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Oral Immunization of Mice with Attenuated *Salmonella typhimurium aroA* Expressing a Recombinant *Mycoplasma hyopneumoniae* Antigen (NrdF)

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***Mycoplasma hyopneumoniae* is the etiological agent of porcine enzootic pneumonia, a commercially expensive respiratory disease of swine. *Salmonella typhimurium* SL3261 was used as a live carrier of plasmid pKF1, which encodes a 15-kDa recombinant *M. hyopneumoniae* protein. This expressed recombinant protein consists of the carboxy-terminal 11 kDa of a 42-kDa *M. hyopneumoniae* NrdF ribonucleotide reductase R2 subunit protein. Rabbit anti-15-kDa serum was able to inhibit the growth of viable *M. hyopneumoniae* J in vitro. When used as a live oral vaccine, *S. typhimurium* SL3261(pKF1) induced a significant secretory immunoglobulin A immune response in the lungs of mice orally immunized against the *M. hyopneumoniae* antigen. Utilization of live oral vaccines expressing potentially protective *M. hyopneumoniae* proteins, such as the NrdF antigen, which can stimulate a lung mucosal response against surface-accessible proteins may provide a cost-effective alternative to the present control strategies used for porcine enzootic pneumonia.**

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP), a chronic respiratory disease of pigs (29) which inflicts severe economic losses on pig producers worldwide (21, 24). This pathogen colonizes the respiratory epithelia of swine and compromises their integrity through the induction of an inflammatory response (3, 25). Without complications from secondary bacterial pathogens, clearance of *M. hyopneumoniae* and resolution of lung lesions can occur after 4 to 5 weeks (1, 19). However, *M. hyopneumoniae* infection predisposes pigs to secondary invaders, such as *Pasteurella multocida* (9) and *Actinobacillus pleuropneumoniae* (38), which exacerbate economic losses and may increase mortality. *M. hyopneumoniae* infection rates within herds can vary between 30 and 80% (29). Current control strategies rely upon animal management procedures (10) and the use of antibiotics. However, as antibiotic usage in food producing animals is increasingly losing favor (37) and as swine are the only hosts for this pathogen, it is feasible and desirable that control of the disease be achieved with strict husbandry practices and an effective vaccination regime. Previous research by our group has shown that the 15-kDa *M. hyopneumoniae* antigen is strongly recognized by swine hyperimmune sera. This recombinant antigen was identified as containing part of a 42-kDa *M. hyopneumoniae* NrdF protein which is the R2 subunit of the essential prokaryotic class I ribonucleotide reductase (12). The carboxy-terminal 11 kDa of the *M. hyopneumoniae* NrdF antigen was expressed as a 128-kDa β-galactosidase fusion protein in *Escherichia coli*. Preliminary pig trials with purified 128-kDa fusion protein delivered intramuscularly resulted in a significant reduction in PEP in vaccinated animals compared with unvaccinated controls following experimental challenge (12), indicating that the recombinant NrdF protein is a potential candidate antigen for use in *M. hyopneumoniae* vaccines.

To date there have been no reports describing the use of attenuated *Salmonella typhimurium aroA* vectors for the delivery of mycoplasmal antigens. Previous studies using *S. typhimurium aroA* (15) have demonstrated that these strains are invasive yet nonvirulent and capable of eliciting immune responses which protect against subsequent virulent challenge (6, 15, 20, 23). After ingestion, *S. typhimurium* attaches to, invades, and proliferates within intestinal enterocytes (7), eventually reaching the basal epithelial cells from which it may traverse the intestinal barrier and colonize the liver, spleen, and mesenteric lymph nodes (13). Of particular importance to live oral immunization strategies is the invasion of the M cells which overlie the Peyer's patches of the intestinal mucosa, important components of the gut-associated lymphoid tissue (GALT) (28). Antigen processing and presentation within the GALT results in the migration of specific B and T cells to distal mucosal regions (4) which are primed for a specific secretory immune response. Attenuated *S. typhimurium aroA* mutants are able to survive for up to 7 days in situ, which is a sufficient period for colonization and limited growth within the invaded intestinal cells (8). After this period the bacteria are no longer viable and cannot avoid the host cell defenses; subsequently, the bacteria are recognized and processed. Studies have also demonstrated that these attenuated strains are able to deliver plasmid-encoded mucosal pathogen antigens via the GALT pathway, resulting in the priming of immune cells in the lung against these heterologous antigens (6, 14, 35). This report describes the expression of a 15-kDa fusion protein containing 11 kDa of *M. hyopneumoniae* NrdF antigen in *S. typhimurium* SL3261(pKF1), and the potential of this live recombinant vaccine strain to elicit mucosal immunoglobulin A (IgA) in mice was examined.

To determine the accessibility and essential nature of the native NrdF protein in *M. hyopneumoniae*, growth inhibition assays were carried out by a procedure described by Stanbridge and Hayflick (30). Sterile discs impregnated with rabbit anti-15-kDa NrdF serum (Fig. 1B) and swine hyperimmune anti-*M. hyopneumoniae* serum (Fig. 1A) inhibited the growth of freshly

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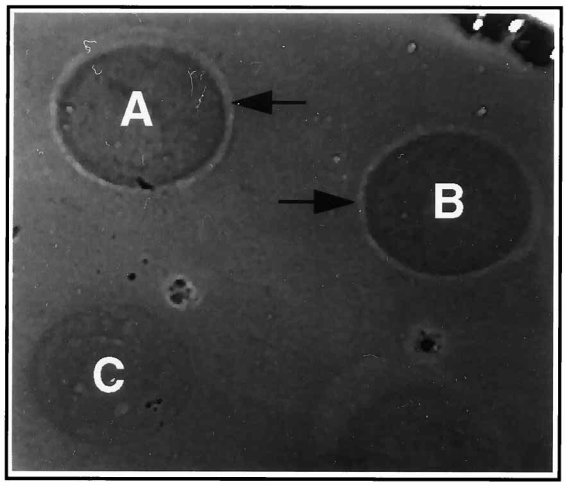

FIG. 1. Growth inhibition assay using sterile paper discs (6 mm in diameter) impregnated with different serum samples overlaid onto a lawn of freshly cultured *M. hyopneumoniae* J. Inhibition of growth can be seen as a halo-like ring (indicated by an arrow) around the darker area where the paper disc was located. Inhibition can be seen around the discs impregnated with hyperimmune pig serum (A) and rabbit 15-kDa NrdF antiserum (B). No zone of clearing was detected around the rabbit prebleed serum (C).

cultured *M. hyopneumoniae*. A distinct clearing zone was observed around the perimeter of the disc (Fig. 1A and B) and is in marked contrast to the absence of such zones around the rabbit prevaccination bleed-impregnated disc (Fig. 1C) and a swine prevaccination prebleed disc (data not shown). Zones of clearance around the 6-mm-diameter disc were found to be approximately 3 mm wide when observed by light microscopy. The zones of inhibition are a product of the inhibited growth of *M. hyopneumoniae*. Clearing increases closer to the disc, presumably due to increased antibody concentrations. Zones of clearance are reduced when stained with Coomassie brilliant blue because the extreme regions of the zones contain limited amounts of mycoplasmal growth which readily stain when this sensitive visualization technique is used.

Plasmid pKF1 containing a 0.8-kb mycoplasmal DNA fragment encoding the NrdF antigen (12) was used to transform (27) *S. typhimurium* SL3261 (15), resulting in the expression of a 15-kDa recombinant protein (Fig. 2) as determined by polyacrylamide gel electrophoresis (17) and Western immunoblotting (5) using rabbit 15-kDa NrdF antiserum. This expressed antigen was purified and consists of 11 kDa from the carboxy-terminal region of the 42-kDa *M. hyopneumoniae* NrdF protein (12), with the remainder being 4 kDa encoded by the polycloning site of pHSG398 (32). The recombinant protein is constitutively expressed in *S. typhimurium* from the lactose promoter because the pHSG398 vector does not contain a lactose repressor gene. Mice immunization trials were performed to assess the ability of the *S. typhimurium* SL3261(pKF1) vaccine strain to elicit anti-*M. hyopneumoniae* secretory and serum antibody responses. Immunization was performed essentially as described by Guzmán et al. (14). Six- to eight-week-old female BALB/c mice were caged separately according to treatment groups. For live oral vector control and vaccine groups, cultures were grown to mid-logarithmic phase, pelleted by centrifugation (2,000 × *g* for 15 min), and subsequently resuspended in ice-cold phosphate-buffered saline (PBS; pH 7.2) to an optical density corresponding to 2×10^9 viable bacteria per 100 μl. Immediately prior to immunization an equal volume of 3% sodium bicarbonate in PBS (pH 7.2) was added to give the final working dilution of 10^9 viable bacteria per 100 μl. Mice in all oral vaccine groups were deprived of water for 2 to 3 h before delivery of the vaccine (100 μl of the bacterial suspension) through an esophageal cannula. For intraperitoneal (i.p.) immunization, a total of 50 μg of 15-kDa NrdF protein was diluted in 50 μl of PBS (pH 7.2) and emulsified in an equal volume of Freund's complete adjuvant to give the final 100-μl injection volume for the primary immunization. In subsequent booster immunizations Freund's incomplete adjuvant was used, while the volume and antigen dose remained identical to those of the primary immunization. All mice received primary immunizations at day 0 and boosters at days 30 and 40 and were sacrificed at day 50. Mice were killed by cervical dislocation prior to exsanguination by the severing of the brachial artery. Sera were collected and stored at −20°C. Lung lavages were collected by pertracheal cannulation by instilling 0.8 ml of PBS containing 2 mM phenylmethylsulfonyl fluoride. Lung lavages were centrifuged briefly (3,000 × *g*) to remove particulate matter and were stored at −20°C. An enzyme-linked immunosorbent assay (ELISA) was used to determine mouse antibody responses for IgA, IgM, and IgG isotypes in sera and lung lavage samples. The primary antigen was either 15-kDa NrdF protein diluted to a final concentration of 1 μg/ml in carbonate buffer (30 mM sodium carbonate, 20 mM sodium hydrogen carbonate, pH 9.6) or *S. typhimurium* SL3261 whole-cell lysate prepared by boiling 10 mg of cell pellet for 10 min in 0.5 M Tris-HCl (pH 6.8) containing 1% glycerol, 0.2% sodium dodecyl sulfate, and 0.5% β-mercaptoethanol. Whole-cell lysate was then pelleted (16,000 × *g* for 3 min at room temperature) to remove debris, and the protein concentration was adjusted to 1 μg/ml in carbonate buffer prior to use. Aliquots (100 μl) of the primary antigen were used to coat 96-well microtiter plates (Nunc) by incubation for 1 h at 37°C. Plates were subsequently washed five times with 0.05% Tween 20 (Sigma) in PBS (pH 7.4). The first antibody was mouse sera diluted 1 in 200 or lung lavage samples diluted 1 in 20; in both cases the diluent was PBS containing 10% fetal bovine serum and 0.05% Tween 20. Aliquots (100 μl) of the first antibody were added in triplicate and incubated for 1 h at 37°C. Following incubation, plates were washed as previously described and 100-μl aliquots of horseradish peroxidase-conjugated rabbit anti-mouse Ig antibodies (Bio-Rad) diluted in PBS (pH 7.4)

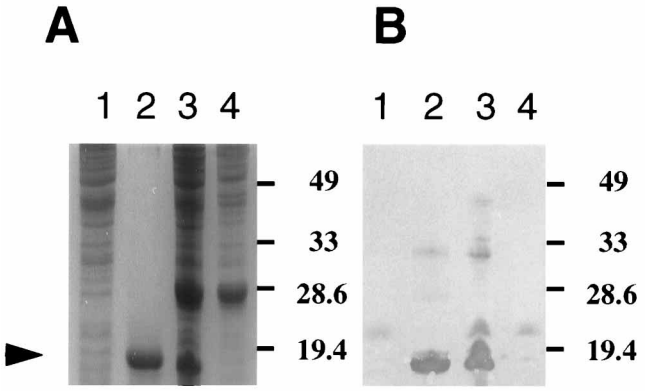

FIG. 2. Expression and identification of *M. hyopneumoniae* NrdF recombinant antigen in *S. typhimurium* SL3261. (A) Coomassie brilliant blue-stained 15% polyacrylamide gel electrophoresis gel. Lane 1, *S. typhimurium* SL3261; lane 2, purified recombinant 15-kDa NrdF antigen; lane 3, *S. typhimurium* SL3261 (pKF1); lane 4, *S. typhimurium* SL3261(pHSG398). (B) Western blot analysis of the same samples described above using rabbit *M. hyopneumoniae* 15-kDa NrdF serum. The sizes of molecular mass markers (in kilodaltons) and that of the recombinant NrdF protein (arrowhead) are indicated.

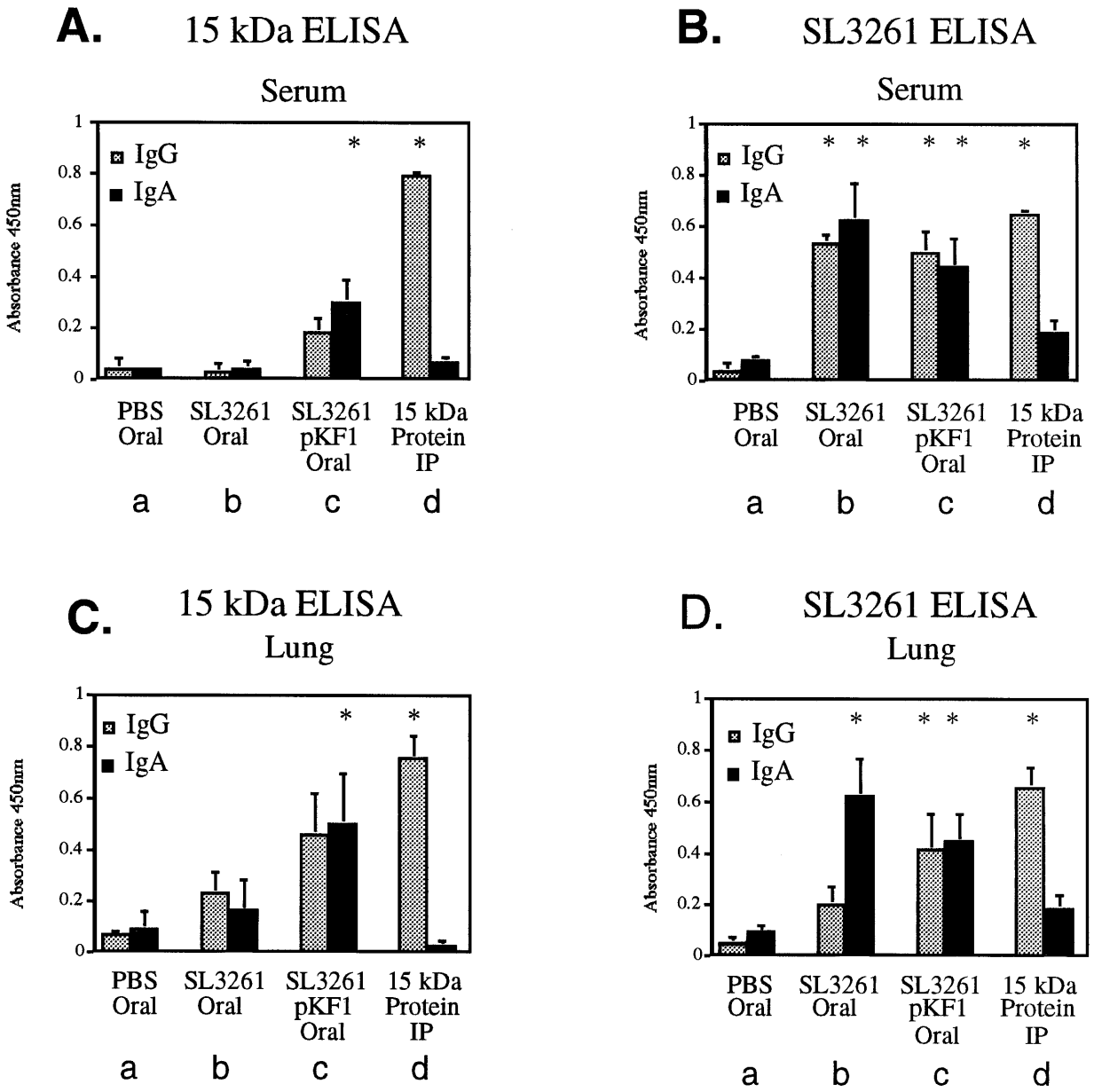

FIG. 3. Levels of recombinant 15-kDa NrdF-specific IgG and IgA antibodies in mouse serum and lung lavage samples after vaccination. (A) Serum antibody levels specific for 15-kDa NrdF protein. (B) Serum antibody levels specific for *S. typhimurium* SL3261 whole-cell proteins. (C) Lung lavage antibody levels specific for 15-kDa NrdF protein. (D) Lung lavage antibody levels specific for *S. typhimurium* SL3261 whole-cell proteins. The standard errors of the means are indicated by vertical lines. Statistical significance ($P < 0.05$) determined by comparing treatments against the PBS oral control group is indicated by asterisks.

containing 10% fetal bovine serum and 0.05% Tween 20 were added to each well. Rabbit anti-IgG (KPL) and anti-IgM (KPL) second antibodies were diluted 1 in 2,000, whereas anti-IgA (KPL) was diluted 1 in 400. Plates were incubated at 37°C for 1 h and were then washed as described above. ELISA plates were developed with 100-μl aliquots of tetramethylbenzidine dihydrochloride (Sigma); development was stopped after 3 min by the addition of 50 μl of 0.1 M phosphoric acid, and the absorbance was read at 450 nm. The plates were blanked against a PBS control, and the variation between plates was standardized with positive control sera, allowing isotype comparisons between groups. Antibody responses were compared by using a Tukey multiple comparison test for unequal sample sizes as described by Zar (39).

Oral delivery of the live *S. typhimurium* SL3261(pKF1) vaccine strain to mice elicited anti-*M. hyopneumoniae* NrdF IgA antibody responses in the lungs of vaccinated mice (Fig. 3C, graph c) but did not elicit significant ($P < 0.05$) IgG (Fig. 3C, graph c) or IgM (data not shown) isotype responses in the serum or lungs. i.p. immunization with the purified 15-kDa NrdF antigen elicited significant serum IgG (Fig. 3A, graph d), IgM (data not shown), and lung IgG (Fig. 3C, graph d) responses against this antigen. The absence of IgM antibody in lung lavage samples precludes the possibility of serum contam-

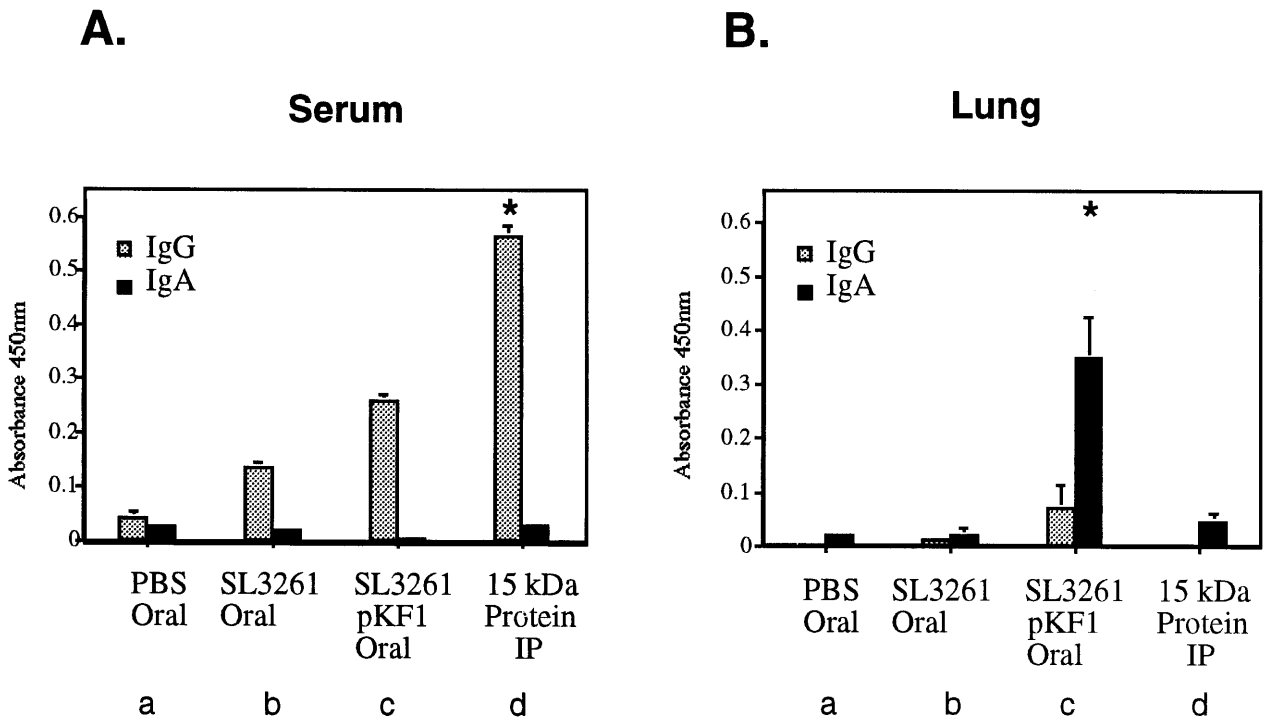

FIG. 4. Levels of recombinant 15-kDa NrdF-specific IgG and IgA antibodies in mouse serum (A) and lung lavage samples (B) detected by whole-cell *M. hyopneumoniae* ELISA. The standard errors of the means are indicated by vertical lines. Statistical significance ($P < 0.05$) determined by comparing treatments against the PBS oral control group is indicated by asterisks.

ination of the mucosal washes (data not shown). Thus, the isotypes detected in the mouse lung samples can be considered an accurate indication of the Ig isotypes present. For mice from groups orally immunized with *S. typhimurium* SL3261, there was a significant ($P < 0.05$) anti-salmonella IgG response in serum (Fig. 3B, graphs b and c) and a significant IgA response in lung lavage samples (Fig. 3D, graphs b and c). These results confirm that the salmonella vector is being recognized and processed by the host immune system. However, the i.p. delivery of the *M. hyopneumoniae* NrdF protein also induced significant serum IgG (Fig. 3B, graph d) and lung IgG *S. typhimurium* antibody responses (Fig. 3D, graph d), indicating cross-reactivity between the recombinant NrdF protein and epitopes found in *S. typhimurium* SL3261 as previously described (12).

The accessibility of the NrdF antigen on the surfaces of intact *M. hyopneumoniae* cells was examined by whole-cell ELISA. The primary antigen was 100 μl of *M. hyopneumoniae* whole-cell suspension (0.03 A_{650} units) in 0.20 mM Tris-HCl (pH 7.2) containing 10% methanol coupled to microtiter plates (Linbro) by centrifugation (2,000 × *g* for 30 min). The supernatant was subsequently removed, 200 μl of blocking solution (2% skim milk in PBS) was added, and the plates were incubated for 1 h at room temperature and then washed and processed as described previously. There were significant secretory IgA levels ($P < 0.05$) in the lung washes of the live orally vaccinated mice; this IgA recognized the cell surface of *M. hyopneumoniae* in a whole-cell ELISA (Fig. 4B, graph c). Significant serum IgG in the i.p. immunized 15-kDa treatment group (Fig. 4A, graph d) was also observed.

Bacterin vaccines are relatively expensive because mycoplasma culture is time-consuming, medium components are expensive, and relatively poor bacterial yields are achieved. In addition, protection against subsequent infection and improvement in growth rates of swine are variable (18, 22). Little is known about the protective mechanism effective against mycoplasmal infection, including the identities of antigens responsible for evoking a protective immune response. Several studies have demonstrated that *M. hyopneumoniae* attaches to cilial tufts which line the epithelium of the bronchioles and trachea of the pig; *M. hyopneumoniae* has never been observed intracellulary (16, 31). The secretion of antibodies to mucosal surfaces is believed to be pivotal in the inhibition of adherence and cytotoxicity and also in the blocking of essential metabolic processes, and as such the generation of mucosal gA against specific antigens is likely to afford protection (26, 36). We have demonstrated that *S. typhimurium* SL3261(pKF1) evoked a mucosal IgA response specifically against a 15-kDa recombinant *M. hyopneumoniae* NrdF antigen. In addition we demonstrated that these antibodies bound to the surface of *M. hyopneumoniae* in a whole-cell ELISA.

Attenuated recombinant vaccines, with *S. typhimurium aroA* as the delivery vector, may provide cheap and easily administrable vaccines. *M. hyopneumoniae* predisposes swine to potentially devastating secondary infections. Targeting the ribonucleotide reductase NrdF protein of *M. hyopneumoniae* may lead to an efficacious vaccine as this enzyme appears to be essential to the metabolic process. NrdF catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, an essential step in DNA replication (34). Inhibition studies of the herpes simplex virus type 1 (33) and *E. coli* (11) ribonucleotide reductase enzymes suggest that the region of the NrdF protein that is targeted (with nonapeptides) causing inhibition is the same carboxy-terminal region that is expressed in the *S. typhimurium* SL3261(pKF1) oral vaccine. In the physiology of *M. hyopneumoniae*, the absence of a cell wall

and outer membrane makes this pathogen particularly vulnerable to neutralizing antibodies (2, 36). Our whole-cell ELISA demonstrates the surface accessibility of the in situ NrdF protein in *M. hyopneumoniae* to specific antibodies raised in orally vaccinated mice (Fig. 4).

The recombinant 15-kDa antigen encoded by plasmid pKF1 has no carrier molecule that would direct the protein to the cell surface or elsewhere. Interestingly, there is no requirement to use strategies to surface express the NrdF antigen in order to promote a lung mucosal IgA response after oral vaccination. Due to a demonstrated 48.4% identical amino acid homology with *S. typhimurium* NrdF, and an 86.6% homology if conserved amino acids are considered, some degree of cross-reactivity is expected between the NrdF *M. hyopneumoniae* antigen and the NrdF protein of *S. typhimurium* (12). This does not present a problem in the detection of NrdF-specific antibody responses. Antibodies raised against the *M. hyopneumoniae* NrdF antigen recognize cross-reactive epitopes in the *S. typhimurium* SL3261 ELISA, resulting in high absorbance values in this ELISA due to the excess of *S. typhimurium* antigen coating in the assay wells. The lack of antibody response in the *S. typhimurium* SL3261 oral control groups against *M. hyopneumoniae* NrdF protein (Fig. 3A and C, graph b) indicates that the bacterial cross-reactive antigen is not immunogenic. This could be because either the *S. typhimurium* protein is not being expressed in sufficiently large quantities or the bacterial antigen is not being processed and presented to the host immune system.

The successful oral immunization against a heterologous *M. hyopneumoniae* antigen in a bacterial vector has not been previously reported. This study is unique not only because it demonstrates that there were no problems associated with the adenosine-tyrosine bias and different codon usage between the selected mycoplasma antigen and salmonella vector but also because it demonstrates that an immune response specific for this antigen can reduce the severity of PEP in swine (12). Although the physiologies of the mucosal immune systems of mice and pigs are different, the induction in mice of a secretory response against an essential metabolic protein from a swine respiratory pathogen leads to the exciting prospect of conducting trials of this oral recombinant vaccine in pigs.

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