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Nasal Immunization with *Lactococcus lactis* Expressing the Pneumococcal Protective Protein A Induces Protective Immunity in Mice[▽]

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Nisin-controlled gene expression was used to develop a recombinant strain of *Lactococcus lactis* that is able to express the pneumococcal protective protein A (PppA) on its surface. Immunodetection assays confirmed that after the induction with nisin, the PppA antigen was predictably and efficiently displayed on the cell surface of the recombinant strain, which was termed *L. lactis* PppA. The production of mucosal and systemically specific antibodies in adult and young mice was evaluated after mice were nasally immunized with *L. lactis* PppA. Immunoglobulin M (IgM), IgG, and IgA anti-PppA antibodies were detected in the serum and bronchoalveolar lavage fluid of adult and young mice, which showed that PppA expressed in *L. lactis* was able to induce a strong mucosal and systemic immune response. Challenge survival experiments demonstrated that immunization with *L. lactis* PppA was able to increase resistance to systemic and respiratory infection with different pneumococcal serotypes, and passive immunization assays of naïve young mice demonstrated a direct correlation between anti-PppA antibodies and protection. The results presented in this study demonstrate three major characteristics of the effectiveness of nasal immunization with PppA expressed as a protein anchored to the cell wall of *L. lactis*: it elicited cross-protective immunity against different pneumococcal serotypes, it afforded protection against both systemic and respiratory challenges, and it induced protective immunity in mice of different ages.

Streptococcus pneumoniae is a common cause of invasive disease and respiratory tract infections in developed and developing countries. Risk groups for diseases caused by pneumococci include children in their first few years of life, elderly people, and patients with immunodeficiencies (7, 8). Because children under 2 years of age have an incompletely developed anatomy and an immature immune system, they are particularly vulnerable to pneumococcal infection by these bacteria (11). The rapid emergence of multidrug-resistant *S. pneumoniae* strains throughout the world has led to an increased emphasis on the prevention of pneumococcal infections by vaccination (19). However, available vaccines present disadvantages associated with their low immunogenicity in populations at risk (i.e., the pneumococcal 23-valent polysaccharide vaccine) or with their high cost as a public health strategy in developing countries (i.e., conjugated vaccine) (6, 7). Some pneumococcal surface proteins are serotype independent and represent a promising alternative for the design of a vaccine (9, 31). However, it seems probable that several pneumococcal proteins are necessary for the production of an effective vaccine against all serotypes. Thus, worldwide research on this subject is focused on the search for possible additional candidates that are antigenically conserved and that elicit antibodies that reduce colonization or protect against systemic disease or both. A novel pneumococcal surface protein, identified as pneumococcal protective protein A (PppA), has been described (15). This protein is antigenically conserved among different serotype strains of *S. pneumoniae*, and it has been reported that nasal immunization of adult mice with recombinant PppA administered with mucosal adjuvants elicits antibodies that are effective in reducing pneumococcal nasal colonization.

Lactic acid bacteria (LAB) are promising candidates as safe vehicles for the in vivo delivery of antigens. Compared with attenuated bacterial vectors, LAB are nonpathogenic and noninvasive gram-positive organisms that are generally recognized as safe. The best-known lactic acid bacterium, *Lactococcus lactis*, has been extensively engineered for the production of heterologous proteins (for a review see reference 20). Recombinant *L. lactis* expressing heterologous antigens has been used successfully to elicit an immune response against bacterial (10, 26) or viral antigens (12). In this sense, there are reports of pneumococcal antigens expressed by recombinant LAB which have been used to improve resistance against *S. pneumoniae* infection (2, 16, 29). In these experiments, nasal immunization was used to evaluate the efficacy of the experimental vaccines,

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TABLE 1. Strains of *Streptococcus pneumoniae* used in this study

S. pneumoniae strain	Capsule type	Source	Reference
T14	14	Blood	26
AV3	3	CSF[a]	This work
AV6	6B	Blood	This work
AV14	14	Blood	This work
AV23	23F	CSF	This work

[a] CSF, cerebrospinal fluid.

since it has been demonstrated that nasal administration of antigens is an efficient route with which to elicit protective immunity in both the mucosal and the systemic immune compartments. However, immunization challenge experiments were performed with adult mice, despite the fact that young individuals are more susceptible to pneumococcal infection. Moreover, those authors used pneumococcal serotypes which are not the most common serotypes in our country (22), and the efficacy of the experimental vaccines against different pneumococcal serotypes was not evaluated. Thus, in the present work, we carried out experiments better suited to our local conditions: we expressed in *L. lactis* NZ9000 a recently characterized antigen, PppA, and assessed its efficacy to induce local and systemic immune responses in mice of different ages. In addition, we determined whether the mucosal administration of the recombinant bacteria increased resistance to systemic and mucosal infections caused by the main *S. pneumoniae* serotypes found in our country (22).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Serotype 14 *Streptococcus pneumoniae* T14 (Table 1) was isolated from the blood of a patient at the Niño Jesús Children's Hospital in Tucumán, Argentina, and serotyped at the Administración Nacional de Laboratorios e Institutos de Salud, Buenos Aires, Argentina. *Streptococcus pneumoniae* T14 was grown in microanaerobiosis in Todd-Hewitt broth at 37°C. *Escherichia coli* DH10B and *E. coli* BL21(D3) (Novagen) were grown with shaking in LB medium at 37°C, and *Lactococcus lactis* NZ9000 was grown in M17 medium supplemented with 1% glucose (M17-glu) at 30°C. $CaCl_2$-competent *E. coli* cells were transformed as described by Sambrook et al. (28), and transformants were selected in LB agar (1.2%) containing neomycin (50 μg/ml; Sigma). Electroporation of *L. lactis* was conducted as indicated by Le Loir et al. (20), and transformants were selected in M17-glu agar (1.2%) with erythromycin (5 μg/ml; Sigma).

Cloning and expression of recombinant PppA in *E. coli* and *L. lactis*. All cloning techniques were carried out as described by Sambrook et al. (28). Restriction endonuclease and ligation reactions were carried out according to the recommendations of the suppliers. The *pppA* gene was amplified from the chromosomal DNA of *S. pneumoniae* T14 (Table 1) with the primers EC1 and EC2 (Table 2), following the conditions described by Green et al. (15). PCR amplifications were performed with a DNA thermocycler (MyCycler 580BR; Bio-Rad) by using 28 cycles consisting of a denaturation step at 94°C for 60 s, a primer annealing step at 52°C for 60 s, and a primer extension step at 72°C for 60 s. The PCR product was purified from agarose gels, using homemade silica beads, and treated with the restriction enzymes NcoI and SalI. Then, the *pppA* gene was ligated into the NcoI and SalI sites of pET28b, using T4 DNA ligase (Promega). A recombinant plasmid containing the *pppA* gene, named pET-PppA, was first recovered into *E. coli* DH10B cells and then transformed into BL21(DE3) cells for expression. BL21(DE3)(pET-PppA) cells were grown with aeration until they reached an optical density at 600 nm (OD_{600}) of 0.8 in LB medium supplemented with neomycin (50 μg/ml) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) for 2 to 4 h. Whole-cell lysates were prepared as indicated by Hebert et al. (18). Cell debris was removed by centrifugation (20 min, 13,000 rpm, 4°C), and the proteins present in the supernatant were run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel to confirm expression of the recombinant PppA (rPppA). rPppA was then purified by using a His-Bind purification kit (Novagen) according to the recommendations of the supplier and visualized by electrophoresis on 12% SDS-polyacrylamide gels. For expression of the *pppA* gene in *L. lactis*, primers LL1 and LL2 were designed (Table 2) and used to amplify, under the same conditions as indicated above (15), *pppA* sequences from the chromosomal DNA of *S. pneumoniae* T14. The PCR product was then treated with the restriction enzymes SalI and EcoRV and subcloned into the SalI and EcoRV sites of pVE5547 (12). Electrocompetent *L. lactis* NZ9000 cells were transformed with the ligation mixture, and a recombinant strain containing plasmid pVE-PppA was selected and named *L. lactis* PppA. Recombinant *L. lactis* PppA was grown in M17-glu plus erythromycin (5 μg/ml) at 30°C until cells reached an OD_{590} of 0.6 and were then induced with 50 ng/ml of nisin for 2 h. Uninduced (*L. lactis* PppA) and induced (*L. lactis* PppA expressing the PppA protein on its cell surface [$PppA^+$]) strains were then used in the immunization experiments. Sequencing of the reactions to confirm the identity of the plasmids pET-PppA and pVE-PppA was carried out at the Sequence Service Facility of GAD, National University of La Plata (Buenos Aires, Argentina).

Western blotting analysis. Purified rPppA protein was used to generate polyclonal antisera in mice. Briefly, 10 μg of rPppA protein was mixed with 50 μl of Freund's complete adjuvant and injected subcutaneously into 20 6-week-old Swiss albino mice (CERELA). The animals were immunized at week 0, given a booster injection at week 4, and then exsanguinated at week 6. The serum samples were pooled and used for further Western blotting analyses.

Whole-cell *L. lactis* PppA lysates were prepared as follows. Cells (5×10^8) were pelleted by centrifugation at 5,000 × *g* for 15 min, and then the supernatant and the pellet were collected. The pellet was resuspended in 500 μl of 50 mM Tris-HCl (pH 7.4), and cell extracts were prepared by adding glass beads (0.15- to 0.25-mm diameter; Sigma) to the bacterial cell suspensions and mixing the suspensions for 7 min at 4°C in a vortex mixer (Reax 2000; Heidolph, Schwabach, Germany) at maximum speed. Glass beads, cell debris, and unbroken cells were removed by centrifugation (10,000 × *g*, 10 min, 4°C), and the lysates were recovered. For protein secretion analysis, 1 ml of supernatant was concentrated by precipitation by the addition of 100 μl of 100% trichloroacetic acid, incubated for 10 min on ice, and centrifuged for 10 min at 7,000 × *g*.

Proteins in lysates and supernatants were separated by a 12% SDS-polyacrylamide gel and then transferred to nitrocellulose (Bio-Rad), using a Mini-Protean 3 cell unit (Bio-Rad). The blots were blocked at room temperature for 30 min in 5% nonfat milk-phosphate-buffered saline (PBS). Membranes were then exposed to pooled mouse antisera (at a 1:500 dilution) for 60 min, followed by three 20-min washes in PBS-0.2% Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (anti-γ chain specific; Sigma-Aldrich) was diluted 1:500 and used to detect bound antibodies. The blots were washed, and peroxidase activity was detected with a 3,3′-diaminobenzidine peroxidase substrate solution (0.05% 3,3′-diaminobenzidine tetrahydrochloride, 0.015% H_2O_2 in 0.01 M PBS [pH 7.2]; Sigma).

Immunofluorescence microscopy. Recombinant *L. lactis* PppA and $PppA^+$ cells were grown as described previously. For immunofluorescence microscopy,

TABLE 2. Sequences of oligonucleotide primers used for PCR amplification and cloning of the *pppA* gene

Primer	Sequence (5′–3′)[a]	Restriction site	Reference
EC1 (forward)	GGGG**CCATGG**CTTGTAGAATTGAAAAAAGAA	NcoI	16
EC2 (reverse)	GGG**GTCGAC**TAAACCAGGTGCTTGTCCAAGTTC	SalI	16
LL1 (forward)	GGGG**GTCGAC**GTAGAATTGAAAAAAGAA	SalI	This work
LL2 (reverse)	GGG**GATATC**TCTAAACCAGGTGCTTGTCCAAGTTC[b]	EcoRV	This work

[a] Restriction sites in each primer are in boldface.
[b] The underlined bases T and C in primer LL2 were included to maintain an in-frame reading of the PppA and cell wall anchor proteins.

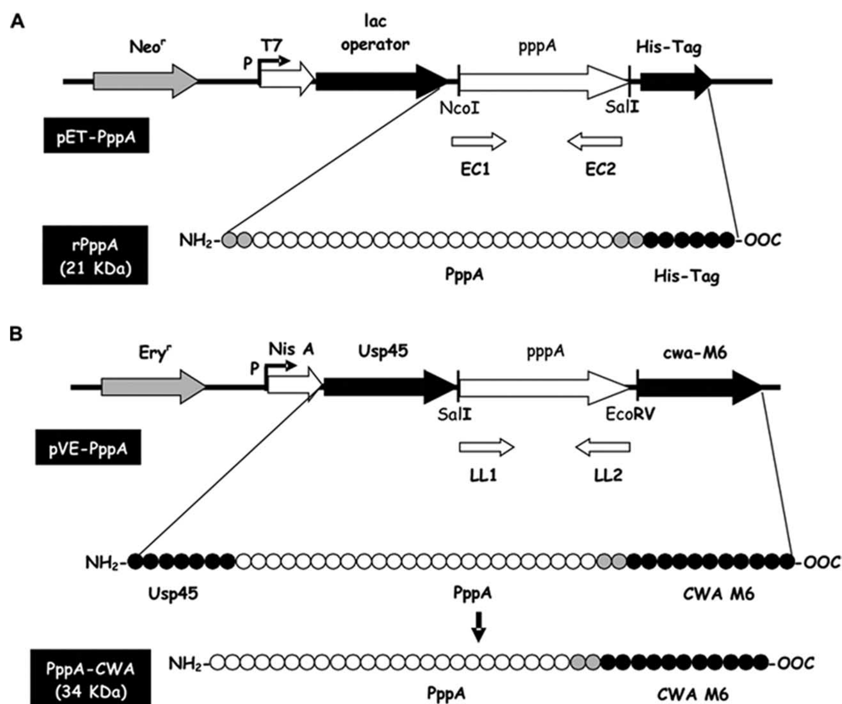

FIG. 1. Plasmids constructed in this work and the expected products. (A) Plasmid pET-PppA derived from the insertion of the *pppA* gene in the NcoI-SalI site of the pET28b vector, which allows the expression of the rPppA in *E. coli*. rPppA has a histidine tag (His-Tag) that allows its purification. The expected molecular mass of rPppA is 21 kDa. (B) Plasmid pVE-PppA derived from the insertion of the *pppA* gene in the SalI-EcoRV site of the pVE5547 vector, which allows expression of the recombinant protein PppA-CWA in *L. lactis*. The recombinant protein is synthesized as a primary transcript with the secretion signal Usp45, which is processed to originate the PppA-CWA protein that is anchored to the cell wall by the anchoring signal CWA-M6. The expected molecular mass of PppA-CWA is 34 kDa.

the cell pellets were labeled sequentially with mouse anti-PppA (1:500) polyclonal antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:1,000) antibodies and then examined by using a fluorescence microscope (Leica DM LS2).

Immunization of mice. Three-week-old (young) and 6-week-old (adult) Swiss albino mice (CERELA) were nasally immunized with 10^8 cells/mouse of recombinant *L. lactis* PppA$^+$ at days 0, 14, and 28 (10 animals per group). The inoculum was slowly instilled into the nostril of each mouse in a 25-μl volume. Negative controls included uninduced *L. lactis* PppA and PBS. Serum and bronchoalveolar lavage (BAL) fluid samples were collected on days 0, 14, 28, and 42 for the determination of specific antibodies. BAL samples were obtained according to the technique described previously (33); briefly, the trachea was exposed and intubated with a catheter, and two sequential bronchoalveolar lavage steps were performed by injecting 0.5 ml of sterile PBS into each mouse. The sample of fluid was centrifuged for 10 min at 900 × *g*, and the supernatant fluid was frozen at −70°C for subsequent antibody analyses.

Systemic challenge of actively and passively immunized mice. Intraperitoneal challenge experiments were carried out 2 weeks after the third immunization of the animals. *S. pneumoniae* T14 was grown until cells reached their exponential phase under the conditions described above, and each mouse was infected with 100 μl of PBS containing 10^8 cells of the pathogen. Survival was closely monitored for 21 days. In addition, two types of passive immunization and challenge experiments were performed as described by Gor et al. (14). In the first series of experiments, the groups of mice to be challenged were passively immunized with an intraperitoneal injection of 100 μl of the pooled serum from adult or young mice previously immunized with *L. lactis* PppA$^+$, *L. lactis* PppA, or PBS. Twenty-four hours after mice were passively immunized, they were challenged intraperitoneally with 10^8 cells of *S. pneumoniae* T14 suspended in PBS, and survival was monitored for 21 days. In a second series of experiments, groups of mice were inoculated with 10^8 cells of *S. pneumoniae* T14 suspended in 100 μl of PBS containing 10% of pooled serum from adult or young mice previously immunized with *L. lactis* PppA$^+$, *L. lactis* PppA, or PBS, and survival was monitored for 21 days.

Respiratory challenge of actively immunized mice. Adult and young mice were challenged with different serotypes of *S. pneumoniae*, which were kindly provided by M. Regueira from the Laboratory of Clinical Bacteriology, National Institute of Infectious Diseases, Argentina. Freshly grown colonies of *S. pneumoniae* strains AV3, AV6, AV14, and AV23 (Table 1) were suspended in Todd-Hewitt broth and incubated at 37°C until the log phase was reached. The pathogens were harvested by centrifugation at 3,600 × g for 10 min at 4°C and washed three times with sterile PBS; cell density was adjusted to 4 × 10^7 cells/ml. Challenge with the different pneumococcal strains was performed on day 42 postimmunization. Mice were challenged nasally with the pathogen by dripping 25 μl of an inoculum containing 10^6 cells into each nostril. To facilitate migration of the inoculum to the alveoli, mice were held in a head-up vertical position for 2 min. The development of pneumococcal disease was evaluated as previously described (25, 33). Briefly, mice were sacrificed 48 h after receiving the challenge, and their lungs were excised, weighed, and homogenized in 5 ml of sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar, and incubated for 18 h at 37°C. *S. pneumoniae* colonies were counted, and the results were expressed as $\log_{10}$ CFU/g of organ. The progression of bacterial growth to the bloodstream was monitored by obtaining blood samples by using cardiac puncture with a heparinized syringe. Samples were plated on blood agar, and bacteremia was reported as negative or positive hemocultures after incubation for 18 h at 37°C.

ELISA for PppA antibodies. Serum and BAL fluid antibodies against PppA protein were determined by a modification of the enzyme-linked immunosorbent assay (ELISA) protocol described by Green et al. (15). Briefly, plates were coated with rPppA (100 μl of a 5-μg/ml stock in sodium carbonate-bicarbonate buffer [pH 9.6] per well). Nonspecific protein binding sites were blocked with PBS containing 5% nonfat milk. Samples were diluted (serum, 1:100; BAL, 1:20) with PBS containing 0.05% (vol/vol) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgM, IgA, IgG, IgG1, or IgG2a (Fc specific; Sigma Chemical) was diluted (1:500) in PBS-T. Antibodies were revealed with a substrate solution (3-3′,5-5′-tetramethylbenzidine [Sigma Chemical]) in citrate-phosphate buffer (pH 5; containing 0.05% H_2O_2), and the reaction was stopped by the addition of H_2SO_4 1 M. OD readings were carried out at 493 nm (VERSAmax Tunable microplate reader), and samples were considered negative for the presence of specific antibodies when OD_{493} was <0.1.

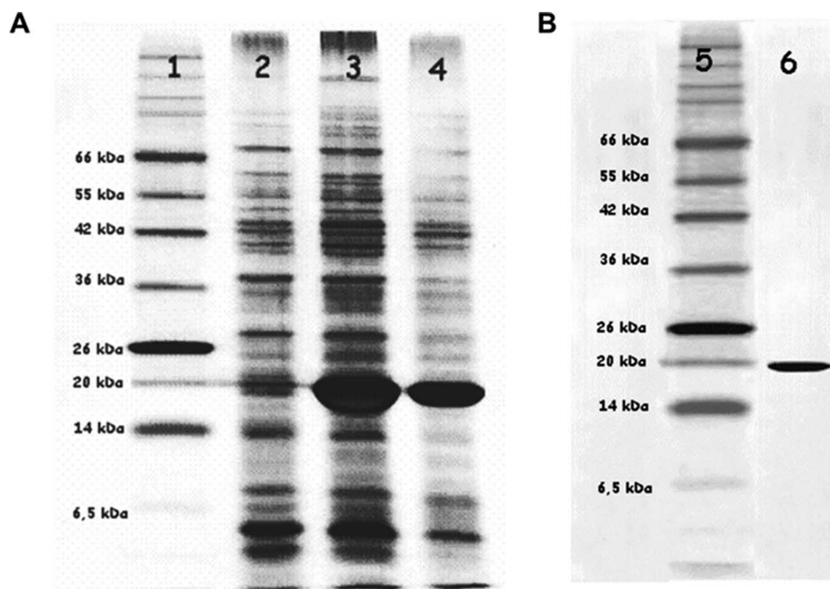

FIG. 2. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of (A) recombinant *E. coli*(pET-PppA) whole-cell lysates before and after induction with IPTG and (B) purified rPppA protein. Samples were loaded on a 12% SDS-polyacrylamide gel and the gel was silver stained. Lanes 1 and 5, molecular mass standards; 2, noninduced *E. coli*; 3, induction for 4 h; 4, induction for 2 h; 6, purified rPppA.

Antibody avidity assay. For the measurement of IgG and IgA antibody avidity, the basic ELISA method was used. After samples were incubated, plates were washed and incubated for 15 min at room temperature with 0.5 M sodium thiocyanate (NaSCN) to induce the dissociation of the antigen-antibody complexes. Plates were washed, and the remaining incubations were performed as described above, without modifications. The avidity index for each sample was determined as follows: the antibody concentration in the presence of the chaotropic agent NaSCN was divided by the antibody concentration in the absence of NaSCN and multiplied by 100 (1).

Statistical analysis. Experiments were performed in triplicate, and results were expressed as means ± standard deviations. For body weight gain, one-way analysis of variance was used. For all other determinations, two-way analysis of variance was used. Tukey's test (for pairwise comparisons of the mean values of the different groups) was used to test for differences between the groups. Differences were considered significant at a P value of <0.05. For survival analysis, the Kaplan-Meier log-rank test was used. Differences were considered significant at a P value of <0.01. In order to compare survival rates between young and adult mice, Cox's F test and Gehay's Wilcoxon test were used.

RESULTS

Cloning and expression of PppA in *E. coli*. A 534-bp DNA fragment containing the *pppA* gene of *S. pneumoniae* T14 was amplified with the primers EC1 and EC2 (Table 2), as described by Green et al. (15). The purified PCR product was cloned into the NcoI and SalI sites of plasmid pET28b to generate pET-PppA, which was recovered by transformation into *E. coli* DH10B. The identity of the *pppA* region present in pET-PppA was confirmed by sequencing. pET-PppA was then transferred to *E. coli* BL21(D3), and the recombinant strain *E. coli* BL21(D3)(pET-PppA) was used to express the rPppA (Fig. 1A). rPppA, a protein of 188 amino acids, with a histidine tag in its carboxyl ends and with a molecular mass of approximately 21 kDa, visualized only when recombinant *E. coli* BL21(D3)(pET-PppA) was induced with 1 mM IPTG (Fig. 2A). Purified rPppA (Fig. 2B) was used to obtain polyclonal anti-PppA serum in mice, which was then used for Western blotting and immunofluorescence analyses.

Expression of PppA in *L. lactis* NZ9000. To facilitate cloning of the *pppA* gene into the pVE5547 expression vector, two new primers, LL1 and LL2, were designed (Table 2). The primer LL1 incorporates a SalI restriction site at its 5′ end, while the primer LL2 incorporates an EcoRV restriction site and two extra bases (T and C [Table 2, underlined in the sequence]) at its 5′ end to ensure that the *pppA* DNA sequence was in-frame with the cell wall spanning the domain sequence present in pVE5547. The 534-bp *pppA* DNA fragment was obtained by PCR amplification using chromosomal DNA from the *S. pneumoniae* T14 strain as a template. The purified PCR fragment was treated with restriction enzymes and cloned into the SalI and EcoRV sites of pVE5547 to generate pVE-PppA (Fig. 1B), which was recovered into the *L. lactis* expression host strain NZ9000 by electroporation. The identity of the recombinant plasmid pVE-PppA was confirmed by PCR analysis (Fig. 3A) and DNA sequencing. When the expression strain *L. lactis* PppA (harboring pVE-PppA) was grown and induced as indicated in Materials and Methods, a fusion protein of the correct size (34 kDa; 314 amino acids) was recognized by anti-PppA antibodies. Immunoblotting experiments showed that, as expected, the PppA-CWA fusion protein was detected only in the cell wall fraction of the induced *L. lactis* PppA (Fig. 3B). The PppA band was not detectable in the soluble cytoplasmic or extracellular fractions or in the respective uninduced recombinant strain (Fig. 3B). The localization of PppA on the surface of *L. lactis* PppA was further verified by immunofluorescence staining that revealed fluorescence only in the previously induced recombinant bacteria harboring pVE-PppA (Fig. 3C) but not in control cells (photos not shown). These results showed that PppA protein was predictably and

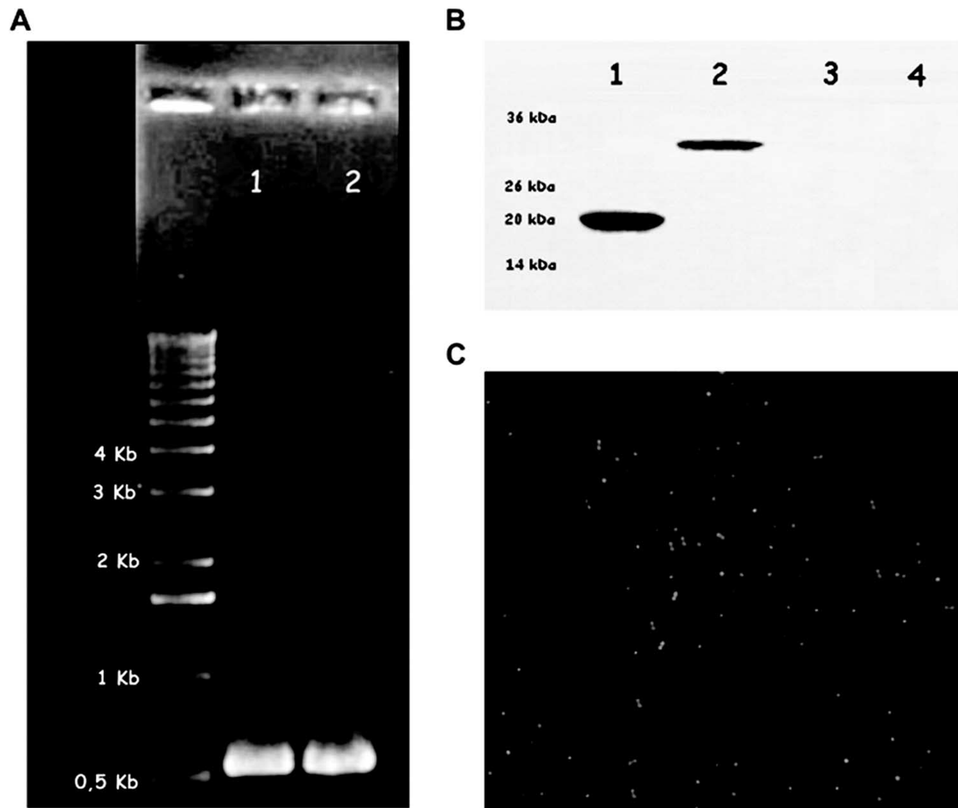

FIG. 3. (A) A fragment of the 534 bp of the *pppA* gene amplified with the boosters LL1 and LL2 from the chromosomal DNA of *Streptococcus pneumoniae* T14 (lane 1) and of the pEV-PppA plasmid obtained from the erythromycin-resistant recombinant strain *L. lactis* PppA (lane 2). (B) Western blotting analysis of whole-cell lysates and culture supernatants of *L. lactis* PppA. An SDS-polyacrylamide gel was blotted onto nitrocellulose for analysis. Polyclonal mouse antiserum to rPppA was used as the primary antibody. Lanes 1, purified rPppA (21 kDa) from *E. coli*; 2, *L. lactis* PppA previously induced with nisin expressing the PppA-CWA protein (34 kDa); 3, *L. lactis* PppA without induction; 4, concentrated culture supernatants from induced *L. lactis* PppA. (C) Detection of PppA at the cell surface of *L. lactis* PppA by immunofluorescence. Recombinant *L. lactis* cells expressing the PppA-CWA protein were treated with specific polyclonal mouse antiserum and then fluorescently stained with goat anti-IgG conjugated with FITC. Magnification, ×100.

efficiently displayed on the cell surface of *L. lactis* after it was induced with nisin.

Nasal immunization with *L. lactis* PppA$^+$ induced local and systemic antibody responses. We evaluated whether nasal immunization with recombinant *L. lactis* PppA$^+$ was able to induce the production of specific antibodies in adult and young mice. For this purpose, we used the immunization protocol described by Green et al. (15), which was shown to elicit strong antigen-specific IgG and IgA antibody responses in bronchial wash fluid and sera. Each mouse received one dose of 10^8 induced recombinant *L. lactis* PppA$^+$, followed by two booster injections on days 14 and 28. Uninduced recombinant *L. lactis* PppA or PBS was used as control. No preimmune reactivity (day 0) was detected with PppA in either adult or young mice (OD_{493}, <0.1). Samples analyzed on days 14, 28, and 42 showed that nasal immunization of young and adult mice with *L. lactis* PppA$^+$ induced specific anti-PppA IgM, IgA, and IgG antibodies in both sera and BAL fluids (Fig. 4A and B and Fig. 5A and B), while no detectable values of these specific antibodies were observed with sera or BAL fluids collected from animals that received *L. lactis* PppA or PBS (data not shown). Anti-PppA serum IgG ($P = 0.003$) and BAL fluid IgA ($P = 0.005$) and IgG ($P = 0.015$) antibodies were significantly higher in immunized young mice than in the adult group (Fig. 4A and B and Fig. 5A and B). The avidity of anti-PppA IgM, IgG, and IgA antibodies was also determined in sera and BAL samples (Table 3), with no significant differences observed between the avidity of young mice and that of adult mice. In addition, the subclasses of IgG anti-PppA antibodies were studied. The production of both IgG1 and IgG2a anti-PppA antibodies was detected in the sera and BAL fluid of young and adult mice (Fig. 4C and D and Fig. 5C and D). To assess the safety of *L. lactis* PppA administration in adult and young mice, weight gain and the number of deaths were monitored daily and compared to those of age-matched mice in the PBS control groups. No deaths occurred, and no effect was observed with weight gain in adult or young mice immunized with *L. lactis* PppA$^+$ or PppA compared to that of PBS controls (data not shown), indicating that the recombinant strain is safe even for subjects in early life.

Enhanced resistance to systemic pneumococcal challenge in actively immunized adult and young mice. In order to determine whether the induction of specific antibodies by nasal immunization with *L. lactis* PppA$^+$ correlated with increased resistance to pneumococcal infection, adult and young mice were intraperitoneally challenged with *S. pneumoniae* T14, and

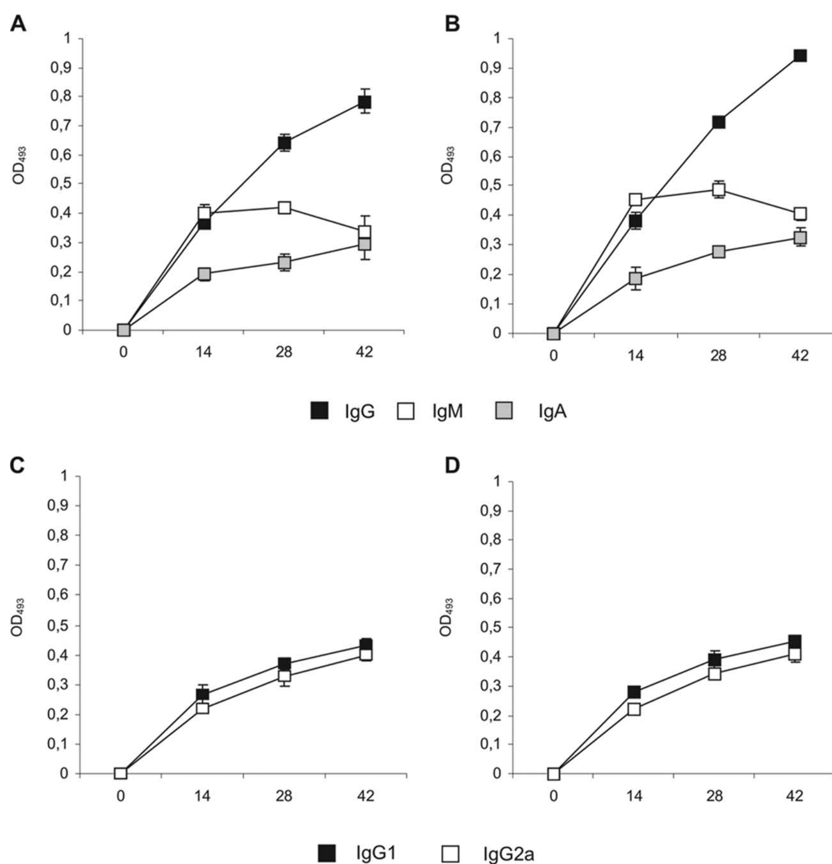

FIG. 4. Antibody immune response to PppA antigen in serum after nasal immunization of adult (A and C) or young (B and D) mice with recombinant *L. lactis* $PppA^+$ (previously induced with nisin). Samples were considered negative for the presence of specific antibodies when $OD_{493} \leq 0.1$.

survival was closely monitored for 21 days. Adult mice immunized with *L. lactis* $PppA^+$ showed a survival percentage of 60% on day 21 after challenge (Fig. 6A), while 100% of the PBS and *L. lactis* PppA adult mice died between days 4 and 7 after challenge. Nasal immunization of young mice with *L. lactis* $PppA^+$ enabled 70% of the mice to survive up to day 21 postinfection (Fig. 6B), while 100% of the young mice in the control groups died on days 3 to 5 postchallenge. These results demonstrate that active immunization with *L. lactis* $PppA^+$ increased resistance to the systemic challenge by *S. pneumoniae* in both groups.

Improved resistance to systemic pneumococcal challenge in passively immunized young mice. Protection against pneumococcal infection is considered to be antibody mediated. Therefore, we evaluated the ability of immune serum generated in *L. lactis* $PppA^+$-immunized adult or young mice to provide protection for naïve young mice. In the first series of experiments, naïve young mice were passively immunized with the immune serum and challenged 24 h later. Passive immunization with pooled serum obtained from *L. lactis* $PppA^+$ adult or young mouse groups significantly increased the survival percentage of the naïve young mice in relation to that of the respective control groups (Fig. 7A and B), with no significant differences between the young and adult groups ($P = 0.91$, Cox's F test). In a second series of experiments, naïve young mice were challenged with *S. pneumoniae* previously opsonized with the immune sera. Mice challenged with pneumococci opsonized with immune serum from *L. lactis* $PppA^+$ adult mice showed a survival percentage of 40% (Fig. 8A), which was significantly higher than that of the control groups. When the pathogen was opsonized with the immune serum from *L. lactis* $PppA^+$ of young mice, the survival percentage was 50% (Fig. 8B), a value significantly higher than the ones for the respective control groups but not significantly different from the values found in the mice that received the immune serum from the *L. lactis* $PppA^+$ adult group ($P = 0.64$, Cox's F test).

Enhanced resistance of actively immunized adult and young mice to the respiratory challenge with different pneumococcal serotypes. Since the pneumococcus bacterium is primarily a respiratory pathogen, we examined the ability of *L. lactis* $PppA^+$ immunization to provide protection against a respiratory challenge. In addition, the ability of the recombinant *L. lactis* $PppA^+$ to elicit cross-protection was evaluated by challenging adult and young mice with different pneumococcal serotypes. In control animals, all *S. pneumoniae* serotypes used were detected in lung cultures and hemocultures 2 days after the intranasal challenge. Nasal immunization with *L. lactis* $PppA^+$ significantly reduced the lung bacterial cell counts of serotypes 3 and 6B (Table 4), while lung cultures were negative for serotypes 14 and 23F (Table 4) in both adult and young mice. Moreover, *L. lactis* $PppA^+$ admin-

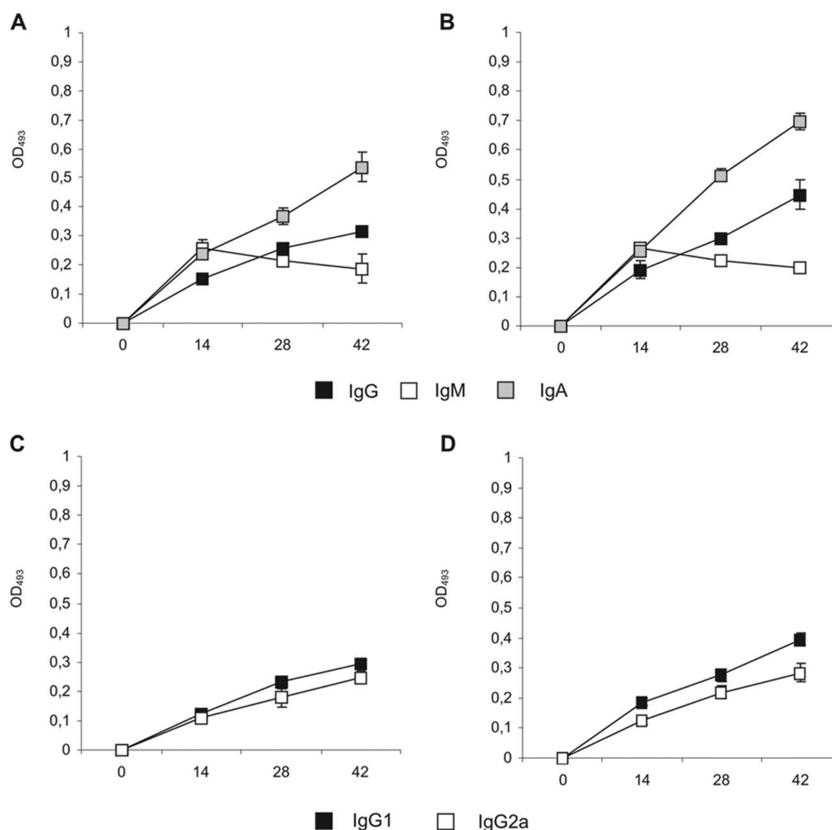

FIG. 5. Antibody immune response to PppA antigen in BAL fluid samples from nasally immunized adult (A and C) or young (B and D) mice with recombinant *L. lactis* $PppA^+$ (previously induced with nisin). Samples were considered negative for the presence of specific antibodies when the OD_{493} was $\leq$0.1.

istration prevented the dissemination of all assayed pneumococcal serotypes, since hemocultures tested negative (Table 4).

DISCUSSION

The PppA is a small *S. pneumoniae* protein that can be recovered in large quantities from the wash fluids of intact pneumococcal cells with PBS (15). PppA is antigenically conserved and exhibits a high level of sequence conservation in serotypes 3, 5, 9, 14, 19, and 23 (15). Although the role of PppA in the pathogenesis of pneumococcal infection is unknown, it has been demonstrated that antibodies against this antigen can reduce nasal colonization of pneumococci. However, the induction of specific anti-PppA antibodies by nasal immunization can be achieved only when the protein is administered with a mucosal adjuvant such as genetically modified cholera toxin (15). Cholera toxin is a potent adjuvant, but direct binding to and accumulation of this toxin in the nervous system following intranasal administration have been reported (13). As this is an important obstacle for the development of effective mucosal vaccines, the search for safe mucosal adjuvants becomes imperative. In this sense, LAB have been tested as potential adjuvants, since they are safe microorganisms, and some of them have been proved to have intrinsic immunoadjuvant properties when they are administered through the mucosal route (25, 33). Several authors have shown that live recombinant LAB can also elicit an efficient immune response, in which the strongest response was mounted when the antigen was bound to the membrane/cell wall compared to that of

TABLE 3. Avidity of PppA-specific antibodies in sera and BAL fluid samples after nasal immunization of adult and young mice[a]

Group	Avidity index ± SD response to serum antibodies			Avidity index ± SD response to BAL fluid antibodies		
	IgG	IgM	IgA	IgG	IgM	IgA
Adult mice						
L. lactis $PppA^+$	62.3 ± 2.8	44.5 ± 1.9	18.3 ± 1.7	61.8 ± 3.9	56.7 ± 3.6	68.4 ± 4.7
Young mice						
L. lactis $PppA^+$	64.1 ± 6.8	45.4 ± 2.3	21.5 ± 2.1	68.5 ± 3.4	57.8 ± 5.3	66.9 ± 3.8

[a] Avidity of PppA-specific antibodies in sera and BAL fluid samples after nasal immunization of adult or young mice with recombinant *L. lactis* $PppA^+$ previous to induction with nisin. The avidity index for each sample was determined by dividing the antibody concentration in the presence of the chaotropic agent NaSCN by the antibody concentration in the absence of NaSCN and multiplying by 100.

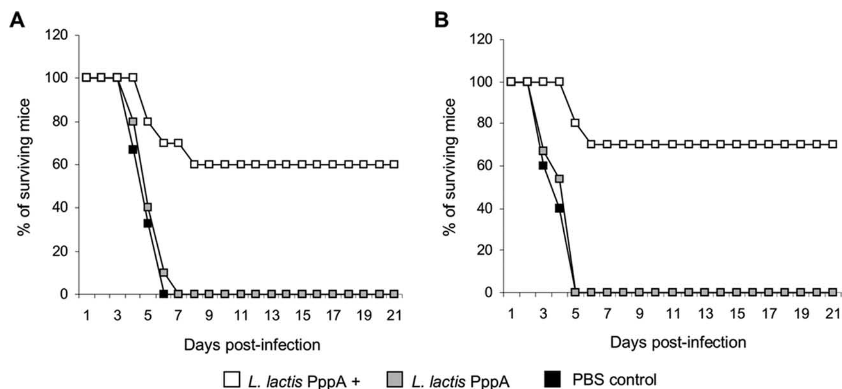

FIG. 6. Survival of adult (A) and young (B) mice after undergoing nasal immunization with recombinant *L. lactis* expressing the PppA protein on its surface (*L. lactis* PppA$^+$) and intraperitoneal challenge with 10^8 cells of *S. pneumoniae* T14. Mice receiving noninduced *L. lactis* (*L. lactis* PppA) or PBS were used as controls.

antigens that were secreted or that of intracellularly located antigens (4, 24, 27). In this work, we determined whether PppA anchored to the cell wall of a recombinant *L. lactis* strain was able to induce a protective immune response in mice of different ages.

The presence and levels of IgG and IgA pathogen-specific neutralizing antibodies are considered one of the most effective defense mechanisms against respiratory infections and a vital attribute of any vaccine designed to prevent these infections (32). In this work, detection of IgM, IgG, and IgA anti-PppA antibodies in the serum and BAL fluid of adult and young mice showed that nasal immunization with the recombinant *L. lactis* PppA$^+$ strain was able to induce a strong mucosal and systemic immune response. The induced production of IgG- and IgA-specific PppA antibodies was significantly higher in young mice. Reviews of preclinical and human vaccine studies indicate that although early life immunization may not lead to early and strong antibody responses, it may result in efficient immunological priming, which can serve as an excellent basis for future responses (3, 30). Our results also confirmed the adjuvant properties of *L. lactis* NZ9000. Moreover, M. Medina, J. Villena, S. Salva, E. Vintiñi, P. Langella, and S. Alvarez (submitted for publication) have shown that nasal administration of nonrecombinant *L. lactis* NZ9000, at the appropriate dose, enhanced the activity of alveolar and peritoneal phagocytes in mouse. This agrees with recent findings that demonstrated that *L. lactis* NZ9000 cells are able to stimulate in vitro the production of tumor necrosis factor-α by murine peritoneal macrophages and to upregulate the expression of costimulatory molecules in bone marrow-derived dendritic cells (2). Thus, the stimulation of antigen-presenting cells by *L. lactis* would be able to induce the development of a stronger specific immune response when administered together with the PppA antigen. In addition, it is important to note that no antibodies against *L. lactis* were found in the serum or the BAL fluid of adult and young mice immunized with the recombinant strain (data not shown). Thus, the host immune response was directed against the protein expressed by *L. lactis* and not against the vector.

We found that both the IgG1 and the IgG2a anti-PppA antibodies were produced. Although data for the role of different IgG subclasses in the protection against pneumococcal infection are limited, it seems that the IgG2a subclass is particularly important, since it is the primary murine IgG subclass

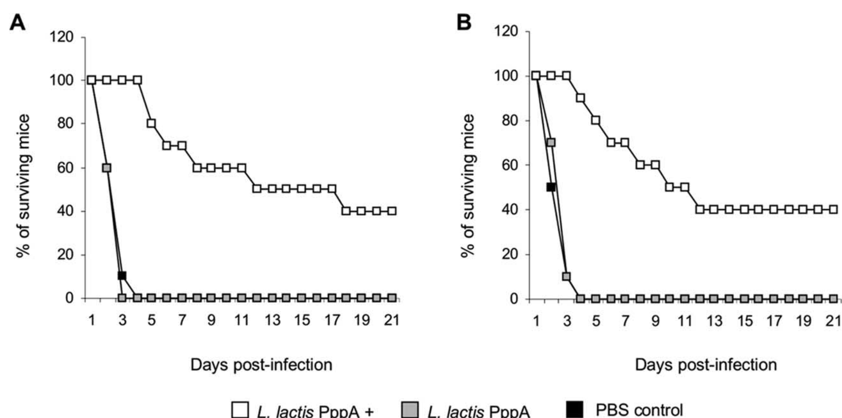

FIG. 7. Survival of naïve young mice passively immunized with pooled serum from adult (A) or young (B) mice nasally immunized with recombinant *L. lactis* expressing the PppA protein on its surface (*L. lactis* PppA$^+$) and challenged 24 h later with 10^8 cells of *S. pneumoniae* T14. Naïve young mice receiving pooled sera from adult (A) or young (B) mice immunized with noninduced *L. lactis* (*L. lactis* PppA) or PBS were used as controls.

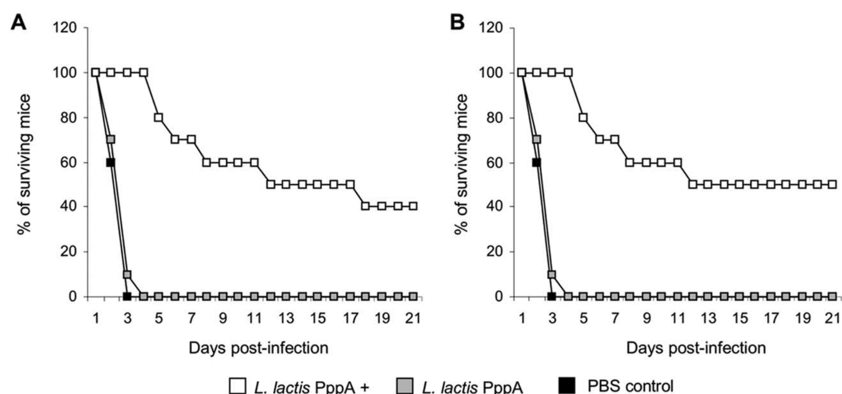

FIG. 8. Survival of naïve young mice challenged with 10^8 cells of *S. pneumoniae* T14 suspended in PBS containing 10% of pooled sera from adult (A) or young (B) mice nasally immunized with recombinant *L. lactis* expressing the PppA protein on its surface (*L. lactis* $PppA^+$). Naïve young mice challenged with *S. pneumoniae* opsonized with the pooled sera from adult (A) or young (B) mice immunized with noninduced *L. lactis* (*L. lactis* PppA) or PBS were used as controls.

that mediates optimal complement fixation (21). In addition, although IgG1 is not efficient at complement fixation, it might also contribute to protection against pneumococcal infection through Fc receptor binding or by preventing attachment and colonization of bacteria at mucosal surfaces. Thus, the production of both types of antibodies would increase resistance against infection. Adult and young mice immunized with *L. lactis* $PppA^+$ showed significantly higher survival percentages than the control groups after systemic challenge with *S. pneumoniae*, which would indicate that anti-PppA antibodies would be able to increase resistance against the pneumococcal invasive disease. The survival of naïve young mice receiving *L. lactis* $PppA^+$ immune serum confirmed the direct correlation between anti-PppA antibodies and protection.

Capsule-specific antibodies have been shown to be highly protective; however, the exact concentration of these serotype-specific antibodies required for protection against disease is still uncertain. Moreover, it is clear that the opsonic activity and avidity of these antibodies are more critical determinants of protection than their concentration, especially in young individuals (5, 23, 31). These findings could also be applied to the antibodies directed against protein antigens on the surface of *S. pneumoniae*. In the present work, the avidity of anti-PppA antibodies elicited in young mice was similar to that found in the adult group. However, in order to understand the mechanisms of antibody action in each group, it would be necessary to perform a comparative analysis of the opsonic activity of the immune sera of adult and young mice; consequently it merits further investigation.

Challenge assays with pneumococcal serotypes 3, 6B, 14 and 23F were performed to evaluate the efficacy of immunization with *L. lactis* $PppA^+$ against respiratory infection produced with different serotypes of *S. pneumoniae*,. These serotypes were selected according to previous epidemiological studies that determined serotype 14 was the most prevalent serotype in our country (22) and also because serotypes 3, 6B, 9, 14, 18, 19y, and 23F are the serotypes most often associated with invasive disease (17). As expected, young mice were more susceptible to respiratory infection than adult mice. However, immunization of young and adult mice with *L. lactis* $PppA^+$ provided protection against the serotypes assessed, although to a higher degree in the latter group. This fact could indicate that

TABLE 4. Lung and blood bacterial cell counts after nasal challenge with different pneumococcal serotypes[a]

Group	Response to serotype							
	3		6B		14		23F	
	Lung (CFU/g)	Blood (CFU/ml)	Lung (CFU/g)	Blood (CFU/ml)	Lung (CFU/g)	Blood (CFU/ml)	Lung (CFU/g)	Blood (CFU/ml)
Adult mice immunized with:								
L. lactis $PppA^+$	3.8 ± 0.3**	<1.5**	3.1 ± 0.2**	<1.5**	<1.5**	<1.5**	<1.5**	<1.5**
L. lactis PppA	6.1 ± 0.4	4.2 ± 0.3	5.6 ± 0.5*	3.9 ± 0.5	5.7 ± 0.4*	4.1 ± 0.4	4.1 ± 0.3	3.6 ± 0.4
Control	6.9 ± 0.3	4.8 ± 0.2	6.3 ± 0.3	4.0 ± 0.2	6.1 ± 0.5	4.1 ± 0.7	4.3 ± 0.4	3.8 ± 0.5
Young mice immunized with:								
L. lactis $PppA^+$	4.2 ± 0.2**	<1.5**	3.3 ± 0.5**	<1.5**	<1.5**	<1.5**	<1.5**	<1.5**
L. lactis PppA	7.9 ± 0.4	4.6 ± 0.3	6.9 ± 0.3*	4.4 ± 0.7	5.9 ± 0.6*	4.3 ± 0.5	5.5 ± 0.5*	4.1 ± 0.7
Control	8.3 ± 0.2	5.2 ± 0.2	7.5 ± 0.3	4.5 ± 0.3	6.9 ± 0.4	4.7 ± 0.4	6.2 ± 0.3	4.4 ± 0.2

[a] Adult and young mice were immunized with recombinant *L. lactis* (pVE-PppA) with *L. lactis* $PppA^+$ or without *L. lactis* PppA previous to induction with nisin. Results are expressed as log CFU/g of lung or log CFU/ml of blood. The lower limits of bacterial detection were 1.5 log CFU/g of lung and 1.5 log CFU/ml of blood. Asterisks represent significant differences from the respective control groups: *, $P < 0.05$; **, $P < 0.01$.

vaccination at an early age would be a better strategy for improved protection of the host against *S. pneumoniae* infection. It seems likely that the BAL fluid anti-PppA antibodies, which were found in *L. lactis* PppA^{+}-immunized young mice at higher levels than in the adult group, were involved in this effect.

In summary, the results presented in this study showed that nasal immunization with PppA anchored to the cell wall of a recombinant *L. lactis* strain elicited cross-protective immunity against different pneumococcal serotypes, afforded protection against both systemic and respiratory challenges, and induced protective immunity in young and adult mice. Our results also suggest that immunization with the recombinant *L. lactis* strain is more effective when the vaccination begins at an early age (young mice).

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