the numbers of spirochetes with identical characteristic are grouped. In addition, organisms with background (R1) or significantly increased fluorescence (R2) are segregated by gating.....….25

4. Western blots of *B. burgdorferi* 297 using anti-OspA monoclonal H5332 antibodies (lane 1) or early Lyme disease serum #4 (lane 2). Lanes 1 and Lane 2 are IgG and IgM Western blots respectively……..…….……..…26

5. SDS-PAGE profile of *B. burgdorferi* 297 after culture in BSK medium (pH 7.4)……....27

6. Fluorescence intensity of *B. burgdorferi* 297 after growth in traditional BSK medium and normal serum (panel A) or early Lyme disease serum #4 (panel B). Panel 1 shows dot plots where the side scatter and fluorescence intensity of each organism are depicted. Panel 2 shows histograms where the numbers of spirochetes with identical fluorescence intensity are grouped……..….……..29

7. Expression of OspC by *B. burgdorferi* 297 cultured in traditional (pH 7.4) BSK at 33 °C (panel A) or 37 °C (panel B) after 1 passage (lane 1), 3 passages (lane 2), 6 passages (lane 3), 9 passages (lane 4), 12 passages (lane 5) or 15 passages (lane 6)……..…....30

8. OspC expression by passaged and temperature-adjusted *B. burgdorferi* 297 after culture in fresh BSK medium adjusted to pH 6.6 (lane 1), pH 7.0 (lane 2), pH 7.4 (lane 3), pH 7.8 (lane 4) or pH 8.2 (lane 5)…….....33

9. OspC and OspA expression by traditional *B. burgdorferi* 297 (A) and passage/temperature/pH-adjusted *B. burgdorferi* 297 (B)……..….34

10. Fluorescence intensity of passage/temperature/pH-adjusted *B. burgdorferi* 297 after combining normal serum (A) or early Lyme disease serum #4 (B) and FITC-labelled anti-human IgM antibodies. Panel 1 shows dot plots where the side scatter and fluorescence intensity of each organism are depicted. Panel 2 shows histograms where the numbers of spirochetes with identical fluorescence intensity are grouped…….....36

LIST OF TABLES

TABLE PAGE

1. Detection of borreliacidal activity and anti-OspC7 antibodies in sera from subjects with early Lyme disease..…...……...…….....…22

2. Detection of IgM anti-OspC7 or anti-OspC antibodies in early Lyme disease serum #4 before and after adsorption with OspC7 peptide……..…….…....24

3. Detection of anti-OspC antibodies in Lyme disease serum #4 using *B. burgdorferi* 50772, traditional 297 or passage/temperature/pH-adjusted *B. burgdorferi* 297 ELISA.……...……..…….…....35

INTRODUCTION

Discovery. Lyme disease, a tick-transmitted bacterial infection caused by *Borrelia* ssp., has become the most commonly reported vector-borne illness in North America (1). The illness was first described in 1975 by Dr. Allen Steere, who noted a cluster of juvenile rheumatoid arthritis cases in children who lived in rural wooded areas near Lyme, Connecticut (2, 3). The cases were unusual because the patients also reported a tick bite and a rash prior to the illness. Subsequently, Dr. Willy Burgdorfer isolated *Borrelia* spp. spirochetes from *Ixodes scapularis* (deer) ticks captured from the region (4), and also demonstrated that the sera from the patients contained antibodies that recognized the spirochetes (5). The combined findings resulted in confirmation of a new illness caused by infection with *Borrelia burgdorferi* spirochetes that was ultimately named Lyme disease (4, 5). The number of cases in the United States (US) now approaches 25,000 annually.

Tick vectors. Lyme disease spirochetes are most often transmitted by the bite of *Ixodes* spp. ticks. For example, deer ticks (*I. scapularis*) are endemic in the upper Midwest and along the eastern seaboard, so most cases of Lyme disease in US patients are caused by transmission of *Borrelia burgdorferi* ss from these arthropods (6). In addition, *I. pacificus*, or black-legged ticks, that can also transmit the spirochetes, are found in western regions of the US (7, 8, 9). The numbers of cases from these ticks lags significantly because the main host for *I. pacificus* is the western fence lizard (*Sceloporus occidientalis*) (8), and the lizard is not an efficient reservoir host because they resist

infection by producing antibodies that target the spirochetes (10). In addition, the primary vectors in Europe and Asia are *I. ricinus* (European sheep tick) and *I. persulcatus* (taiga tick), respectively (11-13). Both species are closely related to *I. scapularis*, but each also transmits *B. afzelii* or *B. garinii* (14) in addition to *B. burgdorferi* ss.

Classification of Lyme disease spirochetes. Since Lyme disease was first discovered, over 20 distinct *Borrelia* genospecies have been associated with the illness (15, 16, 17). As a result pathogenic *Borrelia* spp. are now referred to as *B. burgdorferi* sensu lato until they have been characterized. However, the causative agents recognized most often in the US are *B. burgdorferi* ss and *B. bissettii* (10, 16), while European patients are most often infected with *B. burgdorferi* ss, *B. afzelii, or B. garinii* (14).

Morphology of *B. burgdorferi* ss. *Borrelia burgdorferi* ss are 20 to 30 μm in length and 0.2 to 0.5 μm wide. The organisms have a protoplasmic cylinder surrounded by a thin layer of peptidoglycan and an outer membrane that surrounds a periplasmic space. The protoplasmic cylinder contains cytoplasm and nuclear material, and the periplasmic space contains 8 to 12 flagella comprised of a motor, hook and flagellar proteins (18). The torque from the flagella creates a lashing or corkscrew-type motion that propels the organisms through viscous material. The cytoplasm contains the linear chromosome and approximately 12 linear and 9 circular plasmids (19) that contain genes coding essential proteins. Amino acids, cofactors, and nucleotides necessary for growth *in vivo* are also obtained by parasitizing the host (20). *In vitro, B. burgdorferi* is a fastidious, microaerophilic organism that can be cultured in Barbour-Stoenner-Kelly (BSK) medium (21).

Expression of outer surface proteins. The outer surface proteins (Osps) are typically lipoproteins that perform a variety of functions. For example, OspA is an attachment protein that anchors the spirochetes to tissue in the midgut of the tick vector (22, 23). In addition, OspC is expressed primarily during the early stage of mammalian infection (4, 24) and, while the specific function of this Osp remains unknown, spirochetes that do not express OspC are non-infectious (24). During human infection, OspA is downregulated to only low levels of expression, while expression of OspC is increased to a level that makes it the immunodominant Osp (1). Other Osps, including OspB (25) OspD (26), OspE (26, 27), and OspF (26), have been described, but their function and role during pathogenesis have not been fully elucidated. For example, OspB is suggested to play a role in adhesion to the tick mid gut (25), while OspE is thought to provide complement resistance during mammalian infection (27).

Clinical signs and symptoms. A hallmark of early localized infection with *B. burgdorferi* ss is a "bulls-eye" rash called erythema migrans (EM) that typically appears as an expanding red ring with central clearing. Interestingly, the EM disappears after a week or two, even without antibiotic therapy. Approximately 80% of Lyme disease cases develop an EM lesion within about 10 days after the tick bite (28), and most infected individuals also develop constitutional symptoms that include headache, fatigue, myalgia, and/or arthralgia (28).

If the infection remains untreated, the spirochetes can disseminate from the site of the tick bite to other skin locations, large joints (6, 10), central nervous system tissue (6, 10), or heart muscle (6). Therefore, symptoms of disseminated infections include secondary EM lesions, swollen or painful joints, facial palsy, or cardiac rhythm

abnormalities. In addition, infections that last months to years manifest most often as intermittent swelling of the large joints, including especially the knees (6). In rare instances, a chronic, long-term infection causes neurological abnormalities that include memory loss or paresthesia (10). Moreover, a small percentage of patients develop a condition termed treatment-resistant Lyme arthritis (10), because the patients continue to have joint problems despite long term antibiotic therapy.

Humoral immune response after infection. Infection with *B. burgdorferi* ss induces a typical humoral immune response that includes immunoglobulin (Ig) M and G antibodies. The IgM antibodies are often produced after 7 to 10 days, and the level typically peaks after three to six weeks (29). The IgM antibodies are most often specific for flagellin (41 kDa), OspC (approximately 23 kDa), or a 39 kDa periplasmic protein.

After four to six weeks, IgG antibodies (29) that bind multiple other proteins (18, 28, 31, 45, 58, 66 or 93 kDa) in addition to the proteins that induced IgM antibodies, are produced (30). The IgG antibodies are mostly IgG_1 or IgG_3 subclass that opsonize the spirochetes for recognition by phagocytic cells (31). Some proteins, including OspA, OspB, OspC, decorin binding protein, p66 and p39, also induce IgG antibodies that have been termed borreliacidal since, similar to the IgM antibodies, attachment induces complement to form a membrane attack complex that kills the spirochetes without the necessity of scavenging by phagocytic cells (1, 31-33).

Laboratory confirmation of infection. Infection with *B. burgdorferi* ss may be confirmed by culture, microscopy, or polymerase chain reaction (PCR)-based tests. However, direct detection of the organisms is often difficult, because the spirochetes are typically present in small quantities. Therefore, PCR-based tests have gained the most

acceptance because they detect small amounts of spirochetal DNA in EM lesions (34), synovial fluid (35), or CSF (17, 35). However, availability remains problematic, because the tests require expensive equipment and highly trained personnel.

Lyme disease is therefore most commonly confirmed by serodiagnosis. The current criterion, endorsed by the Centers for Disease Control and Prevention (36, 37), is to perform an initial screening test, such as a sensitive but non-specific whole cell enzyme linked immunosorbent assay (ELISA) or indirect immunofluorescence assay (IFA), followed by a Western blot test with defined interpretation recommendations to ensure high specificity. If the patient has been ill for ≤ 4 weeks, then detection of IgM antibodies that bind at least two of the 23 kDa (OspC), 39 kDa (periplasmic protein), or 41 kDa (flagellar) proteins is considered confirmatory. If the patient has been ill for a longer time period, then detection of IgG antibodies that bind at least 5 of the 18, 23 (OspC), 28, 30, 39, 41, 45, 58, 66, or 93 kDa proteins is necessary for confirmation (17, 30).

Prevention and treatment. SmithKline Beecham introduced a recombinant (r) OspA-based Lyme disease vaccine (LYMErix) to the marketplace in 1998. However, the vaccination was only effective for a few months. In addition, there were concerns that antibodies triggered by the vaccination could also react with human lymphocyte function associated antigen-1 (hLFA-1) in genetically-susceptible recipients (38, 39), and induce an autoimmune phenomenon that caused painfully swollen joints. These concerns caused the public to reject the product, so the vaccine was pulled from the marketplace.

Therefore, the only current treatment option is antibiotic therapy. Most early Lyme disease cases are treated successfully by 7 to 10 days of doxycycline, amoxicillin

or cefuroxime axetil (40-42). However, disseminated infections typically require oral antibiotics for several additional weeks (37, 41-42), and weeks to months of intravenous antibiotic therapy have been necessary to resolve the most complicated infections (37).

STUDY RATIONALE AND OBJECTIVES

Since OspC is the major Osp expressed by *B. burgdorferi* ss during early human Lyme disease (1), there is considerable interest in evaluating the ability of vaccination with OspC to provide protection by inducing antibody-mediated immunity. However, studies to evaluate the feasibility or performance of OspC-based vaccines have been hindered because of considerable sequence variability in the *ospC* gene (43-46), even among *B. burgdorferi* isolates from the same geographic region (43). For example, researchers (47-50) have demonstrated that passive administration of OspC antibodies effectively protected mice from a subsequent needle-challenge with *B. burgdorferi*, but the protection was limited to only spirochetes with identical or highly similar *ospC* sequences (48, 50). This finding has significantly hindered further exploration because of the conclusion that an OspC-based vaccine would therefore not have widespread utility.

Researchers, however, recently postulated two possible strategies to overcome this shortcoming. The first was to induce protective antibodies against multiple regions of OspC, so that each important *ospC* genotype was targeted. For example, Earnhardt and Marconi (51) formulated a chimeric protein comprised of 7 linear epitopes and showed that the immune sera from mice vaccinated with the chimera produced complement-activating antibodies (51, 52) that bound multiple pathogenic *Borrelia* spp. (43, 51-54).

The other option was to induce antibodies specific for a conserved epitope. In support of this option, researchers (55, 56) showed that the immunodominant antibody

response during early human Lyme disease was almost entirely specific for an epitope located within the last 7 amino acids (OspC7) nearest the C-terminus of OspC. More significantly, the region is highly conserved among the pathogenic *Borrelia* spp., and the epitope is expressed on the surface of OspC-expressing spirochetes during human infection (57). Moreover, anti-OspC7 antibodies produced during human infection are borreliacidal (55, 58), which has been a necessary function for effective vaccine-induced antibody mediated immunity (56).

Despite these promising findings, however, additional studies to evaluate the potential of OspC-based Lyme disease vaccines have not been forthcoming. This is likely because of several significant issues that continue to hinder exploration. Most notably, *Borrelia* spp. express abundant amounts of OspA and only small amounts of OspC while they are growing in Barbour-Stoenner-Kelly (BSK) medium, pH 7.4 at 33° C (59, 60). This is especially problematic because the antigenicity then differs significantly from the antigenicity during human infection, where OspC is abundantly expressed and expression of OspA is depressed (50, 55, 58, 59, 60). In addition, laboratory mice are unsuitable as study models because, in contrast to human infection, the spirochetes express little or no OspC during infection (56).

I therefore focused my efforts toward facilitating additional experiments to allow researchers to examine the feasibility or effectiveness of OspC-based vaccines. Specifically, my main goal was to modify the traditional BSK medium and culture conditions so that organisms grown *in vitro* produced levels of OspC that more closely matched the amounts expressed during human infection. An additional goal was to confirm that the increased expression

of OspC also resulted in accessibility of the Osp on the surface of the spirochete. I therefore accomplished the following specific objectives:

1) Detection of an appropriate antibody "marker" for confirming surface expression of OspC.

2) Development of a method for detecting surface expression of OspC.

3) Characterization of OspC expression by *B. burgdorferi* 297 after growth in traditional BSK medium.

4) Upregulation of OspC expression by modifying traditional BSK medium and culture conditions.

5) Confirmation of the surface accessibility of OspC.

METHODS AND MATERIALS

Infectious *B. burgdorferi* ss. Low passage *B. burgdorferi* ss 297, an infectious spirochete originally recovered from human spinal fluid, was used to evaluate the ability of varying culture conditions to increase expression of OspC (1). A clonal population was obtained by limiting dilution, where the spirochetes were grown in BSK medium (23) and the culture recovered from the greatest dilution (approximately 1 organism) was then aliquoted into 200 µl-amounts in 1.5 ml screw cap tubes (Sarstedt, Newton, NC) and stored at -80° C until used.

Control *B. burgdorferi* ss. *B. burgdorferi* ss 50772 was used as a control organism. The spirochete is a unique, but non-infectious isolate that lacks *ospA/ospB* and instead expresses OspC on the surface (61). A clonal population was obtained as described above, and the spirochetes were then aliquoted into 200 µl-amounts in 1.5 ml screw cap tubes (Sarstedt, Newton, NC) and stored at -80° C until used.

Early Lyme disease sera. Archived serum samples (1, 55) from patients with early Lyme disease, characterized by physician-documented single or multiple erythema migrans lesions, were obtained from Gundersen Health System in La Crosse, WI. Serum from a person not exposed to *B. burgdorferi* was used as a normal control.

Monoclonal anti-OspA antibodies. Murine anti-OspA monoclonal antibody H5332 (62) was provided by the Gundersen Health System Microbiology Research Laboratory (La Crosse, WI).

Recombinant (r) OspC. Recombinant OspC was recovered as previously described (55, 56). Briefly, *Escherichia coli* (JM109) transformed with an *ospC* encoding plasmid was grown in 100 ml of 2× TY broth containing ampicillin (100 μg/ml; Sigma-Aldrich, St. Louis, Mo.) for 12 h at 37 °C, diluted 1:10 with 2× TY broth, and incubated for 1 h. Isopropyl-β-D-thiogalactopyranoside (final concentration, 0.1 μM; Sigma-Aldrich, St. Louis, Mo.) was added to the culture, and the culture was incubated for an additional 4 h. The suspension was then centrifuged at 10,000 × *g* for 15 min at 4 °C, and the pelleted cells were resuspended in purification buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100, and lysed with a sonicator (Branson Sonic Power W350, Danbury, CT). The sonicated *E. coli* cells were centrifuged at 10,000 × *g* for 15 min, and the supernatant was passed over a column containing SoftLink resin (Promega, Madison, WI) at a rate of 0.5 ml/min at 4 °C. The column was then washed with 5 column volumes of purification buffer. Finally, OspC was eluted with 5 mM biotin (Sigma-Aldrich, St. Louis, Mo.), and the recovered fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

OspC7 peptide. The OspC7 peptide (63) was synthesized commercially (Genscript, Piscataway, NJ) by biotinylating the amine-terminal end by HBTU (2[1H-benzotriazole-1-yl]-1,1,3,3-tetra-methyl-uronium hexafluorophosphate) activation and purifying the peptide by high-pressure liquid chromatography. The composition was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Mice. Laboratory mice (Harlan Laboratories, Indianapolis, IN) were housed three or four per cage at ambient temperature in the animal facility at the Health Science Center. Food and water were available *ad libitum*. Animal experiments were reviewed by the University of Wisconsin-La Crosse Animal Care and Use Committee prior to implementation.

Vaccination and collection of mouse immune sera. The OspC7 peptide was conjugated to Imject® Blue Carrier® Protein (Thermo Scientific, Carlsbad, CA) and individual mice were vaccinated with 50 µl (0.029 µg) of the peptide-conjugate contained in 20 % v/v Emulsigen® (MVP Laboratories, Inc., Omaha, NE) and boosted after two weeks with the same suspension (55, 56). Two weeks after the booster, the mice were euthanized by CO_2 inhalation and blood was collected by intracardiac puncture. The serum was allowed to clot overnight, and then separated by centrifugation for 5 min at 3,000 x g and stored at -20°C until tested.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. SDS-PAGE was performed using standard procedures (55). Briefly, 7.5 µg of protein was loaded into individual wells of 10-20 % polyacrylamide gels (Criterion™ precast gels, Hercules, CA) and separated by running in an electrophoresis chamber (Criterion™ Cell, Bio-Rad, Hercules, CA) at 200 V for 1 h. The gels were then visualized by staining with Coomassie brilliant blue R-250 and protein sizes were approximated by comparison to molecular weight standards (High range rainbow molecular weight marker, GE Healthcare, Pittsburgh, PA).

Western blotting. Proteins were separated by SDS-PAGE as described above, and then transferred from the polyacrylamide gel to polyvinylidene difluoride (PVDF)

(Perkin-Elmer Life Sciences, Boston, MA) by electrophoresing overnight at 10 V. The PVDF was cut into strips and then blocking buffer (phosphate-buffered saline, pH 7.2 containing 0.1% Tween 20 and 1% bovine serum albumin [PBS-T 1% BSA]) was added to each strip and incubating for 1 hr at 22 °C with gentle rocking. The strips were washed with PBS-T and then incubated for 1 h at 22 °C with either mouse monoclonal anti-OspA antibody diluted 1:400, or human early Lyme patient serum #4 diluted 1:100. Horseradish peroxidase-labeled (HRP) goat anti-human IgM diluted 1:20,000 or a 1:10,000 of anti-mouse IgG heavy and light chains (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to the strips, incubated for 1 hr at 22 °C. Strips were then washed and developed by using the TMB membrane peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, MD).

rOspC7 ELISA. The levels of OspC7-specific antibodies were determined by ELISA as described previously (63). Briefly, individual wells of microtiter plates (Immunolon 2 HB; Thermo Labsystems, Franklin, MA) were coated with 100 µl (4.0 µg/ml) of streptavidin (Pierce, Rockland, IL) in 0.05 M carbonate coating buffer, and incubated overnight at 4 °C. Plates were washed with PBS-T to remove excess streptavidin and 200 µl of blocking buffer (PBS-T 1% BSA) containing 1.0 µg/ml of biotinylated OspC7 peptide was added to each well and incubated for 1 hr at 22° C. The wells were washed and overlaid with serial dilutions (1:200 to 1:409,600) of immune serum and incubated for 1 hr at 22 °C. Then, 100 µl of goat anti-mouse IgG HRP conjugate diluted 1: 10,000, goat anti-human IgM HRP diluted to 1:20,000 or goat anti-human IgG HRP diluted 1:30,000 in PBS were added to each well, and the plates were re-incubated for 1 hr at 22 °C. One hundred microliters of o-phenylenediamine

dihydrochloride (OPD) substrate (Sigma-Aldrich, St. Louis, MO) was added to each well prior to an additional 30 min incubation at 22 °C. After incubation, the reactions were stopped by the addition of 1N H_2SO_4, and the optical density (OD_{490}) was immediately determined by spectrophotometry (VersaMax; Molecular Devices, Sunnyvale, CA).

Removal of anti-OspC7 antibodies. Streptavidin agarose resin (Thermo Scientific, Rockford, IL) was loaded into a 10- by 70-mm polypropylene column and washed with phosphate-buffered saline (PBS; pH 7.2). A 1.0 mg amount of OspC7 was solubilized in 1.0 ml of PBS and then passed over the column. Complete binding was confirmed by protein assay (Bio-Rad, Hercules, CA) prior to passing a 1.0 ml volume of serum diluted ten-fold in PBS over the column. Removal of the antibodies was confirmed by using the OspC7 ELISA.

Recombinant (r) OspC ELISA. Anti-OspC antibodies were detected by ELISA as described previously (1). Briefly, 100 ng/well of rOspC dissolved in coating buffer was added to microtiter plates (Immunolon 2 HB; Thermo Labsystems, Franklin, MA), and the plates were incubated overnight at 4 °C. Two-hundred microliters of blocking buffer (PBS-T 1% BSA) was then added to each well, and the plates were incubated for 1 hr at 22 °C. After incubation, each well was overlaid with 100 µl of human serum diluted 1:200 to 1:409,600, and incubated for 1 hr at 22 °C. One-hundred microliters of anti-human IgM HRP conjugate diluted 1:20,000 (PBS) or anti-human IgG HRP diluted 1:30,000 (PBS) were added to each well prior to an additional incubation for 1 hr at 22 °C. Bound substrate was then detected by adding OPD (Sigma-Aldrich, St. Louis, MO) to each well, incubating for 30 min at 22 °C, and then adding stop solution (1N H_2SO_4).

The OD_{490} was determined by spectrophotometry (VersaMax; Molecular Devices, Sunnyvale, CA).

***B. burgdorferi* 50772 or 297 ELISAs.** *B. burgdorferi* 50772 and 297 ELISAs were performed using standard procedures (55). Spirochetes were cultured in BSK medium until logarithmic growth phase. The spirochetes were then centrifuged at 8,000 × *g* for 15 min, resuspended in PBS, and the suspension was adjusted to a concentration of 1.0×10^9 organisms/ml. The spirochetes were then sonicated (Branson; Danbury, CT) and diluted to 1 µg/ml in coating buffer, and then 100 µl of the suspension was added to each well of a microtiter plate (Immunolon 2 HB; Thermo Labsystems, Franklin, MA) that were subsequently incubated overnight at 4 °C. Plates were washed five times (PBS-T) and 200 µl of blocking buffer (PBS-T 1% BSA) was added to each well, and the plates were incubated for 1 hr at 22 °C. The microtiter plates were then washed with PBS-T, and then overlaid with polyclonal rabbit anti-OspC or mouse anti-OspA antibody diluted 1:200 to 1:409,600 in blocking buffer (PBS-T 1% BSA) and incubated for 1 h at 22 °C. One-hundred microliters of anti-rabbit IgG HRP conjugate diluted 1:10,000 or anti-mouse IgG HRP diluted 1:10,000 were added to each well prior to an additional incubation for 1 hr 22 °C. Bound substrate was then detected by adding OPD (Sigma-Aldrich, St. Louis, MO) to each well, incubating 30 min at 22 °C, and then adding stop solution (1N H_2SO_4). The OD_{490} was determined by spectrophotometry (VersaMax; Molecular Devices, Sunnyvale, CA).

Flow cytometric evaluation of OspC expression. Surface expression of OspC was evaluated by a flow cytometric test using a modification of a previously-described procedure (1). *B. burgdorferi* were grown in BSK medium to logarithmic growth stage,

diluted to a concentration of 1.0×10^6 organisms/ml in BSK and 100 µl amounts were added to separate 100 µl amounts of serially-diluted Lyme disease patient serum or normal serum diluted 1:80 to 1:80,920 in BSK medium. The suspensions were incubated for 30 min at 37 °C to allow antibody binding and then 20 µl of goat anti-human IgM fluorescein isothiocyanate (FITC)-conjugated antibody diluted twenty-fold in PBS was added to each suspension, and re-incubated for 30 min at 37 °C. After incubation, a 100 µl amount was added to 400 µl of PBS, and the suspension was analyzed with a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) and CellQuest Pro software (BD Biosciences, San Jose, CA). Events were collected for 30 seconds and fluorescence intensity and side scatter characteristics of individual spirochetes (dot plots) were evaluated. The data was then converted to histograms to compare the total numbers of organisms with identical characteristics. In addition, non-fluorescing (R1), or fluorescing (R2) spirochetes were segregated by gating.

Detection of borreliacidal antibodies. Borreliacidal antibodies were detected by a flow cytometric test as previously described (58, 64). Briefly, *B. burgdorferi* 50772 in logarithmic growth phase was diluted with fresh BSK to a concentration of approximately 5.0×10^5 spirochetes/ml. Concomitantly, serum samples were diluted 1:40 with BSK and sterilized by passage through a 0.2-µm-pore-size microcentrifuge filter (Costar; Fisher Scientific, Pittsburg, PA). A 200-µl aliquot was then transferred to a sterile 1.5-ml screw-cap microcentrifuge tube (Sarstedt; Newton, NC) and diluted serially from 1:80 to 1:20,480 with BSK. Serum samples were heat inactivated at 56 °C for 10 min, and then a 100-µl aliquot of *B. burgdorferi* 50772 (5.0×10^4 organisms) and 5 µl of

sterile guinea pig complement (Sigma-Aldrich, St. Louis, MO) were added. The assay mixtures were mixed thoroughly and incubated for 16 to 24 h at 35 °C.

Following incubation, 100 µl was removed from each assay and combined with 400 µl of PBS that contained 1 µg/ml acridine orange. Dead, blebbed spirochetes, characteristic of organisms killed by borreliacidal antibodies, were then detected with a FACSCalibur flow cytometer (Becton-Dickinson Immunocytometry Systems). Spirochetes were isolated by gating (CELLQuest Pro software; BD Biosciences, San Jose, CA) and analyzed for 1 to 2 min with the flow rate set at low (12 µl/min). Borreliacidal antibodies were detected by monitoring the increased side scatter and fluorescence intensity that occurs when the acridine orange intercalates into the blebbed spirochetes. Samples that yielded a ≥13% increase in fluorescence intensity compared to the normal serum control were considered to have significant borreliacidal activity (58, 64).

RESULTS

Appropriate antibody "marker" for confirming surface expression of OspC.

It was first necessary to identify an appropriate antibody "marker" for confirming that viable spirochetes were expressing OspC on the surface. I therefore focused my effort on obtaining a source of anti-OspC7 antibodies, because the antibodies bind the C-terminal region of the protein (55, 56) expressed on the surface of intact *B. burgdorferi* (Fig. 1). I initially vaccinated and boosted mice with adjuvanted OspC7 peptide, but the vaccination and booster failed to induce significant amounts of anti-OspC7 antibodies. This was not unexpected, since the finding confirmed a previous report (56) that laboratory mice rarely produce anti-OspC antibodies. However, I considered it a necessary exercise because the results provided additional confirmation that previous OspC-related findings (43, 54) obtained using laboratory mice were potentially suspect.

It was therefore necessary to identify a more reliable source of anti-OspC7 antibodies, so I screened early human Lyme disease sera using the borreliacidal antibody test and rOspC ELISA. Significant amounts of borrelicidal activity and anti-OspC7 antibodies were detected in multiple serum samples (Table 1), and I more critically evaluated Lyme disease serum #4 because the antibody response appeared to be almost entirely anti-OspC antibodies (Fig. 2). The subsequent evaluation showed that removing the antibodies by adsorption to the OspC7 peptide resulted in almost complete elimination of the IgM reactivity detected by the OspC7 ELISA (Table 2). In addition, the serum contained borreliacidal activity against *B. burgdorferi* 50772 that could be

detected in significant amounts to a dilution of 1:5,120. The findings therefore provided strong evidence that Lyme disease serum #4 was appropriate as a reagent for evaluating surface expression of OspC because it was comprised almost entirely of anti-OspC7 IgM borreliacidal antibodies.

Detection of surface expression of OspC by viable *B. burgdorferi.* As final confirmation of the utility of the Lyme disease serum as a surface expression "marker", I next evaluated the ability of the antibodies to bind the *B. burgdorferi* 50772 control organism. No significant difference in fluorescence intensity was detected by flow cytometry after the organisms were combined with the normal human serum and the FITC-labelled anti-human IgM antibodies (Fig. 3, panel A). However, the fluorescence intensity of the spirochetes increased dramatically when the spirochetes were combined with the Lyme disease serum and the anti-human IgM antibodies (Fig. 3, panel B). In addition, the increased fluorescence was no longer detected after the anti-OspC7 antibodies had been removed from the Lyme disease serum (Fig. 3, panel C). The results therefore provided additional confirmation of the validity of the Lyme disease serum #4 as a surface-expression "marker".

OspC expression by *B. burgdorferi* 297 after growth in traditional BSK medium. Coincident with obtaining anti-OspC7 antibodies, I characterized the OspC expression by the *B. burgdorferi* 297 spirochetes after the organisms were cultured in BSK by traditional laboratory methods. Traditional culture methods include growth in BSK medium (pH 7.4) at 33 °C. Western blots using the anti-OspA monoclonal antibodies (Fig. 4, lane 1) or the Lyme disease serum (Fig. 4, lane 2) showed that the

organisms expressed both OspA and OspC. However, the expression of OspC appeared less robust than the expression of OspA (Fig. 5).

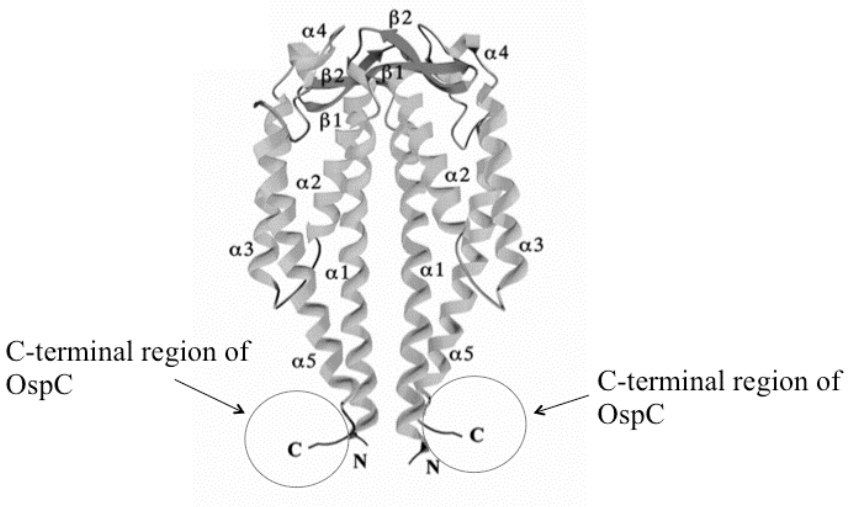

FIG 1 Structure of *B. burgdorferi* OspC with surface expressed C-terminus highlighted.

TABLE 1 Detection of borreliacidal activity and anti-OspC7 antibodies in sera from subjects with early Lyme disease.

		ELISA reactivity[b]	
Early Lyme disease serum	Borreliacidal activity[a]	IgM	IgG
1	3,200	12,800	800
2	25,600	51,200	3200
3	25,600	25,600	400
4	5,120	204,800	3200

[a] Reciprocal of last dilution with ≥13% shift in fluorescence compared to normal serum control.

[b]Reciprocal of last dilution with OD value ≥ 0.200 above normal serum level.

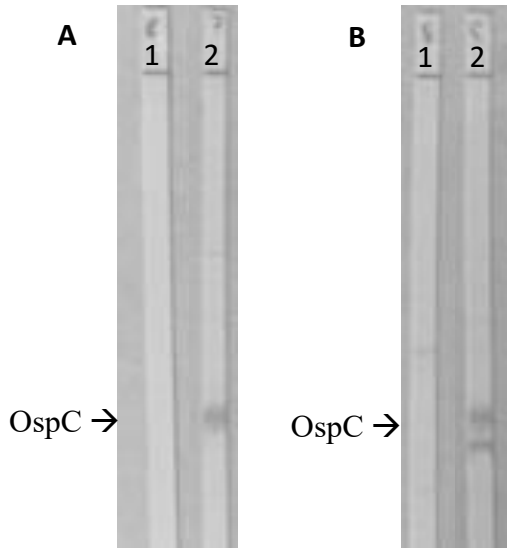

FIG 2 Western blots of antibodies in normal serum (lane 1) or Lyme disease serum number #4 (lane 2) reactive against *B. burgdorferi* 297. Panels A and B are IgM and IgG Western blots, respectively.

TABLE 2 Detection of IgM anti-OspC7 or anti-OspC antibodies in early Lyme disease serum #4 before and after adsorption with OspC7 peptide.

	Last dilution[a] with significant reactivity	
ELISA	Neat	Adsorbed
OspC7	51,200	< 200
rOspC	102,400	800

[a] Reciprocal dilution with OD value ≥ 0.200 above normal serum level

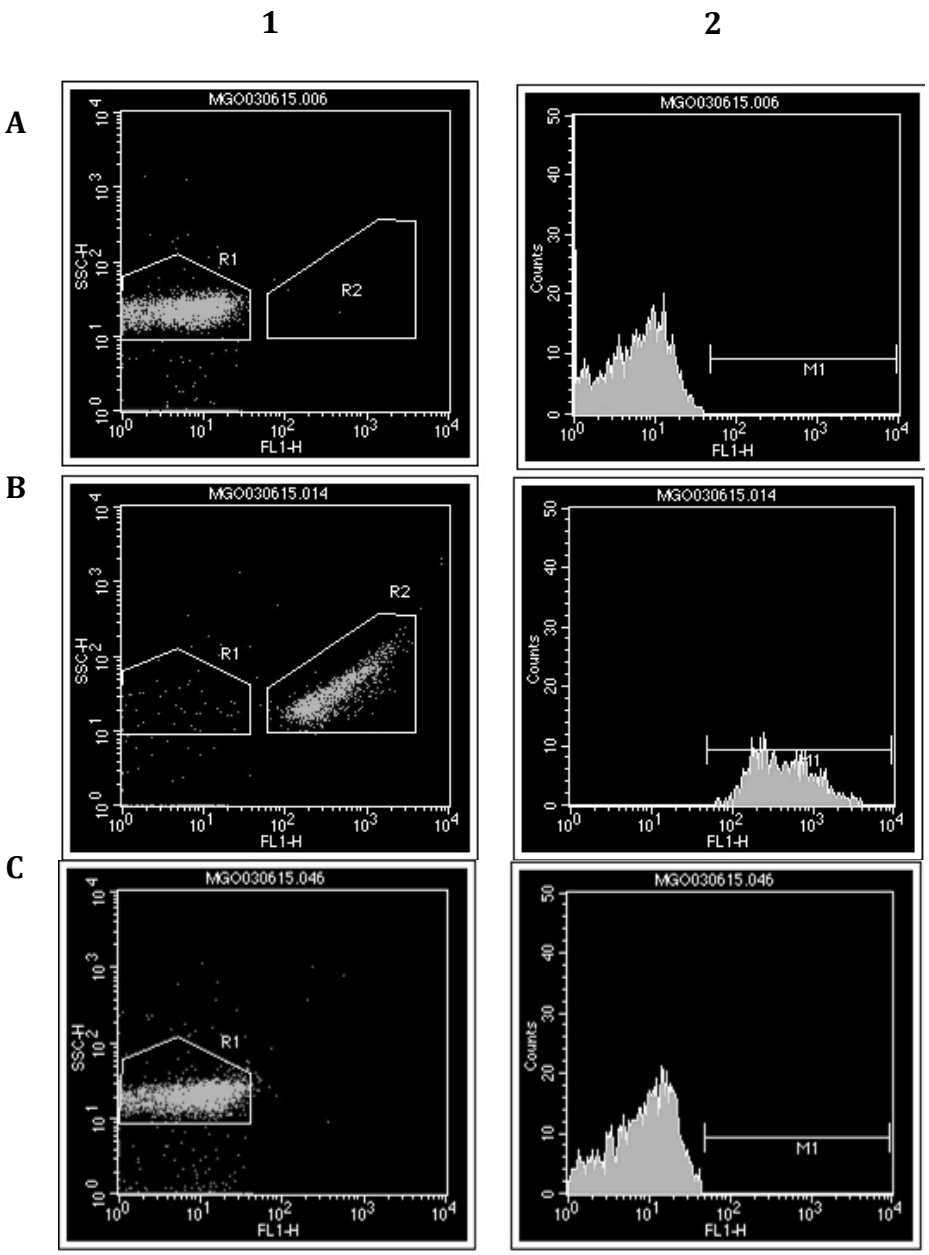

FIG 3 Fluorescence intensity of *B. burgdorferi* 50772 and normal serum (panel A), *B. burgdorferi* 50772 and early Lyme disease serum #4 (panel B) or *B. burgdorferi* 50772 and early Lyme disease serum #4 after removal of anti-OspC7 antibodies (panel C) after the addition of FITC-labelled anti-human IgM antibodies. Panel 1 shows dot plots where the side scatter and fluorescence intensity of each organism are depicted. Panel 2 shows histograms where the numbers of spirochetes with identical characteristic are grouped. In addition, organisms with background (R1) or significantly increased fluorescence (R2) are segregated by gating.

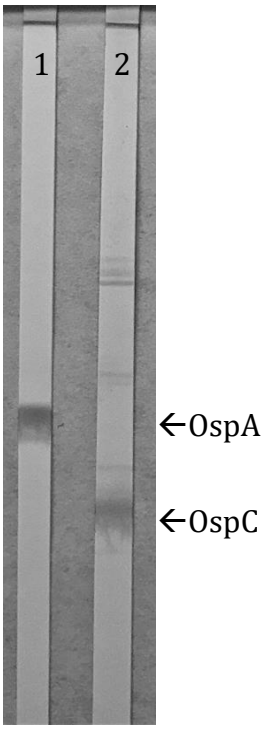

FIG 4 Western blots of *B. burgdorferi* 297 using anti-OspA monoclonal H5332 antibodies (lane 1) or early Lyme disease serum #4 (lane 2). Lanes 1 and Lane 2 are IgG and IgM Western blots, respectively.

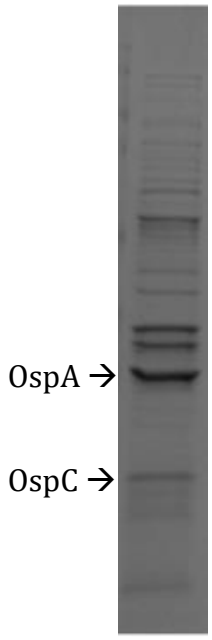

FIG 5 SDS-PAGE profile of *B. burgdorferi* 297 after culture in BSK medium (pH 7.4). Coomassie stain for total protein.

Further evidence of the low level of OspC was indicated by the lack of reactivity of the ELISA and borrelicidal antibody test performed using *B. burgdorferi* 297 organisms. Moreover, increased fluorescence was not detected when the *B. burgdorferi* 297 spirochetes were combined with the Lyme disease serum and the FITC-labelled anti-human IgM antibodies (Fig. 6).

Effect of incubation temperature/passage on OspC expression. After confirming that the *B. burgdorferi* 297 spirochetes expressed only low levels of OspC and OspC was not accessible on the surface, I next determined whether varying the incubation temperature would affect the level of expression. After the spirochetes were cultured in BSK medium at either 33 °C or 37 °C, the level of OspC expression had increased significantly after three passages (Fig. 7). However, the level then decreased significantly with additional passages when the spirochetes were incubated at 37 °C. In contrast, the increased expression remained detectable for nine additional passages when the incubation temperature was kept at 33 °C. Based on these results, I therefore inoculated an aliquot of the original *B. burgdorferi* 297 spirochetes into BSK medium, passaged the organisms seven times by transfer into fresh BSK medium while maintaining the incubation temperature at 33 °C. These organisms were aliquoted into separate 200 µl-amounts and stored at -80 °C.

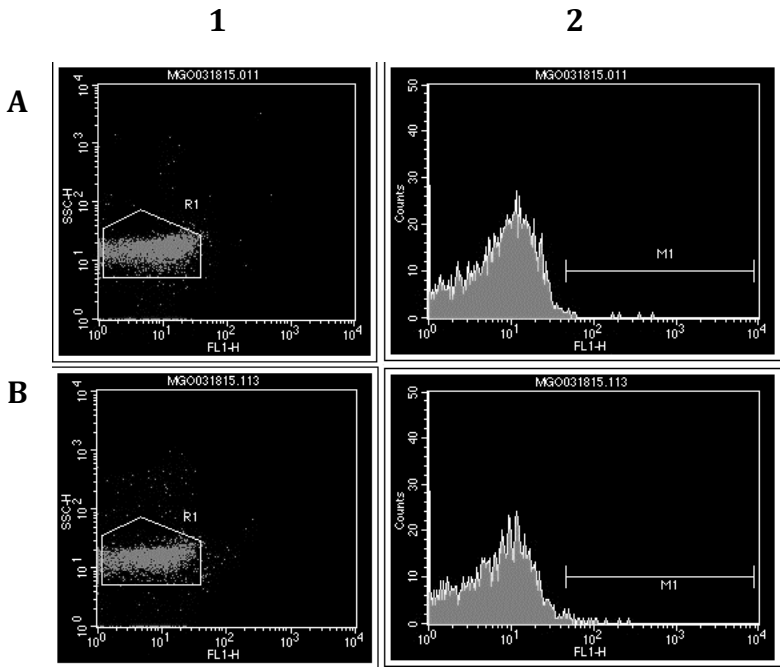

FIG 6 Fluorescence intensity of *B. burgdorferi* 297 after growth in traditional BSK medium and normal serum (panel A) or early Lyme disease serum #4 (panel B). Panel 1 shows dot plots where the side scatter and fluorescence intensity of each organism are depicted. Panel 2 shows histograms where the numbers of spirochetes with identical fluorescence intensity are grouped.

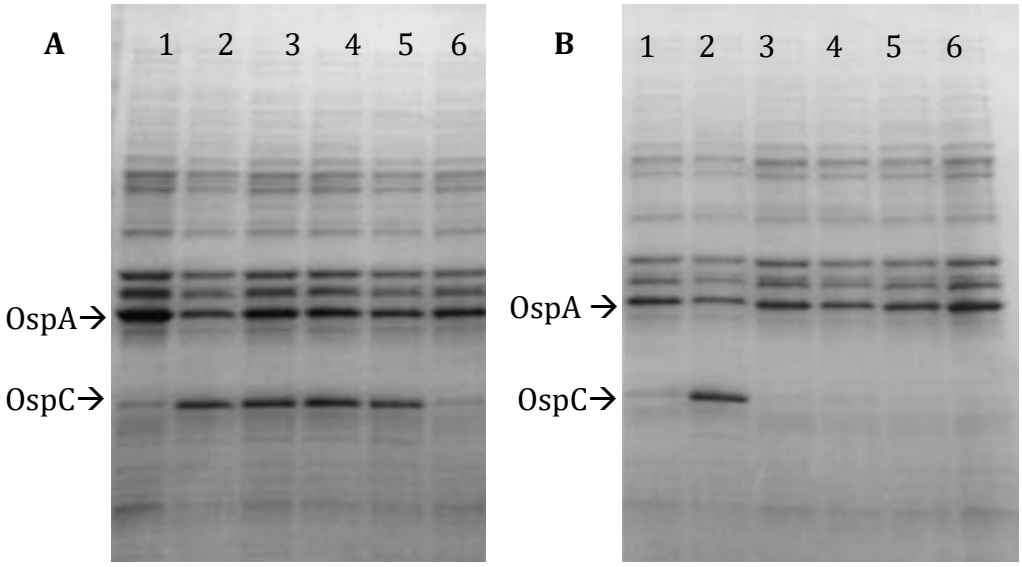

FIG 7 Expression of OspC by *B. burgdorferi* 297 cultured in traditional (pH 7.4) BSK at 33 °C (panel A) or 37 °C (panel B) after 1 passage (lane 1), 3 passages (lane 2), 6 passages (lane 3), 9 passages (lane 4), 12 passages (lane 5) or 15 passages (lane 6).

Effect of pH on OspC expression. I next evaluated the effect of pH by comparing the levels of OspC produced by the passaged spirochetes after they had been inoculated into culture tubes that contained fresh BSK medium adjusted to varying pHs, and then incubated at 33 °C until the spirochetes had reached logarithmic growth phase. The spirochetes in the pH 8.2 medium no longer expressed OspC (Fig. 8). However, the OspC expression by the spirochetes growing in pH 6.6 or 7.0 medium was increased significantly. BSK medium adjusted to a pH lower than 6.6 resulted in death of *B. burgdorferi* 297.

The most significant increase in OspC by *B. burgdorferi* 297 after passage, temperature and pH manipulation was detected at the adjusted growth conditions of passage seven times at 33 °C, followed by transfer into BSK medium at pH 6.6. *Borrelia burgdorferi* 297 grown under these conditions were further analyzed for OspC expression.

Quantitation of OspC expression. A final comparison of the level of OspC expression between the original *B. burgdorferi* 297 spirochetes and the passaged/temperature/pH- adjusted 297 organisms confirmed the increased expression of OspC (Fig. 9). In addition, an ELISA prepared from identical numbers of *B. burgdorferi* 297 organisms that were expressing higher levels of OspC or the *B. burgdorferi* 50772 OspC-expressing control organisms now detected similar amounts of anti-OspC antibodies in the Lyme disease serum (Table 3). Therefore, the combined effect of passage, incubation temperature, and pH was increased expression of OspC to a level similar to the amount produced by *B. burgdorferi* 50772.

Surface accessibility of *B. burgdorferi* 297 OspC after increased expression.

As a final experiment, I evaluated whether the increased expression of OspC also resulted in expression of the Osp on the surface of the spirochetes. In support, a sub-population of *B. burgdorferi* 297 spirochetes now fluoresced significantly after being combined with the Lyme disease serum and FITC-labelled anti-IgM antibodies (Fig. 10), and the increased fluorescence intensity remained detectable even when the serum was diluted 1:81,920. However, there was also a larger population of *B. burgdorferi* 297 spirochetes that failed to bind the anti-OspC7 antibodies. As further proof that OspC was not on the surface of these spirochetes, borreliacidal activity (titer <1:80) was also not detectable when *B. burgdorferi* 297 spirochetes were used with the Lyme disease serum in the borreliacidal antibody test.

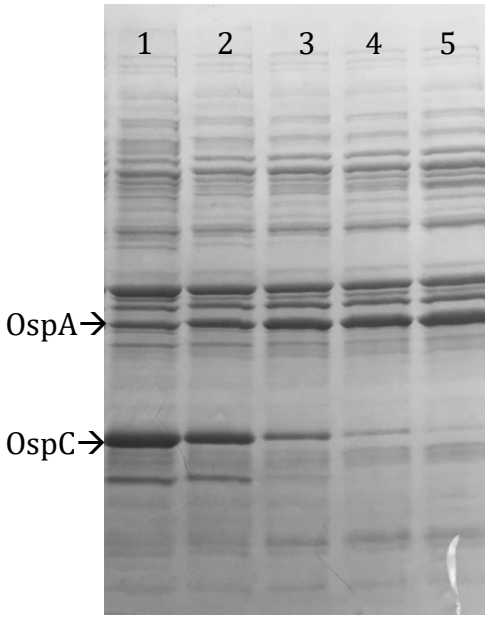

FIG 8 OspC expression by passaged and temperature-adjusted *B. burgdorferi* 297 after culture in fresh BSK medium adjusted to pH 6.6 (lane 1), pH 7.0 (lane 2), pH 7.4 (lane 3), pH 7.8 (lane 4) or pH 8.2 (lane 5).

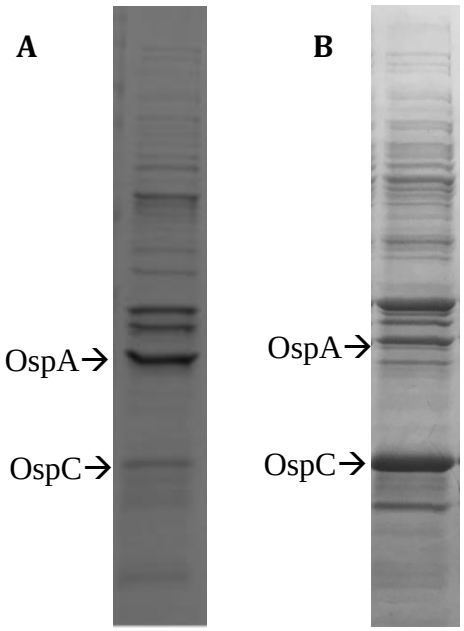

FIG 9 OspC and OspA expression by *B. burgdorferi* 297 by traditional culture methods, low passage growth at 33 °C in BSK medium pH 7.4 (A) and passage/temperature/pH-adjusted *B. burgdorferi* 297 (B) after seven passages at 33 °C and then transferred to BSK medium pH 6.6.

TABLE 3 Detection of anti-OspC antibodies in Lyme disease serum #4 using *B. burgdorferi* 50772, traditional 297 or passage/temperature/pH-adjusted *B. burgdorferi* 297 ELISA.

ELISA	Last dilution with significant[a] reactivity
50772	800
Traditional 297	<50
Passage/temperature/pH-adjusted 297	400

[a] OD ≥0.100 above normal serum control

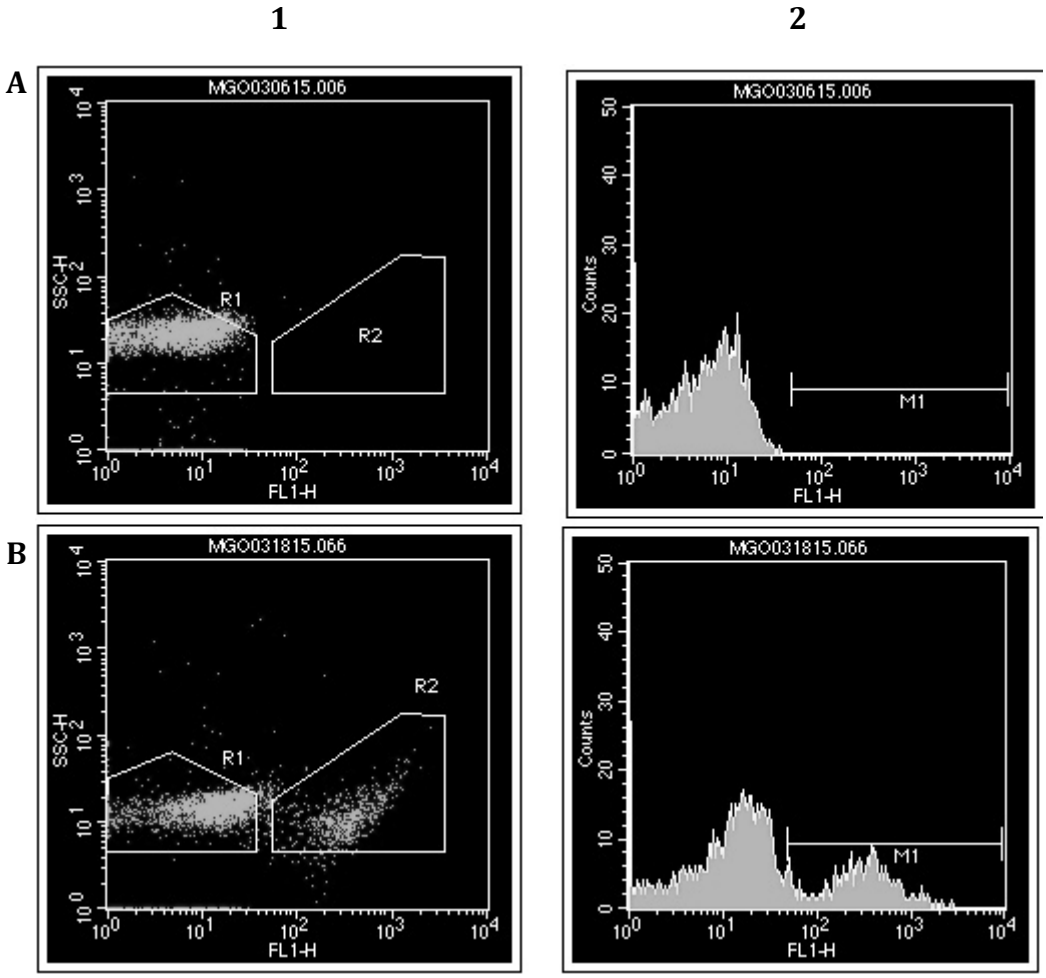

FIG 10 Fluorescence intensity of passage/temperature/pH-adjusted *B. burgdorferi* 297 after combining normal serum (A) or early Lyme disease serum #4 (B) and FITC-labelled anti-human IgM antibodies. Panel 1 shows dot plots where the side scatter and fluorescence intensity of each organism are depicted. Panel 2 shows histograms where the numbers of spirochetes with identical fluorescence intensity are grouped.

DISCUSSION

Lyme disease is the most common vector borne disease in the United States (1) and, while an effective vaccine would be useful for preventing the illness, several problems have hampered evaluation of potential vaccine candidates. For example, OspC-based vaccines offer considerable promise, because the Osp is the immunodominant antigen expressed during early infection (1, 55). In addition, specific epitopes induce protective borreliacidal antibodies (55, 58, 64), and vaccine-induced memory is a possibility. A major drawback, however, is the considerable heterogeneity within *ospC* (43-46), even among isolates from the same endemic focus (43), which must be overcome for an OspC-based vaccine to provide comprehensive protection.

In response, researchers suggested that vaccinating with a chimeric protein comprised of multiple epitopes (51) or a peptide with homology to a highly conserved epitope (55, 56) would overcome this problem. However, additional studies to test these possibilities have not been forthcoming. This is primarily because, in contrast to human infection (50, 55), *Borrelia* spp. spirochetes don't express OspC on the surface when they are grown in traditional laboratory BSK culture medium. In addition, laboratory mice are inappropriate subjects for *in vivo* studies, because the spirochetes don't express OspC during infection (56).

The goal of this study therefore was to manipulate the traditional BSK culture medium and laboratory growth conditions so that infectious *B. burgdorferi* ss 297 were

induced to express more OspC. *Borrelia burgdorferi* 297 organisms were chosen because they were originally recovered from human spinal fluid (1) and a low passage isolate was available in the laboratory. Having a low passage was important, because the organism would be more likely to have maintained the full complement of plasmids necessary for infecting mammals. I therefore first evaluated whether the low passage *B. burgdorferi* 297 organisms expressed OspC after growth in traditional BSK medium, and my findings showed that the spirochetes expressed the Osp, but only in relatively low levels compared to the amount of expressed OspA. In addition, the OspC was not expressed on the surface since it was not susceptible to binding by antibodies specific for the C-terminus of OspC.

However, adjusting the incubation temperature to 33 °C, limiting the number of passages in fresh BSK, and decreasing the pH to 6.6 significantly increased the expression of OspC and, in fact, the level of OspC after these manipulation was similar to the amount expressed by the OspC-expressing *B. burgdorferi* 50772 control organisms (61, 64). The findings therefore supported a previous report (65) that OspC expression differed at varying incubation temperatures and also showed that passage was an important limiting factor. In addition, the results confirmed the previous finding (66) that OspC expression could be increased when the pH of the laboratory medium was adjusted to more closely match the environmental conditions (pH 6.8) encountered during tick feeding.

As an additional possibility, I also hypothesized that the increased production of OspC would result in expression of the Osp on the surface of the 297 spirochetes. This was important because the organisms would then be useful for future *in vitro* and *in vivo*

OspC-based vaccine protection studies. However, only a subpopulation of the OspC-expressing *B. burgdorferi* 297 spirochetes reliably expressed OspC on the surface, and the larger population did not bind the anti-OspC7 antibodies. The small population of OspC-expressing spirochetes would therefore be useful for future studies, but the majority of the spirochetes remained inappropriate. More significantly, the mixed population suggested that the OspC was accessible on the surface of the subpopulation of the spirochetes because these organisms may have lost the plasmid that contained the *ospA/ospB* genes. In support, previous studies (67, 68) have demonstrated that this plasmid is easily lost when the spirochetes are cultured in laboratory growth medium. If this was the case, then the *B. burgdorferi* 297 organisms expressing OspC on the surface were essentially the same as the *B. burgdorferi* 50772 spirochetes, and their use would be limited because the inability to express OspA is typically a characteristic of non-infectious isolates (61). Because of this possibility, additional studies, such as experiments to monitor changes in plasmid profiles, remain necessary.

Additionally, the large numbers of *B. burgdorferi* 297 spirochetes with enhanced OspC expression that still failed to express the Osp on the surface was concerning, especially since the organisms would also not be useful for OspC-based vaccine efficacy studies. A strong possibility for this shortcoming is the concurrent expression of OspA was hindering the ability of the anti-OspC7 antibodies to bind the OspC (56). Therefore, additional studies to refine (decrease) the expression of OspA, so that the dynamics of OspA and OspC expression would more closely match the conditions during human illness, are also critical. One suggestion would be to examine the effects of co-cultivation with tick tissue. In support, Fingerle et al. (69) showed that expression of OspA could be

decreased coincident with increased OspC expression if the Lyme disease spirochetes were co-cultured with a cell line derived from *I. scapularis*. Similarly, Johns et al. (70) reported that adding tick hemolymph to traditional BSK medium caused the expression of OspC to increase significantly.

In summary, OspC-based vaccines offer promise for reducing the incidence of Lyme disease. However, feasibility studies have been hindered significantly by the inability to induce the spirochetes to express OspC correctly in the laboratory. As an important initial step toward overcoming this problem, I show that the expression of OspC can be significantly increased by incubating the spirochetes at 33 °C, limiting the number of passages in fresh BSK medium components, and adjusting the pH of the BSK medium to more closely mimic the environment during tick feeding. Studies to further define additional culture medium or growth requirements to induce OspC expression on the surface of the spirochetes remain necessary, but the modifications highlighted in this investigation should facilitate these efforts.

REFERENCES

1. **Rousselle JC, Callister SM, Schell RF, Lovrich SD, Jobe DA, Marks JA, Wieneke CA**. 1998. Borreliacidal antibody production against outer surface protein C of *Borrelia burgdorferi*. J Infect Dis **178**:733–741.

2. **Steere AC, Malawista SE, Snydman DR, Shope RE, Andiman WA, Ross MR, Steele FM.** 1977. Lyme arthritis: An epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis Rheumatol **20**:7–17.

3. **Steere AC, Schoen RT, Taylor E**. 1987. The clinical evolution of Lyme arthritis. Ann Intern Medicine **107**:725–731.

4. **Tilly K, Rosa PA, Stewart PE**. 2008. Biology of infection with *Borrelia burgdorferi*. Infect Dis Clin North Am **22**:217–234.

5. **Burgdorfer W, Barbour AG, Hayes SF, et al.** 1982. Lyme disease-a tick-borne spirochetosis? Science **216**:1317–1319.

6. **Bacon RM, Kugeler KJ, Mead PS**. 2008. Surveillance for Lyme disease--United States, 1992-2006. Morbid Mortal **57**:1–9.

7. **Castro MB, Wright SA**. 2007. Vertebrate hosts of *Ixodes pacificus* (Acari: *Ixodidae*) in California. J Vector Ecol **32**:140–149.

8. **Lane RS, Mun J, Peribáñez MA, Stubbs HA**. 2007. Host-seeking behavior of *Ixodes pacificus* (Acari: *Ixodidae*) nymphs in relation to environmental parameters in dense-woodland and woodland-grass habitats. J Vector Ecol **32**:342–357.

9. **Pittman W, NP, Emily Taylor**. 2013. Effect of host lizard anemia on host choice and feeding rate of larval western black-legged ticks (*Ixodes pacificus*) – Exp Appl Acarol DOI: 10.1007/s10493-013-9709-3

10. **Steere AC, Coburn J, Glickstein L**. 2004. The emergence of Lyme disease. Clin Investig **113**:1093–1101.

11. **Haugeberg G, Hansen IJW, Skarpaas T, Noraas S, Kjelland V**. 2014. Lyme arthritis in southern Norway--an endemic area for Lyme borreliosis. BMC Infect Dis **14**:185–185.

12. **Platonov AE, Karan LS, Kolyasnikova NM, Makhneva NA, Toporkova MG, Maleev VV, Fish D, Krause PJ**. 2011. Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg Infect Dis **17**:1816–1823.

13. **Movila A, Dubinina HV, Sitnicova N, Bespyatova L, Uspenskaia I, Efremova G, Toderas I, Alekseev AN**. 2014. Comparison of tick-borne microorganism communities in *Ixodes* spp. of the *Ixodes ricinus* species complex at distinct geographical regions. Exp Appl Acarol **63**:65–76.

14. **Reye AL, Hübschen JM, Sausy A, Muller CP**. 2010. Prevalence and seasonality of tick-borne pathogens in questing *Ixodes ricinus* ticks from Luxembourg. Appl Environ Microbiol **76**:2923–2931.

15. **Stanek G, Reiter M**. 2011. The expanding Lyme *Borrelia* complex-clinical significance of genomic species? Clin Microbiol Infect **17**:487–493.

16. **Schutzer SE, Fraser-Liggett CM, Qiu W-G, Kraiczy P, Mongodin EF, Dunn JJ, Luft BJ, Casjens SR**. 2012. Whole-genome sequences of *Borrelia bissettii*, *Borrelia valaisiana*, and *Borrelia spielmanii*. J Bacteriol **194**:545–546.

17. **Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP**. 2005. Diagnosis of Lyme borreliosis. Clin Microbiol Rev **18**:484–509.

18. **Sultan SZ, Manne A, Stewart PE, Bestor A, Rosa PA, Charon NW, Motaleb MA**. 2013. Motility is crucial for the infectious life cycle of *Borrelia burgdorferi*. Infect Immun **81**:2012–2021.

19. **Templeton TJ**. 2004. *Borrelia* outer membrane surface proteins and transmission through the tick. J Exp Med **199**:603–606.

20. **Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb JF, Fleischmann RD, Richardson D, Peterson J, Kerlavage AR, Quackenbush J, Salzberg S, Hanson M, van Vugt R, Palmer N, Adams MD, Gocayne J, Weidman J, Utterback T, Watthey L, McDonald L, Artiach P, Bowman C, Garland S, Fuji C, Cotton MD, Horst K, Roberts K, Hatch B, Smith HO, Venter JC**.1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature **390**:580–586.

21. **Callister SM, Case KL, Agger WA, Schell RF, Johnson RC, Ellingson.** 1990. Effects of bovine serum albumin on the ability of Barbour Stoenner-Kelly medium to detect *Borrelia burgdorferi*. J Clin Microbiol **28**:363-365

22. **Pal U, Yang X, Chen M, Bockenstedt LK, Anderson JF, Flavell RA, Norgard MV, Fikrig E**. 2004. OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. J Clin Invest **113**:220–230.

23. **Battisti JM, Bono JL, Rosa PA, Schrumpf ME, Schwan TG, Policastro PF**. 2008. Outer Surface Protein A protects Lyme disease spirochetes from acquired host immunity in the tick vector. Infect Immun **76**:5228–5237.

24. **Grimm D, Tilly K, Byram R, Stewart PE, Krum JG, Bueschel DM, Schwan TG, Policastro PF, Elias AF, Rosa PA**. 2004. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc Natl Acad Sci U S A **101**:3142–3147.

25. **Skotarczak B**. 2009. Adaptation factors of *Borrelia* for host and vector. Ann Agric Environ Med. **16**:1-8

26. **Lam TT, Nguyen TP, Montgomery RR, Kantor FS, Fikrig E, Flavell RA**. 1994. Outer surface proteins E and F of *Borrelia burgdorferi*, the agent of Lyme disease. Infect Immun **62**:290–298.

27. **Kenedy MR, Akins DR**. 2011. The OspE-related proteins inhibit complement deposition and enhance serum resistance of *Borrelia burgdorferi* the Lyme disease spirochete. Infect Immun. **79**(4): 1451-1457.

28. **Diuk-Wasser MA, Hoen AG, Cislo P, Brinkerhoff R, Hamer SA, Rowland M, Cortinas R, Vourc'h G, Melton F, Hickling GJ, Tsao JI, Bunikis J, Barbour AG, Kitron U, Piesman J, Fish D**. 2012. Human risk of infection with *Borrelia burgdorferi*, the Lyme disease agent, in eastern United States. Am J Trop Med Hyg **86**:320-327.

29. **Craft JE, Fischer DK, Shimamoto GT, Steere AC**. 1986. Antigens of *Borrelia burgdorferi* recognized during Lyme disease-appearance of a new immunoglobulin M response and expansion of the immunoglobulin G response late in the illness. J Clin Invest **78**:934–939.

30. **Dressler F, Whalen JA, Reinhardt BN, Steere AC**. 1993. Western blotting in the serodiagnosis of Lyme disease. J Infect Dis **167**:392–400.

31. **Sigal LH**. 1997. LYME DISEASE: A review of aspects of its immunology and immunopathogenesis. Annu Rev Immunol **15**:63–92.

32. **Hanson MS, Cassatt DR, Guo BP, Patel NK, McCarthy MP, Dorward DW, Höök M**. 1998. Active and passive immunity against *Borrelia burgdorferi* decorin binding protein A (DbpA) protects against infection. Infect Immun **66**:2143–2153.

33. **Kochi SK, Johnson RC, Dalmasso AP**. 1991. Complement-mediated killing of the Lyme disease spirochete *Borrelia burgdorferi*. Role of antibody in formation of an effective membrane attack complex. J Immunol **146**:3964–3970.

34. **O'Rourke M, Traweger A, Lusa L, Stupica D, Maraspin V, Barrett PN, Strle F, Livey I**. 2013. Quantitative detection of *Borrelia burgdorferi* sensu lato in erythema migrans skin lesions using internally controlled duplex real time PCR. PLoS One **8**:e63968–e63968.

35. **Babady NE, Sloan LM, Vetter EA, Patel R, Binnicker MJ**. 2008. Percent positive rate of Lyme real-time polymerase chain reaction in blood, cerebrospinal fluid, synovial fluid, and tissue. Diagn Microbiol Infect Dis **62**:464–466.

36. **Centers for Disease Control and Prevention (CDC).** 1995. Recommendations for test performance and interpretation from the second national conference on serologic diagnosis of Lyme disease. MMWR Morb Mortal Wkly Rep **44**(31):590-591.

37. **Wormser GP, Dattwyler RJ, Shapiro ED, Halperin JJ, Steere AC, Klempner MS, Krause PJ, Bakken JS, Strle F, Stanek G, Bockenstedt L, Fish D, Dumler JS, Nadelman RB**. 2006. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis **43**:1089–1134.

38. **Rahn DW**. 2001. Lyme vaccine: Issues and controversies. Infect Dis Clin N Am **15**:171–187.

39. **Sikand VK, Halsey N, Krause PJ, Sood SK, Geller R, Van Hoecke C, Buscarino C, Parenti D**. 2001. Safety and immunogenicity of a recombinant *Borrelia burgdorferi* outer surface protein A vaccine against Lyme disease in healthy children and adolescents: a randomized controlled trial. JAMA Pediatrics **108**:123–128.

40. **Kowalski TJ, Tata S, Berth W, Mathiason MA, Agger WA**. 2010. Antibiotic treatment duration and long-term outcomes of patients with early Lyme disease from a Lyme disease–hyperendemic area. Clin Infect Dis **50**:512–520.

41. **Dattwyler RJ, Luft BJ, Kunkel MJ, Finkel MF, Wormser GP, Rush TJ, Grunwaldt E, Agger WA, Franklin M, Oswald D, Cockey L, Maladorno D**. 1997. Ceftriaxone compared with doxycycline for the treatment of acute disseminated Lyme disease. N Engl J Med **337**:289–295.

42. **Dattwyler RJ, Wormser GP, Rush TJ, Finkel MF, Schoen RT, Grunwaldt E, Franklin M, Hilton E, Bryant GL, Agger WA, Maladorno D**. 2005. A comparison of two treatment regimens of ceftriaxone in late Lyme disease. Wien Klin Wochenschr **117**:393–397.

43. **Alghaferi MY, Anderson JM, Park J, Auwaerter PG, Aucott JN, Norris DE, Dumler JS**. 2005. *Borrelia burgdorferi* ospC heterogeneity among human and murine isolates from a defined region of northern Maryland and southern Pennsylvania: lack of correlation with invasive and noninvasive genotypes. J Clin Microbiol **43**:1879–1884.

44. **Jauris-Heipke S, Liegl G, Preac-Mursic V, Rossler D, Schwab E, Soutschek E, Will G, Wilske B**. 1995. Molecular analysis of genes encoding outer surface protein C (OspC) of *Borrelia burgdorferi* sensu lato: relationship to *ospA* genotype and evidence of lateral gene exchange of *ospC*. J Clin Microbiol **33**:1860–1866.

45. **Wang IN, Dykhuizen DE, Qiu W, Dunn JJ, Bosler EM, Luft BJ**. 1999. Genetic diversity of ospC in a local population of *Borrelia burgdorferi* sensu stricto. AIMS Genet **151**:15–30.

46. **Lin T, Oliver, Jr. JH, Gao L**. 2002. Genetic diversity of the outer surface protein C gene of southern *Borrelia* isolates and its possible epidemiological, clinical, and pathogenetic implications. J Clin Microbiol **40**:2572–2583.

47. **Scheiblhofer S, Weiss R, Dürnberger H, Mostböck S, Breitenbach M, Livey I, Thalhamer J**. 2003. A DNA vaccine encoding the outer surface protein C from *Borrelia burgdorferi* is able to induce protective immune responses. Microbes Infect **5**:939–946.

48. **Mbow ML, Gilmore RD, Titus RG**. 1999. An OspC-specific monoclonal antibody passively protects mice from tick-transmitted infection by *Borrelia burgdorferi* B31. Infect Immun **67**:5470–5472.

49. **Probert WS, LeFebvre RB**. 1994. Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen. Infect Immun **62**:1920–1926.

50. **Bockenstedt LK, Hodzic E, Feng S, Bourrel KW, Silva A de, Montgomery RR, Fikrig E, Radolf JD, Barthold SW**. 1997. *Borrelia burgdorferi* strain-specific Osp C-mediated immunity in mice. Infect Immun **65**:4661–4667.

51. **Earnhart CG, Marconi RT**. 2007. An octavalent lyme disease vaccine induces antibodies that recognize all incorporated OspC type-specific sequences. Hum Vaccin **3**:281–289.

52. **Earnhart CG, Buckles EL, Marconi RT**. 2007. Development of an OspC-based tetravalent, recombinant, chimeric vaccinogen that elicits bactericidal antibody against diverse Lyme disease spirochete strains. Vaccines **25**:466–480.

53. **Seinost G, Dykhuizen DE, Dattwyler RJ, Golde WT, Dunn JJ, Wang I-N, Wormser GP, Schriefer ME, Luft BJ**. 1999. Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infect Immun **67**:3518–3524.

54. **Earnhart CG, Buckles EL, Dumler JS, Marconi RT**. 2005. Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response. Infect Immun **73**:7869–7877.

55. **Jobe DA, Lovrich SD, Schell RF, Callister SM**. 2003. C-terminal region of outer surface protein C binds borreliacidal antibodies in sera from patients with Lyme disease. Clin Diagn Lab Immunol **10**:573–578.

56. **Lovrich SD, Jobe DA, Schell RF, Callister SM**. 2005. Borreliacidal OspC antibodies specific for a highly conserved epitope are immunodominant in human lyme disease and do not occur in mice or hamsters. Clin Diagn Lab Immunol **12**:746–751.

57. **Kumaran D, Eswaramoorthy S, Luft BJ, Koide S, Dunn JJ, Lawson CL and Swaminathan S.** 2001. Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, *Borrelia burgdorferi*. EMBO J **20**:971-978

58. **Callister SM, Jobe DA, Agger WA, Schell RF, Kowalski TJ, Lovrich SD, Marks JA**. 2002. Ability of the borreliacidal antibody test to confirm Lyme disease in clinical practice. Clin Diagn Lab Immunol **9**:908–912.

59. **Stevenson B, Schwan TG, Rosa PA**. 1995. Temperature-related differential expression of antigens in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect Immun **63**:4535–4539.

60. **Schwan TG, Piesman J, Golde WT, Dolan MC, Rosa PA**. 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc Natl Acad Sci U S A **92**:2909–2913

61. **Anderson JF, Flavell RA Macnarelli LA, Barthold SW, Kanton FS, Wallich R, Persing DH, Mathiesen D, Fikrig E.** 1996. Novel *Borrelia burgdoferi* isolates from *Ixodes scapularis* and *Ixodes dentatus* ticks feeding on humans. J Clin Microbiol **34**(3):524-529.

62. **Barbour AG, Tessier SL, Todd WJ**. 1983. Lyme disease spirochetes and *Ixodid* tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. Infect Immun **41**(2):795-804

63. **Jobe DA, Lovrich SD, Asp KE, Mathiason MA, Albrecht SE, Schell RF, Callister SM**. 2008. Significantly improved accuracy of diagnosis of early Lyme disease by peptide enzyme-linked immunosorbent assay based on the borreliacidal antibody epitope of *Borrelia burgdorferi* OspC. Clin Vaccine Immunol **15**:981–985.

64. **Callister SM, Jobe DA, Schell RF, Pavia CS, Lovrich SD**. 1996. Sensitivity and specificity of the borreliacidal-antibody test during early Lyme disease: a "gold standard"? Clin Diagn Lab Immunol **3**:399–402.

65. **Jutras BL, Chenail AM, Stevenson B**. 2013. Changes in bacterial growth rate govern expression of the *Borrelia burgdorferi* OspC and Erp infection-associated surface proteins. J Bacteriol **195**:757–764.

66. **Yang X, Goldberg MS, Popova TG, Schoeler GB, Wikel SK, Hagman KE, Norgard MV**. 2000. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. Mol Microbiol **37**:1470–1479.

67. **Norris SJ, Carter CJ, Howell JK, Barbour AG**. 1992. Low-passage-associated proteins of *Borrelia burgdorferi* B31: characterization and molecular cloning of OspD, a surface-exposed, plasmid-encoded lipoprotein. Infect Immun **60**:4662–4672.

68. **Schwan TG, Burgdorfer W, Garon CF**. 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of *in vitro* cultivation. Infect Immun **56**:1831–1836.

69. **Fingerle V, Laux H, Munderloh UG, Schulte-Spechtel U, Wilske B**. 2000. Expression of outer surface proteins A and C by individual *Borrelia burgdorferi* in different genospecies. Med Microbiol Immunol **189**:59–66.

70. **Johns RH, Sonenshine DE, Hynes WL**. 2000. Enhancement of OspC expression by *Borrelia burgdorferi* in the presence of tick hemolymph. FEMS Microbiol Letters **193**:137–141.