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Development of non-antibiotic-resistant, chromosomally based, constitutive and inducible expression systems for aroA-attenuated salmonella enterica serovar typhimurium

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Abstract

Live-vaccine delivery systems expressing two model antigens from Mycoplasma hyopneumoniae, F2(P97) (Adh) and NrdF, were constructed using Salmonella enterica serovar Typhimurium aroA (STM-1), and immunogenicity in mice was evaluated. Recombinant plasmid-based expression (PBE) and chromosomally based expression (CBE) systems were constructed. The PBE system was formed by cloning both antigen genes into pJLA507 to create an operon downstream of temperature-inducible promoters. Constitutive CBE was achieved using a promoter-trapping technique whereby the promoterless operon was stably integrated into the chromosome of STM-1, and the expression of antigens was assessed. The chromosomal position of the operon was mapped in four clones. Inducible CBE was obtained by using the in vivo-induced sspA promoter and recombining the expression construct into aroD. Dual expression of the antigens was detected in all systems, with PBE producing much larger quantities of both antigens. The stability of antigen expression after in vivo passage was 100% for all CBE strains recovered. PBE and CBE strains were selected for comparison in a vaccination trial. The vaccine strains were delivered orally into mice, and significant systemic immunoglobulin M (IgM) and IgG responses against both antigens were detected among all CBE groups. No significant immune response was detected using PBE strains. Expression of recombinant antigens in S. enterica serovar Typhimurium aroA from chromosomally located strong promoters without the use of antibiotic resistance markers is a reliable and effective method of inducing a significant immune response.

Keywords

Development, non, antibiotic, resistant, chromosomally, based, constitutive, inducible, expression, systems, for, aroA, attenuated, salmonella, enterica, serovar, typhimurium

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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Development of Non-Antibiotic-Resistant, Chromosomally Based, Constitutive and Inducible Expression Systems for *aroA*-Attenuated *Salmonella enterica* Serovar Typhimurium▽

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Live-vaccine delivery systems expressing two model antigens from *Mycoplasma hyopneumoniae*, $F2_{p97}$ (Adh) and NrdF, were constructed using *Salmonella enterica* serovar Typhimurium *aroA* (STM-1), and immunogenicity in mice was evaluated. Recombinant plasmid-based expression (PBE) and chromosomally based expression (CBE) systems were constructed. The PBE system was formed by cloning both antigen genes into pJLA507 to create an operon downstream of temperature-inducible promoters. Constitutive CBE was achieved using a promoter-trapping technique whereby the promoterless operon was stably integrated into the chromosome of STM-1, and the expression of antigens was assessed. The chromosomal position of the operon was mapped in four clones. Inducible CBE was obtained by using the in vivo-induced *sspA* promoter and recombining the expression construct into *aroD*. Dual expression of the antigens was detected in all systems, with PBE producing much larger quantities of both antigens. The stability of antigen expression after in vivo passage was 100% for all CBE strains recovered. PBE and CBE strains were selected for comparison in a vaccination trial. The vaccine strains were delivered orally into mice, and significant systemic immunoglobulin M (IgM) and IgG responses against both antigens were detected among all CBE groups. No significant immune response was detected using PBE strains. Expression of recombinant antigens in *S. enterica* serovar Typhimurium *aroA* from chromosomally located strong promoters without the use of antibiotic resistance markers is a reliable and effective method of inducing a significant immune response.

The use of live, attenuated bacteria as vaccine delivery systems for heterologous antigens has been extensively studied. In particular, attenuated *Salmonella* strains have been modified to express a wide range of antigens from bacterial, parasitic, and viral sources (reviewed in references 20, 29, and 41). After oral administration, *Salmonella* can penetrate the Peyer's patches via M cells and colonize the mesenteric lymph nodes, which contain various antigen-presenting cells (reviewed in reference 5). This can generate a range of immune responses, including systemic and mucosal responses at local and distal sites. Other advantages of using attenuated *Salmonella* include the ease of oral administration, which bypasses the need for needle administration; increased antigen presentation due to the use of a live-vector delivery system, the induction of both Th1 and Th2 immune responses; and the wide range of attenuated *Salmonella* and recombinant vectors available to researchers (19, 20, 51).

However, there are a number of issues to overcome. Most methods for the expression of heterologous antigens in *Salmonella* use plasmids to express the antigenic proteins. This can have several drawbacks. The stable maintenance of the expression plasmid in vivo can be difficult to achieve. Tightly regulated promoters are often used to increase plasmid stability, and several in vivo-inducible promoters have delivered promising results. Oral delivery of *aroAD*-attenuated *Salmonella enterica* serovar Typhimurium expressing the C fragment of tetanus toxin from *nirBp* was able to protect mice from lethal tetanus challenge (6). Other in vivo-inducible promoters, such as *pagCp*, *sspAp*, and *ssaGp*, have also been used in *aroAD*-attenuated *S. enterica* serovar Typhimurium to generate tetanus toxoid-specific and heat-labile toxin B immune responses in mice (12, 35, 48).

Instability may arise through the extra metabolic burden associated with a high-copy-number plasmid, leading to the selection of variants that have lost the plasmid during growth. In vitro, plasmids can be maintained through the use of antibiotic resistance markers; however, this is not feasible under field conditions, with emerging antibiotic resistance a global health issue. In order for these vaccines to be used in a commercial human or veterinary setting, the antibiotic resistance genes must be removed, although a selection mechanism for the maintenance of plasmids during vaccine production would still be required (48). One method for nonantibiotic maintenance of plasmid vectors is the use of the *asd* vector/Δ*asd* host lethality system, in which the attenuated *S. enterica* serovar Typhimurium has an obligatory requirement for diaminopimelic acid that is complemented by the vector (39). Nonantibiotic resistance markers have also been developed, including *bar*, which encodes resistance to the herbicide DL-phosphinothricin (38); *merA*, which provides resistance to organomercurial compounds (21); and *arsAB*, which express arsenite resis-

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain/plasmid	Characteristics	Source
E. coli		
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^-) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	52
M15(pREP4)	Derivative of *E. coli* K-12; contains kanamycin-resistant pREP4, ensuring the production of high levels of *lac* repressor protein	Qiagen
S. enterica serovar Typhimurium		
STM-1	*aroA*-attenuated strain	1
JA08	Laboratory-passaged STM-1 strain; streptomycin sensitive, rifampin resistant	48
STM-AN1	Chromosomal insertion of the *nrdF-adh* operon into *dps* of STM-1	This study
STM-AN2	Chromosomal insertion of the *nrdF-adh* operon into *glgP* of STM-1	This study
STM-AN3	Chromosomal insertion of the *nrdF-adh* operon into *orf1* of STM-1	This study
STM-AN4	Chromosomal insertion of the *nrdF-adh* operon into *traI* of STM-1	This study
STM-sspA	Chromosomal insertion of the *nrdF-adh* operon with *sspAp* into *aroD* of JA08	This study
M. hyopneumoniae J	Laboratory-passaged nonvirulent strain	44
Plasmids		
pJLA506	Ampicillin-resistant expression vector containing P_LP_R promoters and *atpE* ribosome binding sites	45
pJLA507	pJLA506 with an XhoI-NcoI-EcoRI polylinker inserted into the EcoRI site	This study
pJLA507-A	pJLA507 with *M. hyopneumoniae adh* gene cloned between NdeI and SalI sites	This study
pJLA507-N	pJLA507 with *M. hyopneumoniae nrdF* gene cloned between NdeI and SalI sites	This study
pJLA507-AN	pJLA507-N with *M. hyopneumoniae adh* gene cloned into SalI site	This study
pUC18-NotI	Identical to pUC18 but with NotI-EcoRI-SalI-HindIII-NotI as multiple-cloning site	24
pUC18-AN	pUC18-NotI with promoterless *nrdF-adh* operon cloned into NotI	This study
pUT/Ars	Ap^r; Tn*5*-based delivery plasmid with *arsA* and *arsB*	24
pArs-AN	pUT/Ars with promoterless *nrdF-adh* operon cloned into NotI site	This study
pKKsspATetC	*sspA* promoter cloned into pKK233-2	48
pKKsspA-AN	pKKsspA with *nrdF-adh* operon cloned between NcoI/HindIII sites	This study
pCVDaroDins	pCVD442 with 5′ and 3′ sections of *S. enterica aroD*	48
pCVD-AN	pCVDaroDins with *sspAp-nrdF-adh-rrnBt* cassette cloned into SphI and SacI	This study
$pF2_{P97}$	His-tagged $F2_{P97}$ expression vector based on pQE9	27
pKF1	His-tagged NrdF expression vector based on pQE9	16

tance proteins (9). Plasmid-based expression (PBE) systems can express antigens at high levels; however, high levels of expression of some antigens can have a growth-inhibitory effect (10, 51) and thus can reduce the efficacy of the live delivery system.

To overcome some of the problems associated with PBE systems, heterologous antigen expression cassettes have been integrated into the *Salmonella* chromosome (25, 47). The chromosomally integrated constructs have been examined as vaccines in several studies and were shown to elicit a protective immune response, although generally the level of antigen expression is much lower than in plasmid-based systems (20).

In this study, we used two *Mycoplasma hyopneumoniae* antigens, $F2_{P97}$ (hereafter referred to as Adh) and ribonucleotide reductase (NrdF), in a screen to identify novel promoters useful for antigen expression in *S. enterica* serovar Typhimurium from single-copy chromosomal constructs. *M. hyopneumoniae* is a pathogen of swine that colonizes the ciliated epithelial cells of the respiratory tract and causes significant economic losses (11). Adh and NrdF have both been previously studied in vaccination experiments when expressed from plasmid-based systems in *S. enterica* serovar Typhimurium *aroA* (7, 8, 15, 16, 17). Oral vaccination with NrdF-expressing *S. enterica* serovar Typhimurium *aroA* has resulted in significant immunoglobulin A (IgA) responses in murine lungs (16), increased murine splenocyte NrdF-specific gamma interferon (IFN-γ) production (7), and primed the porcine respiratory tract for an NrdF-specific secretory IgA response (17). Adh-stimulated splenocytes from mice orally vaccinated with *S. enterica* serovar Typhimurium *aroA* expressing Adh showed increased IFN-γ production (8). The constructs generated in this study, which expressed both antigens in tandem, were used to orally vaccinate mice, and the immune responses were evaluated.

MATERIALS AND METHODS

Growth of bacterial strains and isolation of genomic and plasmid DNA. The bacterial strains and plasmids used in this study are listed in Table 1. *M. hyopneumoniae* was grown in Friis medium as previously described (18). *Escherichia coli* and *S. enterica* serovar Typhimurium strains were grown in Luria-Bertani (LB) medium supplemented with antibiotics, where appropriate, at 37°C. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml: kanamycin, 50 μg/ml: rifampin (rifampicin), 100 μg/ml: and streptomycin, 100 μg/ml. Growth curve analysis with minimal medium supplemented with aromix was performed as previously described (33).

Plasmid DNA was isolated using the Wizard Plus SV Miniprep kit (Promega, Australia) according to the manufacturer's instructions. Isolation of genomic DNA was achieved using the DNeasy Blood and Tissue Kit (Qiagen, Australia) according to the manufacturer's instructions.

For protein expression, His_6-tagged Adh (27) and NrdF (16) expression vectors were used. *E. coli* cultures were grown to an optical density at 560 nm (OD_{560}) of 0.8 at 37°C, induced with isopropyl thiogalactopyranoside at a final concentration of 1 mM, and grown for a further 4 h. The cells were pelleted at 5,000 × *g* for 10 min, and the recombinant protein was purified using nickel-nitrilotriacetic acid resin (Qiagen) as specified in the manufacturer's instructions.

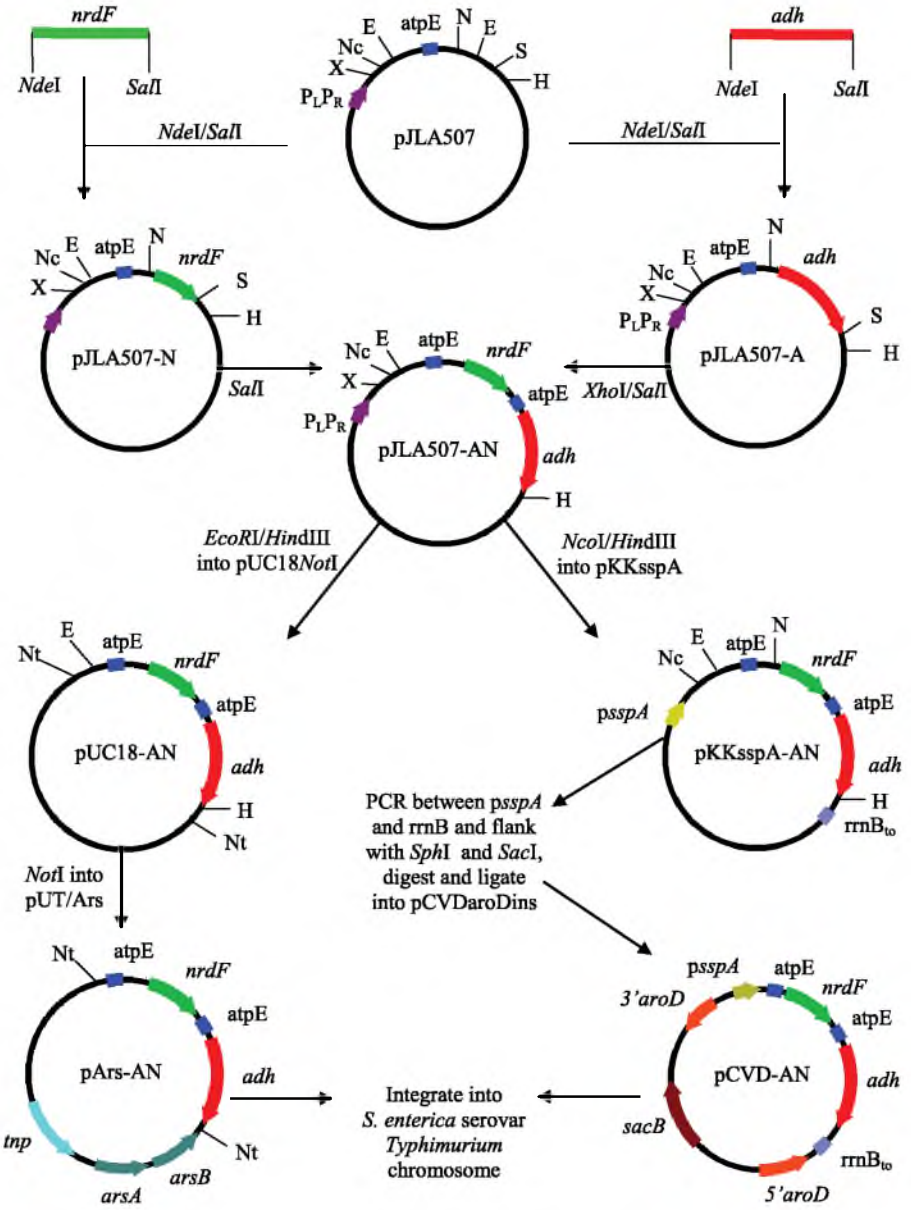

FIG. 1. Cloning schematic for the production of PBE and CBE constructs. E, EcoRI; H, HindIII; N, NdeI; Nc, NcoI; Nt, NotI; S, SalI; X, XhoI; atpE, atpE ribosome binding site; rrnBto, *E. coli* rRNA terminator; *tnp*, transposase. The diagram is not to scale.

Construction of recombinant plasmids. Restriction enzyme digestions and DNA ligations were performed according to standard procedures (43). To construct pJLA507-AN, *nrdF* was PCR amplified from *M. hyopneumoniae* strain J DNA using the primers 5′-GGCATATGGATCTATTATATAAACTAATT-3′ and 5′-GGGTCGACTTAAAACTCCCAATCTTCATG 3′. This PCR product encompassed the DNA encoding the 11-kDa carboxy terminus of NrdF. The *adh* gene was PCR amplified from *M. hyopneumoniae* strain J DNA using the primers 5′-GGCATATGAAATTAGACGATAATCTTCAG-3′ and 5′-GGGTCGACT TAAGGATCACCGGATTTTGAA-3′. This PCR product encompassed the DNA encoding an approximately 36-kDa segment of P97 incorporating both C-terminal repeat regions and is referred to as Adh. The PCR products and pJLA507 were digested with NdeI and SalI, ligated, and transformed into *E. coli* JM109. Potential clones were screened using digoxigenin (Dig)-labeled probes in a colony hybridization screening procedure according to the manufacturer's instructions (Roche, Australia). Plasmid pJLA507-A was then digested with XhoI and SalI to release the *adh* gene, which was then subcloned into the SalI site of pJLA507-N to yield pJLA507-AN. To construct pARS-AN, pJLA507-AN was digested with EcoRI and HindIII to release the *nrdF-adh* operon, which was then subcloned into pUC18NotI to create pUC18NotI-AN. Plasmid pUC18NotI-AN was digested with NotI, and the *nrdF-adh* operon was cloned into the NotI site of pUT/Ars (24) to form pARS-AN and subsequently transformed into *E. coli* CC118 λ*pir* (Fig. 1).

Construction of the *sspA* chromosomal expression strain began with digesting pJLA507-AN with NcoI and HindIII and then subcloning the promoterless *nrdF-adh* operon into the NcoI and HindIII sites of pKKsspATetC to form pKKsspA-AN. The region from *sspAp* to the *rrnB* terminator sequence (including the *nrdF-adh* operon) was PCR amplified using primers 5′-GTCGAAAGC TTGTCGACTTAAGGA-3′ and 5′-GACTCCCATGGATCTATTATATAAAC T-3′. pCVDaroDins was digested with SphI and SacI, and the PCR product was ligated into these sites to form pCVD-AN.

PCR amplification of the *nrdF-adh* operon for screening purposes was performed using 5′-GGCATATGGATCTATTATATAAACTAATT-3′ and 5′-GGGTCGACTTAAGGATCACCGGATTTTGAA-3′.

Conjugation and arsenic resistance screening. pARS-AN was transformed into *E. coli* SM10 λ*pir*. *S. enterica* serovar Typhimurium STM-1 was mated with SM10 λ*pir*(pARS-AN) overnight on LB agar at 37°C. Prior to being plated onto

selective arsenite [As(III)] medium, the mating mixture was subjected to phosphate starvation. This was achieved by growing the mating mixture for 24 h with shaking at 37°C in 200 ml of minimal phosphate buffer (50 mM MOPS [morpholinepropanesulfonic acid], 50 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM $CaCl_2$, 0.3 mM KH_2PO_4, 0.5 g/liter sodium citrate, 1 g/liter $[NH_4]_2SO_4$, 36 μM $FeSO_4$). As(III)-resistant colonies were then selected on LB agar plates supplemented with streptomycin, 100 μM 2,2′ bipyridyl, and 2 mM As(III) for 96 h at 37°C. To discriminate between genuine transposition events and illegitimate recombination events, arsenite-resistant colonies were screened for the loss of the ampicillin resistance marker by examining sensitivity to ampicillin.

Conjugation of pCVD-AN into *S. enterica* serovar Typhimurium JA08 (a rifampin-resistant, streptomycin-sensitive derivative of STM-1) was performed as previously described (48). Selection for double crossovers using *lacZ* color selection was performed as previously described (48). The double crossovers were confirmed via PCR, and the double-crossover strain was designated STM-sspA.

Colony immunoblotting screening for antigen expression. Colony immunoblotting was performed essentially as previously described (43). The colonies to be screened were picked and patched onto the appropriate agar plates and grown overnight at 37°C. The colonies were lifted onto a nitrocellulose membrane. The nitrocellulose was exposed to chloroform vapor for 15 min in an airtight glass container. The colonies were lysed overnight at room temperature with gentle agitation in lysis buffer (100 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, 1 μg/ml DNase I, 40 μg/ml lysozyme). The nitrocellulose membranes were then washed twice for 30 min each time in phosphate-buffered saline (PBS) (8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na_2HPO_4, 0.2 g/liter KH_2PO_4) and blocked with PBS-5% skim milk for 1 h at room temperature with gentle agitation. The primary antibody was diluted 1:1,000 in PBS-1% skim milk and applied to the membranes for 2 to 4 h at room temperature with gentle agitation. The membranes were then washed three times in PBS for 10 min per wash, and the appropriate secondary antibody was diluted 1:1,000 in PBS-1% skim milk. The membrane was incubated with the secondary-antibody solution for 2 h at room temperature with gentle agitation, followed by three 10-min washes in PBS. The membrane was equilibrated in 100 mM Tris-HCl (pH 7.6) for 2 min and developed in diaminobenzidene developing solution (50 mg diaminobenzidine, 50 ml Tris-HCl, pH 7.6, 30 μl H_2O_2) until sufficient color was obtained. The reaction was then stopped by immersing the membrane in distilled H_2O.

Inverse PCR and Southern hybridization analyses. Chromosomal DNA was isolated from *S. enterica* serovar Typhimurium strains using the DNeasy Blood and Tissue Kit (Qiagen, Australia) according to the manufacturer's instructions. DNA was digested with the appropriate restriction enzymes (Fermentas, Australia), followed by heat inactivation according to the manufacturer's instructions. Chromosomal DNA was then religated overnight in a final volume of 100 μl as described previously. Inverse PCR was then performed using the primer sequences 5′-TCAATTAGTTTATATAATAGATCC-3′ and 5′-TTAGTCAAT TATCGGCTCG-3′, which were outward-facing sequences based on the *adh* and *nrdF* genes, respectively. The PCR products were DNA sequenced as previously described (37).

Southern transfer was performed as previously described (43). Dig-labeled *nrdF* PCR products were used as probes, and Southern hybridization analysis was performed according to the manufacturer's instructions (Roche, Australia).

Induction of PBE strains and expression stability. *S. enterica* serovar Typhimurium STM-1 strains harboring the pJLA507 series of constructs were grown in a 10-ml LB starter culture overnight at 37°C. The entire starter culture was used to inoculate a 50-ml LB culture, and the OD_{560} was adjusted to 0.4 with LB. The strains were grown for 1 h at 37°C. Induction was achieved by culturing the strains at 42°C. Growth curves in minimal medium supplemented with aromix were performed as previously described (33).

SDS-PAGE and Western blot analyses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were run as described by Sambrook et al. (43) and stained with Coomassie brilliant blue. For Western blot analysis, SDS-PAGE gels were electrophoresed but not stained. The samples were normalized by culture amount and visual analysis on Coomassie-stained SDS-PAGE so that equivalent amounts were loaded in each lane. Western transfer and membrane blotting were performed as previously described (15). The membrane was then developed in diaminobenzidene developing solution (50 mg diaminobenzidine, 50 ml Tris-HCl, pH 7.6, 30 μl H_2O_2) until sufficient color was obtained. The reaction was then stopped by immersing the membranes in distilled H_2O. Alternatively, membranes were developed using a SuperSignal West Pico chemiluminescent substrate kit (Pierce, Australia) according to the manufacturer's instructions. For pooled sera, the highest-responding mouse in each chromosomally based expression (CBE)-immunized group (as determined by enzyme-linked immunosorbent assay [ELISA] IgG response against Adh) was selected. Equal volumes of sera from these mice were then pooled, diluted 1:50, and used in Western blot analysis.

Animal immunization procedures. Six-week-old female BALB/c mice were caged separately according to treatment groups (10 mice per group). For live oral vaccinations, CBE cultures were grown to an OD_{560} of 1.0 and pelleted at 5,000 × *g* for 10 min. PBE cultures were induced at 42°C for 4 h and pelleted at 5,000 × *g* for 10 min. All oral vaccination strains were then resuspended in ice-cold PBS with 5% sucrose to an OD corresponding to 1×10^9 viable cells per 100 μl. The mice were deprived of drinking water for 3 h prior to oral immunization. After 3 h, the mice were orally immunized with a single 100-μl dose containing 1×10^9 viable cells delivered behind the incisors using a pipette tip. For intraperitoneal immunization, 50 μg of purified Adh or NrdF was diluted to a total volume of 50 μl in PBS and then mixed with an equal volume of Freund's incomplete adjuvant. The mice were then immunized with a 100-μl dose.

A total of three immunizations were performed for both the oral and intraperitoneal groups, each given 2 weeks apart. Two weeks after the final immunization, five mice in each group were euthanized with CO_2 and exsanguinated by severing the brachial artery (day 42). Sera were collected and stored at −20°C. Lung wash samples were taken using 500 μl PBS containing 2 μM phenylmethylsulfonyl fluoride and stored at −20°C. The remaining five mice in each group were given an intranasal antigen challenge 2 weeks later (day 56) consisting of 5 μg of Adh and 5 μg of NrdF in PBS (total volume, 10 μl). These mice were exsanguinated as described above 2 weeks after antigen challenge (day 70).

The generation of rabbit polyclonal NrdF and Adh antisera was performed as previously described (31).

To determine the in vivo stability of the STM-1 strains, five mice per group were orally inoculated with 1×10^9 CFU as described above. Ten days after inoculation, the mice were exsanguinated as described above and the spleens were removed. The spleens were homogenized in 5 ml ice-cold PBS and plated onto LB-rifampin (STM-sspA) or LB-streptomycin (all other strains). The total CFU were calculated, and 50 colonies (10 colonies per mouse) were randomly selected for further analysis. Southern blotting was performed as described above using a Dig-labeled *nrdF* probe to detect the presence of the operon. Western blotting was performed as described above with rabbit polyclonal Adh antisera.

ELISA protocols and statistical analysis. ELISA was performed as previously described (16), and the results were analyzed using Softmax Pro 4.0 software (Molecular Devices, Australia). For whole-cell ELISA, STM-1 was streaked onto an agar plate and grown overnight at 37°C. A single colony was then used to inoculate 10 ml of LB and was grown in a 37°C shaking incubator until an OD_{600} of 1 was reached. The cells were pelleted at 5,000 × *g* for 10 min at 4°C, and the supernatant was discarded. The pellet was then resuspended in 10 ml of PBS-10% methanol. The cells were dispensed into 1-ml aliquots and stored at −20°C until they were required. The cells were thawed on ice, and 96-well ELISA plates (Interpath, Australia) were then coated with 50 μl of cells per well. The 96-well plates were centrifuged at 420 × *g* for 10 min at room temperature. The solution in the wells was removed, and the plates allowed to air dry. The plates were then blocked, incubated, and developed as described above.

Statistical significance was determined using a Student *t* test by comparison of each trial group to the PBS-negative control group. A P value of <0.05 was considered to be significant.

RESULTS

Construction of PBE strains. The goal of this study was to identify promoters useful for antigen expression in *S. enterica* serovar Typhimurium. We devised a genetic screen using two *M. hyopneumoniae* antigens, Adh ($F2_{P97}$) and NrdF. The *adh* gene encodes a domain containing both repeat regions of P97 (27). P97 is a proteolytically processed adhesin protein capable of binding swine cilia (11, 53). NrdF has been extensively studied as a putative vaccine candidate, and previous vaccination studies have demonstrated that NrdF can invoke Th1 and Th2 immune responses and improve the average daily weight gain of swine challenged with *M. hyopneumoniae* (7, 16, 17).

The cloning scheme of the PBE constructs is outlined in Fig. 1. *NrdF* and *adh* antigens were PCR amplified from *M. hyopneumoniae* chromosomal DNA and flanked with NdeI and SalI restriction sites, which facilitated cloning into pJLA507 to produce pJLA507-A (containing *adh*) and pJLA507-N (con-

taining *nrdF*). The pJLA507-A vector was digested with XhoI/SalI, and *adh* was subcloned into the SalI site of pJLA507-N to produce pJLA507-AN. Plasmid pJLA507-AN is capable of expressing both antigens as individual proteins from the temperature-inducible P_LP_R promoter (45).

Construction of CBE strains. In order to attain antigen expression from single-copy non-antibiotic-resistant chromosomal constructs in *S. enterica* serovar Typhimurium, CBE systems were engineered. Initially the promoterless *nrdF-adh* operon from pJLA507-AN was subcloned into pUC18*Not*, which flanked the *nrdF-adh* operon with NotI sites. Plasmid pUC18NotI-AN was digested with NotI, and the *nrdF-adh* operon was subcloned into pUT/Ars to produce pARS-AN (Fig. 1). Plasmid pARS-AN was then transformed into SM10 λ*pir*, and a promoter-trapping experiment was performed whereby SM10 λ*pir*(pARS-AN) was mated with *S. enterica* serovar Typhimurium STM-1. A total of 1,200 arsenite-resistant, ampicillin-sensitive STM-1 transconjugants were screened for the expression of Adh via colony immunoblotting (results not shown), and four highly expressing transconjugants were selected for further characterization. The presence of the *nrdF-adh* operon in the chromosomes of arsenite-resistant, Adh-expressing STM-1 colonies was confirmed using PCR and Southern hybridization analyses (Fig. 2).

To construct the *sspA* chromosomal expression strain, pJLA507-AN was digested with NcoI and HindIII, and the promoterless *nrdF-adh* operon was subcloned into the NcoI and HindIII sites of pKKsspA to form pKKsspA-AN. The region from p*sspA* to *rrnBt*, including the *nrdF-adh* operon, was PCR amplified and flanked with SphI and SacI sites. pCVDaro-Dins was digested with SphI and SacI, and the PCR product was cloned between these sites to form pCVD-AN (Fig. 1). pCVD-AN was transformed into *E. coli* S17.1 λ*pir* and mated with STM-1 JA08. Double crossovers were selected using *lacZ* color screening, putative double crossovers were confirmed via PCR (Fig. 2), and a selected strain was designated STM-sspA.

Characterization of STM-1 expression strains. Inverse PCR was performed to examine the insertion point of the *nrdF-adh* operon in the chromosome of STM-1 to determine which promoters were driving antigen expression. Once inverse-PCR products were generated, DNA sequence analysis was used to determine the point of insertion (Fig. 2). Briefly, expression of NrdF and Adh in STM-AN1 was under the control of the *dps* promoter. The *dps* gene encodes a DNA binding protein, which provides starvation-induced resistance to hydrogen peroxide (22). In STM-AN2, the *nrdF-adh* operon has been inserted into the glycogen metabolism operon, namely, the glycogen phosphorylase gene, *glgP* (2). In STM-AN4, the *nrdF-adh* operon has been inserted into a gene required for conjugal transfer, *traI*.

DNA sequence analysis of the inverse-PCR product obtained for STM-AN3 revealed that the construct had been inserted into a novel DNA sequence that showed no homology to any known *Salmonella* genome sequence. In order to confirm the existence of this novel sequence in STM-1, PCR primers were designed based on the sequence, and a PCR product was amplified from the genome of the original parental strain, STM-1 (data not shown). The PCR product was sequenced and was a 100% match to the original inverse-PCR product from STM-AN3 (data not shown). The sequence was assessed

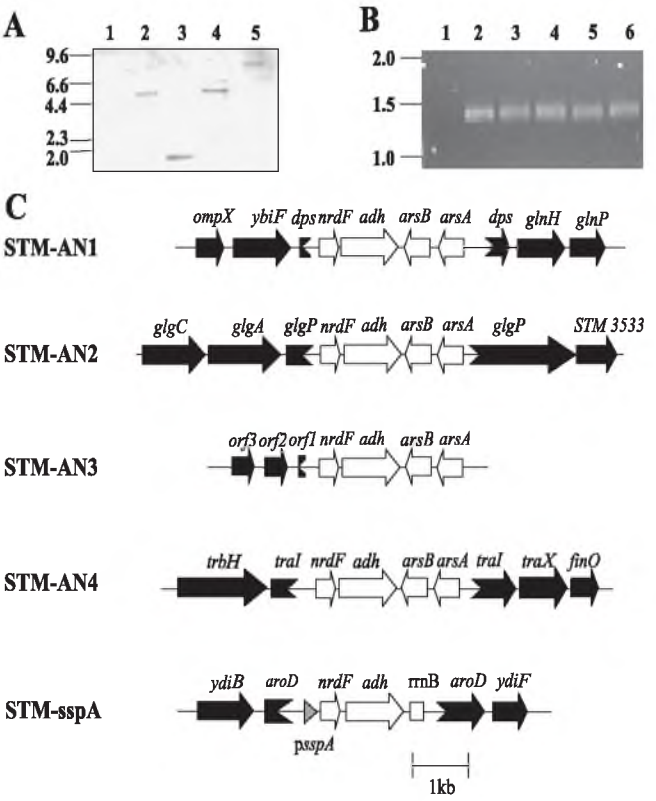

FIG. 2. Chromosomal locations of antigen gene constructs. (A) Southern hybridization analysis of HindIII-digested chromosomal DNA from STM-1 vaccine strains using a Dig-labeled *nrdF* probe. Lanes: 1, STM-1; 2, STM-AN1; 3, STM-AN2; 4, STM-AN3; 5, STM-AN4. Molecular size markers in kb are indicated on the left. (B) PCR of the *nrdF-adh* operon from the chromosomes of STM-1 vaccine strains. Lanes: 1, STM-1; 2, STM-AN1; 3, STM-AN2; 4, STM-AN3; 5, STM-AN4; 6, STM-sspA. Molecular size markers in kb are indicated on the left. (C) Chromosomal locations of the *nrdF-adh* operon within STM-1 vaccine strains as determined using inverse PCR. Gene names are indicated. *adh*, *M. hyopneumoniae* $F2_{p97}$; *aroD*, 3-dehydroquinase; *arsA*, arsenite-translocating ATPase; *arsB*, arsenite efflux membrane protein; *dps*, stress response DNA binding protein; *finO*, FinP binding protein; *glgA*, glycogen synthase; *glgC*, glucose-1-phosphate adenylyltransferase; *glgP*, glycogen phosphorylase; *glnH*, high-affinity glutamine transport protein; *glnP*, glutamine transport permease protein; *nrdF*, *M. hyopneumoniae* ribonucleotide reductase; *ompX*, outer membrane protein X; *orf*, hypothetical open reading frame; p*sspA*, promoter for stringent starvation protein A; rrnB, *E. coli* rRNA terminator; STM3533, putative transcriptional regulator; *traI*, OriT nickase/helicase; *traX*, pilin subunit acetylation; *trbH*, conjugative-transfer protein; *ybiF*, putative permease; *ydiB*, quinate/shikimate dehydrogenase; *ydiF*, putative acetyl-coenzyme A (CoA)/acetoacetyl-CoA transferase beta subunit. The scale in kb is indicated.

for putative open reading frames. A putative open reading frame (*orf1*) was found, and a Blastx search was performed. The sequence was 100% homologous to a hypothetical protein from *Ralstonia solanacearum* (accession number AL646052.1) (42). Analysis of other putative open reading frames (*orf2* and *orf3*) revealed significant amino acid matches (99% and 100%, respectively) to other hypothetical *Ralstonia pickettii* proteins.

Expression profiles of PBE and CBE strains. A comparison of the expression profiles of the recombinant constructs was performed. The expression of NrdF and Adh from STM-1(pJLA507-AN) is presented in Fig. 3. SDS-PAGE revealed a

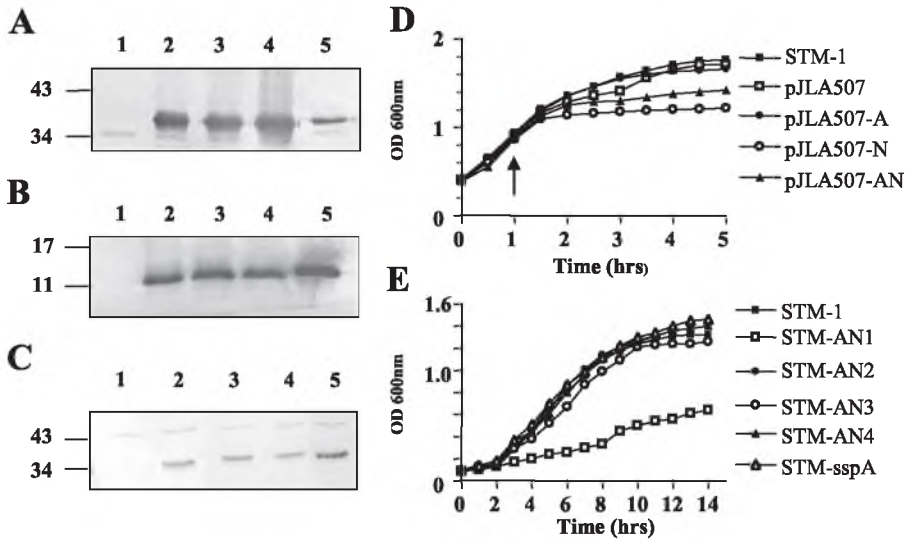

FIG. 3. Growth curve and Western blot analyses of expression from STM-1(pJLA507-AN) and CBE strains. (A and B) Western blot of whole-cell lysates of STM-1(pJLA507-AN) using rabbit polyclonal Adh antisera (A) and rabbit polyclonal NrdF antisera (B). Lane 1, STM-1(pJLA507-AN) preinduction; lane 2, STM-1(pJLA507-AN) 1 h postinduction; lane 3, STM-1(pJLA507-AN) 2 h postinduction; lane 4, STM-1(pJLA507-AN) 4 h postinduction; lane 5, purified His-tagged Adh (200 ng) (A) or purified His-tagged NrdF (5 to 10 μg) (B). (C) Western blot analysis of whole-cell lysates of CBE strains using rabbit polyclonal Adh antisera. Lane 1, STM-1; lane 2, STM-AN1; lane 3, STM-AN2; lane 4, STM-AN3; lane 5, STM-AN4. Molecular mass markers in kDa are shown on the left. (D) Growth curve analysis of PBE strains performed in LB. The strains were grown at 37°C for 1 h prior to induction at 42°C, indicated by the arrow. (E) Growth curves of CBE strains performed in minimal medium supplemented with aromix.

protein with an apparent molecular mass of 36 kDa evident 1 h postinduction (data not shown). Western blot analysis showed that the protein reacted with polyclonal Adh antiserum. Expression of the 11-kDa C terminus of NrdF was not apparent using SDS-PAGE (data not shown). However, Western blotting with polyclonal NrdF antiserum detected the expression of an 11-kDa band, confirming the expression of NrdF in STM-1(pJLA507-AN).

The expression profiles of the CBE strains are also displayed in Fig. 3. Neither Adh nor NrdF expression could be visualized using SDS-PAGE with Coomassie brilliant blue (data not shown). Western blot analysis with polyclonal Adh antisera revealed the presence of a 36-kDa band corresponding to the correct mass of Adh in all CBE strains, with the exception of STM-sspA (data not shown). NrdF expression was faintly detected in the majority of the CBE strains, indicating that expression of NrdF was occurring but that the level of expression was at the very limit of detection (data not shown).

In vitro growth and stability of expression. Growth curves of the CBE and PBE strains were performed to determine what effect the genetic manipulations had on the various strains' abilities to replicate in vitro (Fig. 3). The PBE strains were grown at mid-log phase for 1 h before induction at 42°C. Shortly after induction, two of the PBE strains, STM-1(pJLA507-N) and STM-1(pJLA507-AN), had static levels of growth. Both of the strains express NrdF, suggesting that the production of NrdF had a growth-inhibitory effect on STM-1. CFU counts were conducted, which confirmed that the overexpression of NrdF had a bacteriostatic effect on STM-1(pJLA507-N) and STM-1(pJLA507-AN) (data not shown).

Growth curves of the CBE strains were performed in minimal medium supplemented with aromix to ensure that the insertion of the *nrdF-adh* operon into the chromosome of STM-1 did not further attenuate the strains. Analysis of growth revealed that one strain, STM-AN1, replicated at a much lower rate than the other strains (Fig. 3). This indicated that the insertion of the *nrdF-adh* operon into the *dps* gene attenuated the growth of this strain in minimal medium, making it unsuitable for subsequent vaccination trial experiments.

The stability of antigen expression from the various PBE and CBE strains in vivo was examined. Mice were orally inoculated with 1×10^9 CFU, and the spleens were harvested 10 days postinoculation. Fifty colonies (10 per mouse) were randomly selected for each strain and analyzed by Southern blotting for the presence of the operon and by Western blotting for heterologous antigen expression (Table 2). Colonies were not detected in the spleens for any of the PBE strains or STM-AN2. The *nrdF-adh* operons in colonies that were detected for all other CBE strains showed 100% stability, and all colonies examined were capable of heterologous antigen expression.

Immune responses of mice following oral vaccination. Mouse oral vaccination trials were conducted using the PBE and CBE strains. Mice were given either three oral immunizations with *S. enterica* serovar Typhimurium strains or three intraperitoneal vaccinations with purified Adh or NrdF. Fourteen days after the final vaccination, five mice in each group were sacrificed (day 42). As *M. hyopneumoniae* is not capable of infecting mice, immune recall was tested using purified recombinant antigens. Two weeks later, the remaining five mice were intranasally challenged with purified Adh and NrdF. Serum and lung wash responses were determined using ELISA (Fig. 4). Statistically significant ($P < 0.05$) serum IgM responses against Adh, NrdF, and STM-1 whole cells were detected for all CBE and purified intraperitoneal antigen mice at

TABLE 2. Stability of the *nrdF-adh* operon and heterologous antigen expression in various *S. enterica* serovar Typhimurium *aroA* vaccine strains following 10 days of in vivo passage in mice

Strain	Mouse no.	Total no. of CFU recovered[a]	Operon presence	Antigen expression
STM-AN2	1	0	ND[b]	ND
	2	0	ND	ND
	3	0	ND	ND
	4	0	ND	ND
	5	0	ND	ND
STM-AN3	1	216	10/10	10/10
	2	102	10/10	10/10
	3	36	10/10	10/10
	4	421	10/10	10/10
	5	712	10/10	10/10
STM-AN4	1	37	10/10	10/10
	2	65	10/10	10/10
	3	65	10/10	10/10
	4	167	10/10	10/10
	5	16	10/10	10/10
STM-sspA	1	25	10/10	10/10
	2	40	10/10	10/10
	3	11	10/10	10/10
	4	54	10/10	10/10
	5	15	10/10	10/10
STM(pJLA507)	1	0	ND	ND
	2	0	ND	ND
	3	0	ND	ND
	4	0	ND	ND
	5	0	ND	ND
STM(pJLA507-A)	1	0	ND	ND
	2	0	ND	ND
	3	0	ND	ND
	4	0	ND	ND
	5	0	ND	ND
STM(pJLA507-AN)	1	0	ND	ND
	2	0	ND	ND
	3	0	ND	ND
	4	0	ND	ND
	5	0	ND	ND

[a] In vivo stability determined by orally inoculating groups of five mice with 1×10^9 CFU of a single strain. Ten days after inoculation, the spleens were removed. *S. enetrica* serovar Typhimurium *aroA* was selected by plating homogenized spleens onto Rif LB agar (STM-sspA) or Sm LB agar (all other strains). Colonies were confirmed as *S. enterica* serovar Typhimurium by streaking them onto MacConkey agar. Ten colonies per mouse were randomly selected for further analysis.

[b] ND, no data.

day 42, but not day 70. No IgM response against Adh or NrdF was detected among PBE mice, although there was an IgM STM-1 whole-cell response at day 42 (data not shown). Serum IgG responses against NrdF and Adh are shown in Fig. 4. At day 42, only the purified-intraperitoneal-antigen- and STM-sspA-vaccinated mice had significant IgG responses ($P < 0.05$) against Adh and NrdF. After intranasal challenge (day 70), all of the CBE and intraperitoneally vaccinated mice displayed significant serum IgG responses ($P < 0.05$) against both antigens, indicating that a systemic response had been induced. A serum IgG response against Adh or NrdF could not be detected at any time point for the PBE mice, although an IgG response against STM-1 whole cells was detected. The lung wash samples from the mice were analyzed for the presence of NrdF- and Adh-specific IgA and IgG; however, no significant response was detected at any time point (data not shown).

Pooled sera from orally immunized mice prior to intranasal challenge (day 42) were used in a Western blot against *M. hyopneumoniae* (strain J) whole-cell lysate, purified Adh, and purified NrdF (Fig. 4). The antiserum recognized a protein of approximately 95 kDa in the *M. hyopneumoniae* whole-cell lysate, which corresponds to the mature adhesin protein (27, 50). The antiserum also recognized the adhesin cleavage products, displaying the characteristic ladder pattern previously observed (28, 50, 53). This antiserum recognized a protein corresponding in mass to full-length NrdF (42 kDa) in the *M. hyopneumoniae* whole-cell lysates (15). The antiserum recognized both of the purified antigens, indicating that oral immunization of mice with *S. enterica* serovar Typhimurium is capable of eliciting specific serum IgG responses against both of the antigens.

DISCUSSION

Oral vaccination of recombinant, attenuated live bacterial expression systems is a promising method for heterologous antigen delivery. In this study, CBE of *M. hyopneumoniae* antigens in *aroA*-attenuated *S. enterica* serovar Typhimurium elicited significant systemic immune responses. This approach to vaccine design has a number of advantages that allow it to be used for commercial applications. They include the use of non-antibiotic resistance markers, a stable expression system, low cost of production, and easy administration.

The PBE system used in this study failed to elicit any significant antigen-specific immune responses. Induction of expression in the PBE system was performed at 42°C so that the PBE strains would be loaded with overexpressed antigens upon administration. This is in contrast to the CBE strains, which expressed NrdF and Adh in vitro at much lower levels. Nonetheless, the CBE strains did elicit significant systemic (IgG and IgM) responses in vivo. Lowering the level of heterologous antigen expression may reduce the physiological stress on the host *Salmonella* strain, thus increasing the level of colonization and immune response (46). Colonies were not detected after 10 days of passage in vivo for any of the PBE strains examined. Expression of the antigens and the extra metabolic burden associated with maintaining the plasmid in vivo appear to have severely impacted the abilities of these strains to survive in the murine spleen, which also may have contributed to the lack of an antigen-specific immune response. Dunstan et al. (13) examined the abilities of *aroA*- and *aroD*-attenuated *S. enterica* serovar Typhimurium harboring a range of tetanus toxin fragment C-expressing plasmids to survive in various murine organs after a single inoculation. The authors found colonies in the spleen up to 20 days postinoculation for all vectors examined, although they used an inoculum 10-fold greater than those examined here. This, along with the choice of plasmid vector, may have contributed to these contrasting results.

Overexpression of NrdF in the PBE strains had a bacteriostatic effect on *S. enterica* serovar Typhimurium, which most likely would have had a negative impact on its ability to invade and replicate in vivo. Such a bacteriostatic effect has been

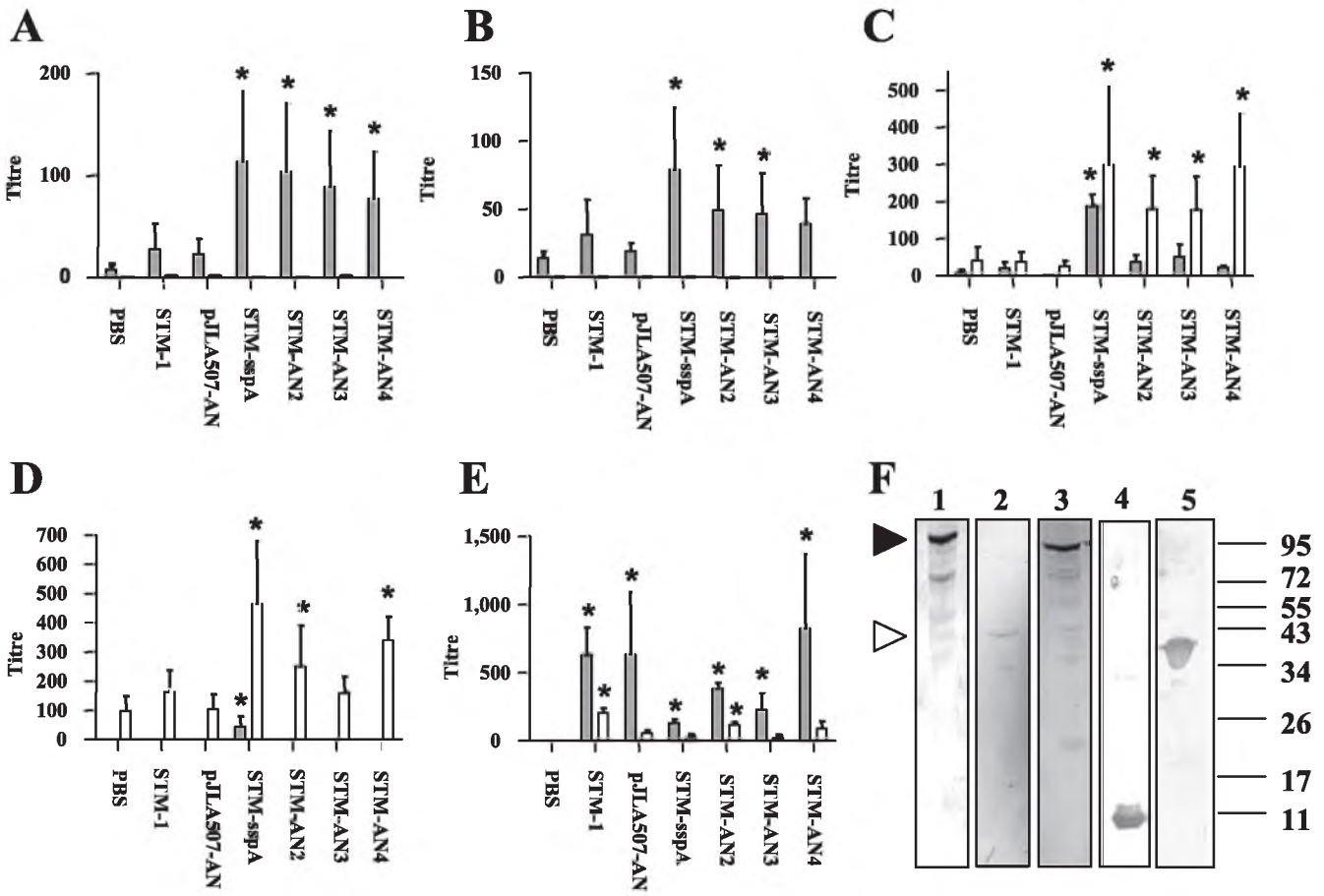

FIG. 4. Murine serum immunoglobulin responses against purified Adh, NrdF, and whole-cell STM-1 as determined by ELISA and Western blotting. Day 42 responses (shaded bars) and day 70 responses (white bars) are shown. The *y* axis represents reciprocal titer values. Standard errors are indicated. Statistical significance ($P < 0.05$) determined by comparison to the PBS group is indicated by asterisks. (A) Serum IgM responses against purified Adh. (B) Serum IgM responses against purified NrdF. (C) Serum IgG responses against purified Adh. (D) Serum IgG responses against purified NrdF. (E) Serum IgG responses against whole-cell STM-1. (F) Western blot analysis of pooled CBE orally immunized-mouse sera (day 42; diluted 1:50) and intraperitoneally immunized-mouse sera (day 42) against *M. hyopneumoniae* (strain J) whole-cell lysate and purified antigen. Lane 1, CBE orally immunized-mouse sera against *M. hyopneumoniae* whole-cell lysate; lane 2, NrdF intraperitoneally immunized-mouse sera against *M. hyopneumoniae* whole-cell lysate; lane 3, Adh intraperitoneally immunized-mouse sera against *M. hyopneumoniae* whole-cell lysate; lane 4, CBE orally immunized-mouse sera against purified NrdF; lane 5, CBE orally immunized-mouse sera against purified Adh. Molecular mass markers in kDa are shown on the right. The filled arrowhead indicates the molecular mass of intact adhesin protein, and the unfilled arrowhead indicates the molecular mass of intact NrdF protein.

shown during the expression of a *Mycoplasma arthritidis* superantigen (10), which decreased the viability of the host *E. coli* cells. Therefore the results presented in this study suggest that the ability of live, *aroA*-attenuated *S. enterica* serovar Typhimurium-based vaccines to replicate and express stably in vivo is more important than the level of antigen expression at immunization in generating systemic immune responses.

Characterization of STM-AN1 revealed the presence of the *nrdF-adh* operon within the *dps* gene. *dps* encodes a DNA binding protein that provides starvation-induced resistance to hydrogen peroxide, and expression is upregulated when *Salmonella* comes under oxidative stress during invasion of macrophages (22, 30). In *E. coli*, *dps* transcription is regulated in a sigma S- and IHF-dependent manner, and the IHF protein has been shown to bind upstream of the *dps* promoter (3). In this study, insertion of the *nrdF-adh* operon into *dps* further attenuated STM-AN1 in minimal medium supplemented with aromix. Given this result and the importance of *dps* during macrophage invasion, STM-AN1 was not used in the vaccine trial.

The *nrdF-adh* operon in STM-AN2 was inserted into *glgP*. This was the only CBE strain for which colonies could not be detected in the murine spleen 10 days after in vivo passage. Previous infection studies in chickens with a glycogen mutant *S. enterica* serovar Typhimurium had shown that glycogen metabolism has a minor role in colonization and pathogenesis but a more significant role in survival (36); this observation agrees with our findings. In this experiment, dual attenuation of *aroA* and *glgP* reduced the ability of *S. enterica* serovar Typhimurium to survive in vivo. Control of the glycogen metabolism pathway is thought to be allosterically regulated by ADP-glucose pyrophosphorylase (40). In *E. coli*, the binding of CsrA to *glgCAP* transcripts promotes *glgCAP* mRNA degradation, which inhibits glycogen metabolism (4). Transcriptional regulation of the operon in *E. coli* also appears to be controlled by RpoS (23).

Recent microarray data suggest the *glgP* transcript was downregulated during *Salmonella* intracellular infection of murine macrophages, although it was below the significant threshold value (14). Despite this observation and the reduced ability of STM-AN2 to survive in the spleen, it was still able to elicit an antigen-specific IgG response.

The position of the *nrdF-adh* operon in STM-AN3 was able to be identified; however, the sequence did not match any known *Salmonella* sequence. The putative open reading frame (*orf1*) driving expression of the antigens had 100% amino acid homology to a hypothetical protein from the plant pathogen *R. solanacearum* (42) and a 99% match to a hypothetical protein from the human pathogen *R. pickettii*. Analysis of the translated sequence using the Pfam and Expasy databases revealed no matches with known proteins. As this sequence has not previously been reported in *Salmonella*, and given the strain's ability to survive in vivo, it is unlikely to be critical for pathogenesis.

STM-AN4, which contains the *nrdF-adh* operon inserted within the conjugal-transfer gene *traI*, displayed significant IgG titers after intranasal challenge. *traI* encodes OriT nickase/helicase (34) and catalyzes the unwinding of the DNA duplex while also acting as a sequence-specific DNA transesterase that provides the site/strand-specific nick required to initiate DNA transfer (32). Microarray analysis performed on the *S. enterica* serovar Typhimurium transcriptome during intracellular infection of murine macrophages did not examine *traI* transcript levels; however, the conjugal-transfer genes on either side (*trbH* and *traX*) both displayed significantly elevated transcript levels (14), suggesting that *traI* may also be upregulated during in vivo infection.

The importance of in vivo expression is further highlighted by the fact that STM1-sspA, which is upregulated in vivo (48), produced the only statistically significant CBE-generated response at day 42 against either Adh or NrdF. The expression of *sspA* in *E. coli* is induced during stationary phase while the bacterium is undergoing starvation for either carbon, amino acids, nitrogen, or phosphate (49). It has been previously demonstrated that *S. enterica* serovar Typhimurium *aroA*, expressing tetanus toxoid under the control of either single-copy (chromosomal) or multicopy (plasmid) *sspAp*, can generate significant immune responses in mice (48). The use of in vivo-inducible promoters for *S. enterica* serovar Typhimurium expression of heterologous antigens appears to be a promising approach, with the use of other promoters, such as *ssaGp* and *pagCp*, increasing the immunogenicity of heterologous antigens in murine models in comparison to native constitutive promoters (13, 35).

The strains capable of surviving for 10 days in vivo all displayed 100% stability of the *nrdF-adh* operon. Husseiny and Hensel (26) reported that integration of a heterologous antigen into *purD* of *S. enterica* serovar Typhimurium was stable after 9 days in vivo; however, they did not report whether the colonies were still capable of expression. All recovered colonies examined in this experiment were capable of heterologous antigen expression, demonstrating that the integrated operon was highly stable.

Previous studies were conducted using attenuated *S. enterica* serovar Typhimurium *aroA* to express single NrdF (7, 16, 17) or Adh (8) antigens. Fagan et al. (16) orally immunized mice with *aroA*-attenuated *S. enterica* serovar Typhimurium SL3261 expressing NrdF from pHSG398. This construct elicited NrdF-specific serum IgA and secretory IgA but failed to produce a significant serum IgG response. Chen et al. (7) expressed NrdF using plasmid-based prokaryotic and eukaryotic expression vectors in *S. enterica* serovar Typhimurium *aroA* CS332, and they failed to elicit a humoral immune response in orally vaccinated mice. However, the splenocytes from the mice produced a significant level of IFN-γ when stimulated with NrdF, indicating the induction of a cell-mediated immune response (7). The varying results from this work and the previous trials indicate the importance of the choice of expression system and *Salmonella* strain for immunization purposes.

This study has demonstrated the ability of native *Salmonella* promoters to stably express heterologous, single-copy antigens from the chromosome and to generate systemic immune responses via the oral immunization route. The main advantages of this technique are that it can be used to generate immune responses against bacteriostatic antigens and can do so without the use of antibiotic resistance markers. The integration of the heterologous expression operon into the *S. enterica* serovar Typhimurium chromosome via a promoter-trapping technique allows many novel promoters and attenuation sites to be simultaneously assessed. This technique also allows the production of stable, cheap, easily administered vaccines that may be used in a commercial setting.

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