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Oral Immunization with Recombinant *Lactobacillus plantarum* Induces a Protective Immune Response in Mice with Lyme Disease▽

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Mucosal immunization is advantageous over other routes of antigen delivery because it can induce both mucosal and systemic immune responses. Our goal was to develop a mucosal delivery vehicle based on bacteria generally regarded as safe, such as *Lactobacillus* spp. In this study, we used the Lyme disease mouse model as a proof of concept. We demonstrate that an oral vaccine based on live recombinant *Lactobacillus plantarum* protects mice from tick-transmitted *Borrelia burgdorferi* infection. Our method of expressing vaccine antigens in *L. plantarum* induces both systemic and mucosal immunity after oral administration. This platform technology can be applied to design oral vaccine delivery vehicles against several microbial pathogens.

Lactic acid bacteria are naturally associated with mucosal surfaces, particularly the gastrointestinal tract, and are also indigenous to food-related habitats, including plants, wine, milk, and meat. These gram-positive bacteria include both important pathogens, e.g., several *Streptococcus* species, and extremely valuable nonpathogenic species that have been used since ancient times for food and feed fermentation (11, 37, 58). The host is highly adapted to the presence of commensal intestinal bacteria (36). There is evidence that some strains of lactic acid bacteria have a favorable influence on physiologic and pathological processes of the host due to their specific health-promoting probiotic characteristics that relate to modulation of the immune system (15, 36, 41). Some strains of lactic acid bacteria polarize the naïve immune system by skewing it toward Th1 responses (41, 56).

There have been a number of reports of oral vaccine candidates established from genetically modified pathogenic bacteria, such as *Salmonella* and *Listeria* species (1, 2, 30, 45, 52), or commensal bacteria, such as *Lactococcus lactis* and *Lactobacillus* species (27, 31, 32, 60). The latter are food-grade bacteria that have GRAS status (*g*enerally *r*egarded *a*s *s*afe). While both pathogenic and commensal bacteria have advantages and disadvantages as mucosal delivery vehicles, lactic acid bacteria are preferable in terms of safety and a lower risk of side effects (27, 47). Presentation of antigens on the surface of lactobacilli is attractive for vaccine design, especially because the peptidoglycan layer of some strains appears to exhibit natural immuno-adjuvanticity (33, 35, 44, 46). Thus, these species are excellent candidates for the development of safe mucosal delivery vehicles of prophylactic and therapeutic molecules. Of the *Lactobacillus* strains previously used for vaccine delivery, we chose *L. plantarum* because there is evidence that this strain is a better agent for vaccination with tetanus toxin fragment C (TTFC) than *L. casei* or *L. lactis* (22, 53).

Lyme disease is the most prevalent vector-borne infectious disease in the United States. A vaccine administered via needle inoculation and based on outer surface protein A (OspA) has proved effective in preventing *Borrelia burgdorferi* infection in animals and humans (7, 10, 16–18, 55). Although a controversial autoantigenic epitope identified in OspA (8, 24) hampered its acceptance for human use, a second-generation OspA-based subcutaneous vaccine has been developed (59). This vaccine works in an unconventional manner. As *B. burgdorferi* expresses OspA mostly at lower temperatures while undergoing the tick stage of its enzootic cycle (49, 50), OspA-specific antibodies in the serum from vaccinated mice block or neutralize *B. burgdorferi* in the tick midgut, thereby preventing its transmission to the mouse upon tick feeding (10).

We used the Lyme disease mouse model to develop a live oral vaccine based on recombinant *Lactobacillus plantarum*. In order to determine vaccine efficacy we challenged vaccinated mice with infected field ticks, which are the natural vectors of infection.

MATERIALS AND METHODS

Construction and characterization of vaccine candidates. The antigen was cloned in an expression vector, pLAC613. This vector has the replication region of pSH71 (*repA* and *repC*) and the chloramphenicol acetyltransferase gene (*cat*) from pC194 for selection purposes. A detailed description of the plasmid features can be found in reference 47. Based on the coding sequence of *Borrelia burgdorferi* OspA, primers were designed with SphI and BamHI restriction sites that allowed for direct cloning of the gene under the inducible Pxyl promoter. The vector was transformed into *Lactobacillus plantarum* strain 256, and protein expression was checked by immunoblotting as follows: recombinant *L. plantarum* was mechanically broken with a French press (SLM-Aminco Instruments), and supernatants were run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and electrotransferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) for analysis with OspA-specific monoclonal antibodies LA2.2 and 336.1.

Preparation of vaccine antigen. *L. plantarum* expressing the target antigen was cultured in Lactobacillus medium (1% proteose peptone [wt/vol], 1% beef extract [wt/vol], 0.5% yeast extract [wt/vol], 0.5% lactose [wt/vol], 9 mM ammonium citrate, 61 mM sodium acetate anhydrous, 0.4 mM magnesium sulfate, 0.3 mM manganese sulfate, 11.2 mM dipotassium phosphate, 0.5% Tween 20 [vol/vol]), supplemented with 10 μg/ml of chloramphenicol (Cm) and 0.5% lactose and grown at 30°C to an optical density at 600 nm (OD_{600}) of 1.0. That is the

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equivalent of 1×10^9 cells/ml, corresponding to approximately 125 μg of total protein. The cells were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C and resuspended in 10% glycerol–phosphate-buffered salt solution (Gibco, Grand Island, NY) in 10% of the initial volume. Cell suspensions in aliquots of 2 ml were quickly frozen in a dry ice bath and stored at −80°C. Aliquots were thawed at 4°C, and 400 μl (4×10^{10} cells) was placed in a ball-tipped syringe for oral gavage inoculation.

Immunization regimen. Three groups of four female C3H-HeJ mice (6 to 8 weeks old; Charles River, Boston, MA) were immunized by intragastric inoculation of 4×10^{10} OspA-expressing lactobacilli (LpA antigen), OspAα (LpAα antigen), or the control, *L. plantarum* carrying the empty vector pLAC613 (Lp antigen). A total of three independent experiments were performed. Mice received the first immunization (priming), twice daily, for 8 days (days 1 to 4 and 8 to 11). After resting for 2 weeks the mice were bled (day 28), and on days 30 to 33 they received the first oral boost. On day 45, they were bled for the second time, and on days 52 to 55 they received the second boost. On day 64, mice were bled for the third time. Serum was tested by indirect enzyme-linked immunosorbent assay (ELISA) for the presence of immunoglobulin G (IgG) to OspA. Stool samples for determination of anti-OspA IgA were collected on the same days as the serum. Challenge was performed on day 67, via *B. burgdorferi*-infected *Ixodes scapularis* nymph inoculation. One month later (day 97), mice were euthanized and blood, heart, and bladder tissues were obtained to assess protection or spirochete dissemination.

Enumeration of viable lactobacilli in the gut. Approximately 100 mg of the luminal contents was placed in tubes containing 1 ml of 1% bovine serum albumin (BSA) in phosphate-buffered saline with protein inhibitor mixture (Roche, NJ) for homogenization by vortexing. Numbers of viable lactobacilli in stool were determined by plating serial dilutions of the suspension on MRS/Cm^+ agar (Oxoid, Cambridge, United Kingdom) followed by incubation for 3 days at 37°C. The number of colonies was counted as CFU per gram of luminal contents. Presence of the *ospA* gene in the *Lactobacillus* colonies was confirmed by PCR.

Challenge with *B. burgdorferi*-infected field ticks. *Ixodes scapularis* ticks that were collected in areas where Lyme disease is endemic (from New York state; ticks were kindly provided by D. Brisson and R. S. Ostfeld) and maintained in our laboratory (21) were checked for *B. burgdorferi* infection by PCR. Tick challenge of mice was performed as follows: we placed 8 to 10 *B. burgdorferi*-infected nymphal field ticks on the back of the mouse heads and restrained the mice for 2 h to allow enough time for ticks to attach. Three days later, ticks that were engorged after taking a blood meal were collected after naturally falling off and counted, and a daily record was kept for each mouse.

Mucosal and systemic immune responses. (i) Extraction of mucosal IgA antibodies. OspA-specific IgA antibodies were extracted from stool pellets. Briefly, 100 mg of stool was dissolved in 1 ml of phosphate-buffered saline–1% BSA (Sigma) supplemented with protease inhibitor cocktail (Complete; Roche, Germany). The suspension was mixed vigorously and incubated for 16 h at 4°C and then centrifuged at 16,000 × *g* to remove insoluble material.

(ii) Antibody assays. Purified recombinant lipidated OspA was used to coat Nunc MaxiSorp flat-bottom ELISA plates (eBioscience, San Diego, CA), and an indirect ELISA was performed using either extracted stool samples (undiluted) or serum (1:400) from immunized mice to identify OspA-specific IgA or IgG antibodies, respectively. We used as secondary antibodies anti-mouse IgA conjugated to horseradish peroxidase or anti-mouse IgG conjugated to alkaline phosphatase (1:1,600; Jackson ImmunoResearch, West Grove, PA). To further characterize the IgG response, subclass isotyping was done in serum from immunized mice by capture ELISA, using the mouse IgG1, IgG2a, and IgG2b ELISA quantitation kit (Bethyl Laboratories Inc., Montgomery, TX) according to the manufacturer's instructions. We checked for anti-*B. burgdorferi* antibodies in serum from immunized mice (1:100) by immunoblotting (Virablot; Plannegg, Germany). We considered a pattern of 5 out of 10 bands positive as preliminary evidence of infection. Immunized mice showing a single OspA band were considered potentially protected.

Determination of vaccine efficacy: assessment of *B. burgdorferi* dissemination. Mice orally immunized with recombinant *L. plantarum* were challenged with *B. burgdorferi*-infected field ticks. One month after challenge, mice were sacrificed and spirochete dissemination was detected by immunoblotting of serum against whole-cell sonicate of *B. burgdorferi* (Virablot) and by culture of *B. burgdorferi* from heart and bladder tissues in BSK-H medium with an antibiotic mixture for *Borrelia* (Sigma) at 34°C for up to 6 weeks. Cultures were checked for the presence of spirochetes by dark-field microscopy, and results were confirmed by PCR amplification of the *B. burgdorferi ospC* gene from DNA extracted from the same tissues.

Statistics. Student's *t* test and McNemar's exact test for correlated proportions were used to analyze differences between immunized and control groups. *P* values of <0.05 are considered statistically significant.

RESULTS

Construction and characterization of the vaccine candidates. We developed two constructs for our vaccine study. For wild-type OspA-expressing *L. plantarum* (LpA antigen), full-length *ospA* was subcloned from a plasmid kindly provided to us by John Dunn (Brookhaven National Laboratory). The second vaccine candidate (LpAα antigen) was constructed by replacing the controversial autoantigen sequence in *ospA* (Fig. 1A). In order to do this substitution we had to consider the maintenance of charge parity. Changing T170 to K would require changing V179 to E on the next β-strand. To address both issues we replaced residues 161 to 190 in OspA from *B. burgdorferi* with the analogous region from a nonarthritogenic European species (*Borrelia afzelii*) that had these compensation changes. To further stabilize the C terminus of the mutant OspA molecule, which is extremely important in inducing a protective immune response, we replaced it with the analogous C-terminal sequence from the same species used to do the previous substitution. This construct, comprised of OspA $B31_{1–164}PGau_{165–189}B31_{190–218}Pko_{219–273}$ was then used to generate *L. plantarum* expressing the mutant OspA (LpAα). Cloned genes were confirmed by sequencing. To evaluate protein expression, recombinant *L. plantarum* clones were evaluated by immunoblotting, using anti-OspA monoclonal antibodies. As expected, monoclonal antibody LA2.2 recognized the C-terminal sequence of *B. burgdorferi* OspA in LpA and monoclonal antibody 336.1 recognized the C-terminal sequence of *B. afzelii* OspA in LpAα, in contrast to the control (Fig. 1B).

Enumeration of viable lactobacilli in the gut after immunization. Throughout the immunization regimen, stool samples were collected on days 0, 2, 4, 7, 9, 11, 14, 28, 30, 32, 34, 49, 51, 53, and 55 to determine the viability of *L. plantarum* and its ability to colonize the digestive tract. Suspensions of stools collected from mice immunized with LpA or LpAα contained on average 10^6 cells/g of luminal contents, in contrast to the control. The number of *L. plantarum* able to grow on MRS/Cm^+ agar increased within 24 h after the first inoculation and decreased 48 h after the last inoculation (Fig. 2). PCRs done to confirm the presence of *ospA* in recombinant *L. plantarum* colonies were positive only for LpA or LpAα.

Mucosal and systemic immune responses after oral immunization. To assess the mucosal and systemic immune response induced by the oral vaccine, we tested stool and serum levels of total OspA-specific IgA and IgG antibodies, respectively, by indirect ELISA (Fig. 3). Mice vaccinated orally with *L. plantarum* expressing both wild-type and mutant OspA had detectable anti-OspA IgA and IgG antibodies 4 weeks postimmunization (day 28). As a result of boosting, this response increased considerably from day 28 until day 64. Results for determination of OspA-specific IgA antibody in gut luminal material and IgG antibody in serum on day 64, 3 days before tick challenge, are shown in Fig. 3A and B, respectively. All vaccinated mice showed a significant difference ($P < 0.0001$) in OspA-specific IgA and IgG antibody titers in comparison to the control,

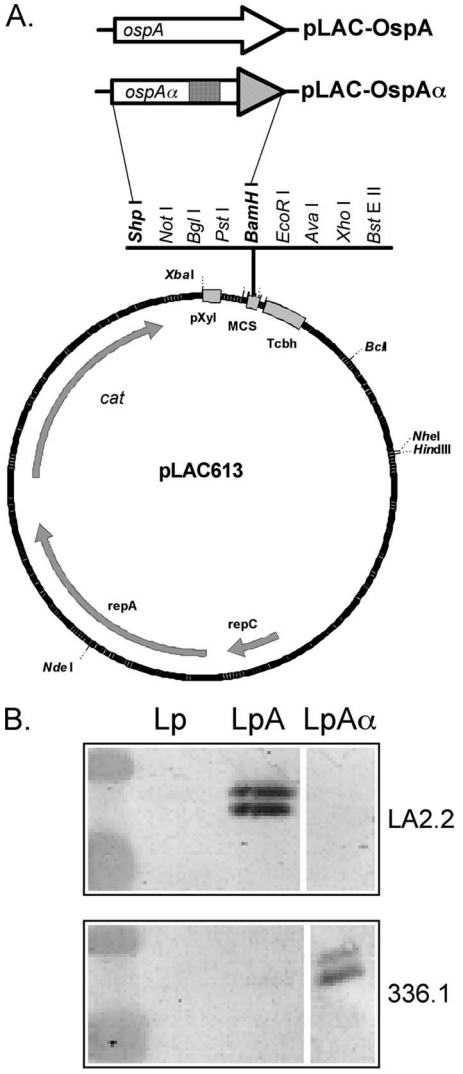

FIG. 1. Graphical representation and characterization of the vaccine constructs, showing cloning design of the vaccine candidates (A). The vector pLAC613 comprises the replication region of pSH71 (*repA* and *repC*) and the *cat* gene from pC194 for selection purposes. OspA (pLAC-OspA) and OspAα (pLAC-OspAα) were cloned in the SphI and BamHI sites, using primers with the appropriate restriction sites. Expression of recombinant proteins by *L. plantarum* was confirmed by immunoblot assay (B). Whole-cell extracts of control, OspA-, and OspAα-expressing *L. plantarum* (Lp, LpA, and LpAα antigens) were run on a 15% sodium dodecyl sulfate-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and tested by immunoblotting with OspA-specific monoclonal antibodies LA2.2 and 336.1.

except for one mouse immunized with mutant LpAα that did not have significant levels of IgA.

We determined the OspA-specific antibody isotype distribution with a capture ELISA by testing threefold serial dilutions of serum collected on day 64. Oral immunization with *L. plantarum* expressing wild-type OspA (LpA) resulted in equivalent levels of the three IgG subclasses with a bias toward IgG2a production, and oral immunization with the mutant OspA (LpAα) resulted in an IgG subclass distribution biased toward IgG2a and IgG1 (Fig. 4).

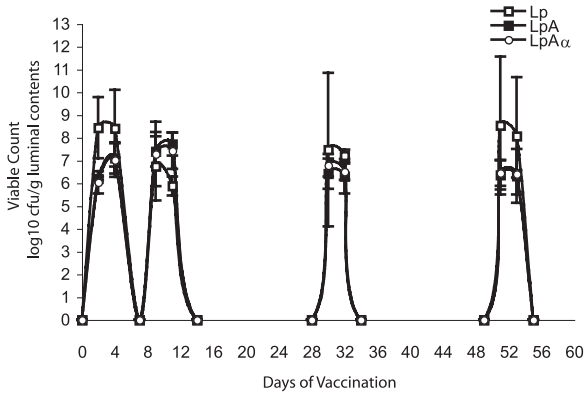

FIG. 2. Recombinant *L. plantarum* does not colonize the gut. Four mice per group were immunized by intragastric inoculation of recombinant OspA- and OspAα-expressing *L. plantarum* (LpA or LpAα) or with *L. plantarum* carrying the empty vector pLAC613 (Lp antigen). Stool samples were collected 24 h before and during and after vaccine inoculations and plated on MRS/Cm^+ agar to screen for recombinant *L. plantarum*. The results are expressed as the mean $\log_{10}$-transformed CFU/g of luminal content, and the error bars are standard errors of the means.

Evaluation of vaccine efficacy. Next, we wanted to determine if the systemic anti-OspA immune response elicited by this oral vaccine could protect mice from *B. burgdorferi* infection in vivo. In vaccinated mice, protection or infection was determined by the absence or presence of *B. burgdorferi* dissemination, respectively, after challenge by infestation with *I. scapularis* nymphs carrying *B. burgdorferi* (in vivo correlate of natural infection). The infection rate of the laboratory-maintained field ticks used for challenge was determined at 80% by PCR. A total of 36 mice were immunized orally with *L. plantarum* expressing either the wild-type antigen (LpA; $n = 12$) or the mutant antigen (LpAα; $n = 12$) and the parental strain (Lp antigen; $n = 12$). Results from one of three independent experiments are shown in Fig. 5. Data from three experiments are summarized in Table 1. In the groups orally immunized with either the wild-type or the mutant vaccine (LpA or LpAα), we observed that all mice were free of spirochetes as determined by immunoblotting (Fig. 5A) and by dark-field microscopy analysis of *B. burgdorferi* cultures from tissues (Fig. 5B). Culture results were confirmed by PCR (Fig. 5C). None of the mice in the control group developed antibodies to OspA, and all had an immunoblot profile indicative of *B. burgdorferi* dissemination that was confirmed both by culture and PCR.

Taken together, these results demonstrate that oral immunization with *L. plantarum* expressing either wild-type or mutant OspA resulted in an OspA-specific seroconversion that protected vaccinated mice from *B. burgdorferi* infection. Differences between the control and the wild-type or mutant vaccines were statistically significant by McNemar's exact test for correlated proportions ($P < 0.05$) (Table 1). The percentage of infected mice observed in the group of control mice also showed that the number of infected ticks used to challenge mice was appropriate and effective in transmitting *B. burgdorferi* and therefore validated the results observed in the vaccinated groups.

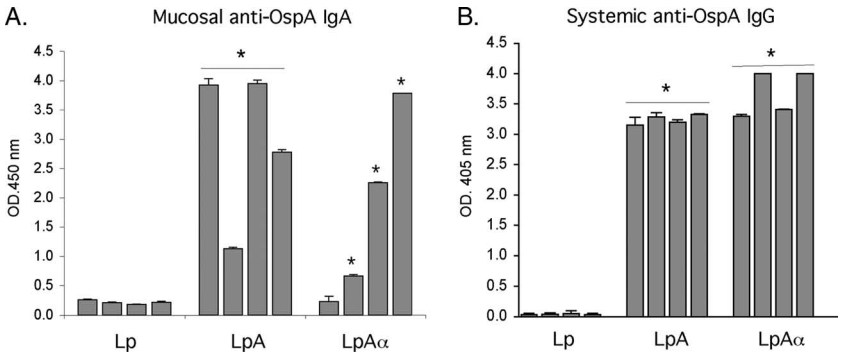

FIG. 3. Oral immunization with OspA-expressing *L. plantarum* induces a mucosal and systemic anti-OspA immune response. Four mice per group were immunized by intragastric inoculation with recombinant OspA- or OspAα-expressing *L. plantarum* (LpA or LpAα) or *L. plantarum* carrying the empty vector pLAC613 (Lp antigen). On day 64, stool and serum samples were collected and OspA-specific IgA (A) and IgG (B) antibodies were determined by ELISA, respectively. The results are expressed as the OD at 450 nm (IgA) or 405 nm (IgG) of the mean end point titer. The average of triplicate samples per mouse was determined, and the error bars indicates standard deviations. Cutoff values are the averages of the negative control plus 3 standard deviations. Differences between vaccinated and control responses were statistically significant ($P < 0.0001$) as determined with Student's *t* test. Results are representative of one of three independent experiments.

DISCUSSION

The main goal of this study was to develop a platform delivery system using safe lactic acid bacteria to design oral vaccines against a number of maladies that affect humans. In this report we describe the development of an effective, recombinant *Lactobacillus*-based, live oral vaccine against a vector-borne infectious disease.

Mucosal immunization has several advantages over other routes of antigen delivery, including convenience, cost-effectiveness, and more importantly, induction of both local and systemic immune responses (38, 51, 52). Mucosal vaccines using commensal bacteria rely on its endogenous regulation being transferred to the vaccine antigen (40). Vaccines based on attenuated bacterial pathogens, especially live *Salmonella*-based oral vaccines, have demonstrated the ability to induce protective mucosal and systemic immune responses (6, 14, 52, 61, 63). However, public fear of using an attenuated pathogen as a vaccine carrier has deterred its acceptance. Recent studies have shown that oral administration of a number of lactobacilli expressing recombinant immunogens induces local mucosal (42) and systemic antibody responses (62). Other studies have explored this further and showed that immune responses induced via lactobacilli delivered through oral vaccination confer some protection against challenge with the respective pathogen (27, 31, 32, 60). Because of its GRAS status, a live vaccine based on *Lactobacillus* will be more readily acceptable to the public than an oral vaccine based on a well-recognized pathogen.

We chose *L. plantarum* because it is a better agent for oral vaccination than *L. casei* or *L. lactis* (22, 53) due to its higher intrinsic antigenicity. Data that support these findings were recently reported from a study which analyzed the differences between these species on immunization and found that *L. plantarum* was 10-fold more immunogenic (9). Furthermore, *L. plantarum* can activate human myeloid dendritic cells through upregulation of costimulatory molecules (CD40) on the cell surface (3, 4, 28, 57).

L. plantarum survives gastrointestinal passage, and its transit time was monitored in mice that received the vaccine throughout the immunization regimen. Transit dynamics of *L. plantarum* revealed that the number of live bacteria in mouse luminal contents increases 2 h after the first inoculation, reaches its highest level 2 h later, and returns to preinoculation levels within 24 h (39, 43). In addition, evidence has accumulated that suggests that more doses are required to obtain efficient priming and boosting of antibody responses via the intragastric route than via the intranasal route of administration (58). Thus, we designed an immunization regimen that included inoculating the mice twice a day for a longer priming period to allow enough time for the immunogen to be presented to the intestinal immune system. This strain of *Lactobacillus plantarum* did not colonize the gut for extended periods of time. Stimulation of the intestinal immune system with the vaccine antigen merely during our predetermined immunization timetable seems to be a crucial factor in the induction of a protective systemic immune response rather than induction of oral tolerance. Thus, we believe that both the immunization schedule and dose administered contribute to the outstanding efficacy of this vaccine.

In mouse models, IgG2a and IgG2b are induced primarily by Th1 cytokines, while IgG1 is induced by Th2 cytokines. Studies done with parenteral and mucosal administration of TTFC vaccines have shown that the route of vaccination determines the immune response phenotype. TTFC vaccine antigen injected with alum induces an immune response dominated by an IgG1/Th2 response, whereas mucosal delivery of TTFC-expressing *L. plantarum* induces both the IgG1 and IgG2a subclasses of antibody to TTFC (22, 23, 58). Similarly, OspA antigen administered subcutaneously, such as the previous human Lyme disease vaccine, induces equivalent levels of the three IgG subclasses with a bias toward IgG1 (59). In this study, we found that oral immunization with *L. plantarum* expressing wild-type OspA (LpA) induced primarily IgG2a, a Th1-driven immune response, and immunization with the mutant induced IgG2a/IgG1, a mixed Th1/Th2-driven immune response. Thus, we too determined that *Lactobacillus* vaccines promote a mixed Th-cell response. The ability of *Lactobacillus* spp. to shift cellular immune responses toward Th1 may be

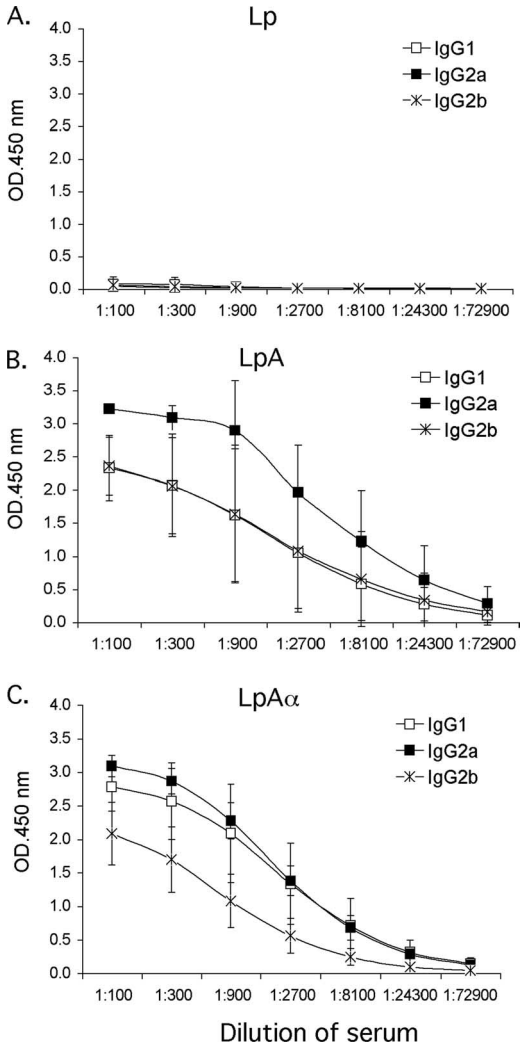

FIG. 4. OspA-expressing *L. plantarum* induces a systemic IgG response with a subclass distribution skewed toward IgG2a, and OspAα-expressing *L. plantarum* induces IgG2a and IgG1. We determined the OspA-specific antibody isotype distribution by testing threefold serial dilutions of sera collected on day 64 from mice immunized with OspA- or OspAα-expressing *L. plantarum* (LpA or LpAα) or *L. plantarum* carrying the empty vector, pLAC613 (Lp antigen), by ELISA. Horseradish peroxidase-labeled secondary antibody was used. The results are expressed as the OD at 450 nm of the mean end point titer. The average of triplicate samples from four mice is represented (each data point), and standard errors of the means were determined. Results are representative of one of three independent experiments.

advantageous for vaccination strategies, as was recently shown in a model of pneumococcal infection (26). *Lactobacillus* species have several components that elicit innate immune responses through molecular pattern recognition receptors of mammalian cells, such as peptidoglycan, lipoteichoic acids, and bacterial oligodeoxynucleotides (29, 54). Furthermore, there is evidence that some *Lactobacillus* strains per se can have an adjuvant effect (27). Our data support the finding that the bacterial vehicle itself can influence the immune response due to its immunomodulatory properties. All together these data suggest that IgG class switching is determined by the OspA antigen, by the delivery system, and by the route of vaccination.

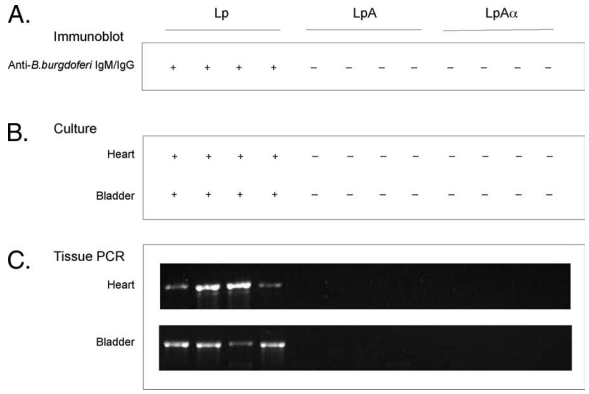

FIG. 5. *B. burgdorferi* does not infect mice immunized orally with *L. plantarum* expressing OspA. Immunized mice were sacrificed 1 month after tick challenge, and blood and tissues were collected to assess spirochete dissemination. Serum was tested against whole-cell extract of *B. burgdorferi* by immunoblotting using the Virablot test (A). Heart and bladder cultures were checked for the presence of spirochetes by dark-field microscopy (B), and results were confirmed by PCR of the same tissues (C). Results are representative of one of three independent experiments.

Because of our extensive experience developing immunoprophylactic agents for *B. burgdorferi*, we used the Lyme disease mouse model as a proof of concept. OspA was the immunogen of choice because it is a proven vaccine candidate against *B. burgdorferi* via the parenteral route (16, 18) in addition to inducing a protective immune response when administered orally as purified antigen (34) or as recombinant *Escherichia coli* (17, 21). Although there have been many studies to find additional vaccine candidates for Lyme disease (5, 12, 13, 19, 20, 25, 48), OspA is still the most effective immunogen against this pathogen. Following the discovery of a controversial autoantigenic epitope in OspA (24), we substituted this epitope in its sequence before cloning it into *L. plantarum*. In this study, we demonstrated that orally delivered, live *L. plantarum* expressing either wild-type OspA or its mutant are equally effective in blocking transmission of *B. burgdorferi* in the tick, thereby preventing infection. Neutralization of *B. burgdorferi* within the tick is dependent on OspA-specific IgG present in the serum of orally vaccinated mice, and intestinal OspA-specific IgA responses to recombinant *L. plantarum* could be a controlling factor in the uptake of bacteria and the ability of luminal bacteria to interact with the systemic immune system.

We report the development of an oral, live vaccine delivery vehicle based on a bacterium generally regarded as safe by the FDA. Our method of expressing vaccine antigens in *L. plantarum* induces both systemic and mucosal immunity after oral administration. Standard parenteral vaccines do not induce mucosal immunity, and the failure to do so has led to vaccine failures even in the face of strong systemic immunity. Our platform technology, in addition to the effective oral vaccine that has been described here for Lyme disease, can be expanded upon and applied to design oral vaccines against several microbial pathogens and possibly some allergens. Some examples of potential mucosal vaccines include targets to *Yersinia pestis*, *Bacillus anthracis*, and *Francisella tularensis*. These protection strategies for airborne, category A bioterrorism

TABLE 1. Overall efficacy of an oral anti-*B. burgdorferi* vaccine based on *L. plantarum*[a]

Antigen	No. of mice	No. of mice positive by test method/total no. tested						
		OspA ELISA	Immunoblot assay	Culture		Tissue PCR		Overall (%I/P)[b]
				Heart	Bladder	Heart	Bladder	
Lp	12	0/12	11/11	10/11	10/11	4/4	4/4	11/11 (100/0)
LpA	12	12/12	0/11	0/11	0/11	0/4	0/4	0/11 (0/100)*
LpAα	12	12/12	0/12	0/12	0/12	0/4	0/4	0/12 (0/100)*

[a] Antigens administered were derived from *L. plantarum* control (Lp), *L. plantarum* expressing OspA (LpA), or *L. plantarum* expressing OspAα (LpAα). Discrepancies in numbers of mice per group for the various assays are due to anesthesia-related deaths, except for tissue PCR.
[b] %I/P, percent infected/percent protected. *, significantly different ($P < 0.05$) from Lp control, by McNemar's exact test for correlated proportions.

agents would greatly benefit from a mucosal and systemic double-edged immune response. Other examples include respiratory syncytial virus, enteropathogenic *Escherichia coli*, and malaria vaccines.

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