Evaluation of the immunogenicity of the P97R1 adhesin of *Mycoplasma hyopneumoniae* as a mucosal vaccine in mice

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The immunogenicity of P97 adhesin repeat region R1 (P97R1) of *Mycoplasma hyopneumoniae*, an important pathogenesis-associated region of P97, was evaluated in mice as a mucosal vaccine. Mice were immunized orally with attenuated *Salmonella typhimurium aroA* strain CS332 harbouring a eukaryotic or prokaryotic expression vector encoding P97R1. Local and systemic immune responses were analysed by ELISA on mouse sera, lung washes and splenocyte supernatants following splenocyte stimulation with specific antigens *in vitro*. Although no P97R1-specific antibody responses were detected in serum and lung washes, significant gamma interferon was produced by P97R1-stimulated splenocytes from mice immunized orally with *S. typhimurium aroA* harbouring either expression system, indicating induction of a cell-mediated immune response. These results suggested that live bacterial vectors carrying DNA vaccines or expressing heterologous antigens preferentially induce a Th1 response. Surprisingly, however, mice immunized with the vaccine carrier *S. typhimurium aroA* CS332 induced serum IgG, but not mucosal IgA, against P97R1 or *S. typhimurium aroA* CS332 whole-cell lysate, emphasizing the importance of assessing the suitability of attenuated *S. typhimurium* antigen-carrier delivery vectors in the mouse model prior to their evaluation as potential vaccines in the target species, which in this instance was pigs.

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INTRODUCTION

Porcine enzootic pneumonia (PEP) caused by *Mycoplasma hyopneumoniae* is a chronic, non-fatal respiratory disease. It affects pigs of all ages, resulting in slow growth and reduced feed efficiency, and causes significant economical losses in the pig industry worldwide (Sheldrake *et al.*, 1991; Thacker *et al.*, 1998). PEP is often followed by secondary infections by *Porcine reproductive and respiratory syndrome virus* and opportunistic bacterial pathogens (Calsamiglia *et al.*, 1999; Sheldrake *et al.*, 1991; Thacker *et al.*, 1998, 1999). As current market vaccines (bacterins prepared by chemical inactivation of whole-cell *M. hyopneumoniae*) do not protect pigs completely from establishment of *M. hyopneumoniae* infection and/or secondary infection (Kristensen *et al.*, 1981; Maes *et al.*, 1999; Murphy *et al.*, 1993; Pallares *et al.*, 2000; Thacker *et al.*, 1998), development of an improved vaccine is desirable.

P97 adhesin has probably been the most-studied and best-defined potential protective antigen of *M. hyopneumoniae* since it was identified as an important adhesin responsible for the adherence of *M. hyopneumoniae* to respiratory ciliated epithelial cells in swine (Zhang *et al.*, 1995). Furthermore, a repeat region of P97 called R1 (P97R1) has been identified as containing both cilium- and antibody-binding sites (Hsu & Minion, 1998; Hsu *et al.*, 1997) and has been reported to function independently from other P97 regions (Minion *et al.*, 2000). P97R1 has been shown to induce significant P97R1-specific serum IgG titres in both mice and pigs (Chen *et al.*, 2001), indicating that it is immunogenic. However, the immunogenicity of P97R1 has yet to be evaluated as a DNA vaccine. Evaluation of this immunization strategy is important, as DNA vaccines have been reported to induce both humoral and cell-mediated immune responses (Gurunathan *et al.*, 2000). Since P97R1 is an adhesin, generation of anti-P97R1 immune responses at the mucosal site may facilitate the prevention of colonization of *M. hyopneumoniae* on respiratory epithelial cells (Sheldrake *et al.*, 1993; Thacker *et al.*, 2000). Such a goal may be achieved by using live attenuated pathogens as antigen carriers, such as aromatic-dependent *Salmonella* and

Abbreviations: AP, alkaline phosphatase; IFN- γ , gamma interferon; P97R1, P97 repeat region R1; PEP, porcine enzootic pneumonia.

The GenBank/EMBL/DBJ accession number for the P97 sequence of *M. hyopneumoniae* is AY957500.

Shigella species, *Mycobacterium bovis* Bacille Calmette-Guérin and *Listeria monocytogenes*, to deliver DNA vaccine plasmids with a view to generating mucosal antibodies (Darji *et al.*, 1997; Dietrich *et al.*, 1998; Fennelly *et al.*, 1999; Paglia *et al.*, 1998; Pasetti *et al.*, 1999; Sizemore *et al.*, 1995, 1997). The aim of this study was to evaluate humoral, local and cell-mediated immune responses against P97R1 as a bacterial DNA vaccine delivered orally via an attenuated *S. typhimurium aroA* in a mouse model. As a comparison, the immunogenicity of *S. typhimurium aroA* expressing P97R1 as a recombinant protein was also investigated.

METHODS

Bacterial strains. *M. hyopneumoniae* strain A518/1 was provided by Ms J. Forbes-Faulkner (Oonoonba Veterinary Laboratory, Queensland, Australia). $r^- m^+$ *S. typhimurium* strain P9121 was provided by Dr Bruce Stocker (Stanford University, California, USA) and *S. typhimurium aroA* strain CS332 was obtained from McMaster Laboratory, Sydney University Campus, CSIRO Division of Animal Health, Australia.

Growth and isolation of genomic DNA of *M. hyopneumoniae*. *M. hyopneumoniae* was grown in modified Friis mycoplasma broth as described by Friis (1975). The culture was grown at 37 °C at 100 r.p.m. until acidity (yellow-brown colour indicating a pH change) was observed. Genomic DNA was extracted using a Quantum Prep AquaPure Genomic DNA kit (Bio-Rad).

Construction of P97R1 expression plasmids. Based on the published P97 gene sequence (GenBank accession no. U50901), the P97 gene was obtained by PCR from strain A518/1 genomic DNA using primers 5'-ATGAGTAAAAATCAAAAACATTTAAATTTGGTT-3' and 5'-TTATTTAGATTCTGGTTCCTGATTATTATTACTTTG-3'. The P97 PCR product was purified using the High Pure PCR Product Purification kit (Roche) and cloned into the prokaryotic expression vector pTrcHis2 (Invitrogen) according to the manufacturer's instructions. The pTrcHis2-P97 plasmid was purified using a QIAprep Spin Miniprep kit (Qiagen) and sequenced. The P97 sequence has been submitted to GenBank under accession no. AY957500. P97R1 was PCR amplified from pTrcHis2-P97 and cloned into pTrcHis2 using forward primer 5'-GAAGGTAAAAGAGA-AGAAGTAGATAAAAAAGTTAAGG-3' and reverse primer 5'-TGTAAGTGAAAAGCCAGTATTAGTAGCAACTG-3', and into the eukaryotic expression vector pcDNA3.1 (Invitrogen) using the same primers except for the addition of a Kozak sequence (ATTATGG) to the 5' end of the forward primer. The resultant plasmids, pTrcHis2-P97R1 and pcDNA3.1-P97R1, were then purified and sequenced.

Expression of the P97R1 fusion protein in *S. typhimurium aroA* and purification. The plasmid pTrcHis2-P97R1 purified from *Escherichia coli* TOPO 10 cells was first electrotransformed into $r^- m^+$ *S. typhimurium* P9121; the purified plasmid was subsequently electrotransformed into *S. typhimurium aroA* CS332 using a 1 mm cuvette at 129 Ω , 25 μ FD and 1.7 kV (Electro Cell Manipulator ECM600 Electroporation System). SDS-PAGE was performed with a 12% gel using the Bio-Rad Mini Protein II Cell apparatus according to the manufacturer's instructions. Western blot analysis was carried out according to the protocol for the use of anti-His(C-terminal)-HRP antibody (Invitrogen). The P97R1 fusion protein was visualized using 5 ml TMB stabilized substrate (Promega). P97R1 was purified under denaturing conditions using a QIAexpress kit for high-level expression and purification of

6 $\times$ His-tagged proteins (Qiagen). Protein concentration was determined using the Coomassie Plus Protein Assay (Pierce) according to the manufacturer's instructions.

Transient expression of the P97R1 fusion protein in mammalian cells. Transient transfection of COS-7 cells with pcDNA3.1-P97R1 was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Detection of the P97R1 fusion protein was performed using a WesternBreeze Novex Chromogenic Western Blot Immunodetection kit (Invitrogen) following the manufacturer's instructions. The antibody used to detect P97R1 was alkaline phosphatase (AP)-conjugated antibody anti-V5 (Invitrogen), as P97R1 was fused to the C-terminal V5 epitope on the vector.

In vitro invasion assay for *S. typhimurium aroA* CS332. An *in vitro* invasion assay for *S. typhimurium aroA* CS332 into the human embryonic intestinal cell line HI-407 was carried out as described by Walker *et al.* (1992). Both *S. typhimurium aroA* and *S. typhimurium aroA* carrying heterologous plasmids were tested for their invasiveness.

Immunization of mice with *S. typhimurium aroA* CS332 live vector vaccines. Preparation of live *Salmonella* carrier vaccines and mouse immunization were carried out as described by Fagan *et al.* (1997). The vaccine groups comprised: (i) *S. typhimurium aroA* CS332 carrying pTrcHis2-P97R1 [*Salmonella*(pTrcP97R1)]; (ii) *Salmonella*(pcDNA3.1-P97R1); (iii) *Salmonella*(pTrcHis2 vector); (iv) *Salmonella*(pcDNA3.1 vector); and (v) PBS (pH 7.2). Each group contained five mice. On day 0, 6–8-week-old female BALB/c mice were each immunized orally with 0.25 ml *Salmonella* suspension (2×10^8 c.f.u.) for groups 1–4. On day 28, mice were given a second dose of 0.25 ml *Salmonella* suspension (3×10^8 c.f.u.) orally. For the PBS group, each mouse was given 0.25 ml PBS orally on days 0 and 28. On day 56, all mice were sacrificed. The molecular microbiological manipulations carried out in this investigation were approved by the Institutional Biosafety Committee and the experiments carried out in mice were approved by the Animal Ethics Committee of the University of Southern Queensland.

Estimation of antibody responses. Sera and lung washes collected as described elsewhere (Fagan *et al.*, 1997) were stored at –20 °C. *S. typhimurium* CS332 whole-cell lysate was prepared by adding formalin to the cell culture to a final concentration of 1%, incubating at 37 °C for 2–4 h and checking for sterility. The culture was pelleted by centrifuging for 10 min at 2000 g and resuspended in bicarbonate buffer (30 mM sodium carbonate, 20 mM sodium hydrogen carbonate, pH 9.6). The suspension was sonicated (5×20 s on/off cycles) at a 60% duty cycle, adjusted to an OD₆₀₀ of 0.4 and stored at –20 °C. ELISA was performed according to Desolme *et al.* (2000) with the following modifications. Microtitre plates were coated with either 50 μ l purified P97R1 (5 μ g ml^{–1}) or 50 μ l *S. typhimurium* whole-cell lysate. The secondary antibody used was horseradish peroxidase-conjugated goat anti-mouse IgG or IgA (Sigma). The variation between plates was normalized against positive control sera by multiplying the test sample titre by the ratio of the mean positive control titre to the positive control titre for a particular plate (Mukkur *et al.*, 1995). The antigen-specific antibody titres were determined as the reciprocal of the dilution at a cut-off point of 2.5 times the absorbance of the lowest dilution of the sera from non-immunized mice.

Determination of cytokine production. The spleens from each vaccine group were removed aseptically and pooled into 5 ml Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 100 U penicillin/streptomycin ml^{–1} and 50 μ M 2-mercaptoethanol on ice. Splenocytes were isolated using a cell constrainer (Becton Dickinson) and centrifuged at 800 g for 10 min at room temperature. The supernatant was removed, the cell pellet was resuspended

in 10 ml of the above medium and a 10 µl aliquot was removed for cell counting before the solution was centrifuged again as described above. The washed cell pellet was resuspended in 10 ml DMEM containing 100 U penicillin/streptomycin ml⁻¹, 10 % fetal bovine serum and 50 µM 2-mercaptoethanol and diluted to 5 × 10⁶ cells ml⁻¹. Cells (2 ml) were added to each well of a 24-well plate. The antigen stimulant (purified P97R1 fusion protein) was then added at a concentration of 1 µg ml⁻¹ to wells in duplicate. The plate was incubated at 37 °C in 5 % CO₂ for 72 h. The splenocyte culture supernatants were collected and stored at -20 or at -70 °C for long-term storage. Cytokine production in the splenocyte supernatants was measured using a Mouse Cytokine ELISA kit (Pierce) according to the manufacturer's instructions.

Statistical analysis. Values for antibody titres and cytokine production were compared using one-way ANOVA. Results were considered significant for values of $P \leq 0.05$.

RESULTS

Analysis of the P97R1 sequence

DNA sequencing analysis showed that the P97R1 fragment from *M. hyopneumoniae* strain A518/1 was 285 bp and contained 11 (A/T)KP(E/V)(A/T) amino acid repeats, which are characteristic of P97 (Wilton *et al.*, 1998), and one VKPVA sequence (Fig. 1).

Expression of P97R1 in *S. typhimurium* aroA and protein purification

Expression of P97R1 in *S. typhimurium* aroA CS332 was observed by Western blotting following IPTG induction or simply by growing the bacteria overnight in Terrific broth containing 100 µg ampicillin ml⁻¹. The P97R1 fusion protein was detected by anti-His(C-terminal)-HRP antibody in the Western blot. The size of P97R1 was estimated to be ~17 kDa including the 3–4 kDa C-terminal tag from the vector (Fig. 2).

Expression of P97R1 in mammalian cells

Plasmid pcDNA3.1-P97R1 purified from *S. typhimurium* aroA CS332 was transfected into COS-7 cells. Expression of

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GAAGGTAAGAGAGAGAGTAGATAAAAAAGTTAAGGAATTAGATAATAAATAAAGGT
1  E G K R E E V D K K V K E L D N K I K G
ATATTACCTCAGCCCCAGCGGCGAAACCGGAAGCGGTAAACAGTAGCGGCTAAACCT
61  I L P Q P P A A K P E A V K P V A A K P
GAAGCAGCTAAACCTGAAGCAGCAAAACAGTAGCAGCTAAACCTGAAGCAGCAAAACCA
121 E A A K P E A A K P V A A K P E A A K P
GTAGCAGCTAAACCGGAAGCAGCAAAACAGTTGCTGCCAAGCCAGAAGCGGCAAAACCA
181 V A A K P E A A K P V A A K P E A A K P
GTAGCAGCAAAACAGTTGCTACTAATACTGGCTTTTCACTTACA
241 V A A K P V A T N T G F S L T -
    
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Fig. 1. DNA and amino acid sequences of the P97R1 fragment from *M. hyopneumoniae* strain A518/1.

Fig. 2. Expression of the P97R1 fusion protein in *S. typhimurium* aroA CS332. Lanes: 1, BenchMark Protein Ladder; 2, pTrcHis2 vector; 3, induction of P97R1 expression by IPTG; 4, expression of P97R1 in *S. typhimurium* aroA CS332 in Terrific broth containing 100 µg ampicillin ml⁻¹ after overnight incubation. P97R1 was detected using anti-His(C-terminal)-HRP antibody in the Western blot.

the P97R1 fusion protein (fused to the C-terminal V5 epitope) was examined by Western blotting using anti-V5-AP antibody (Fig. 3). The size of the P97R1 fusion protein expressed in COS-7 cells was similar to that expressed in bacteria.

In vitro invasiveness of *S. typhimurium* aroA CS332

An investigation was carried out to test the effect of the presence of recombinant plasmids on the *in vitro* invasiveness of *S. typhimurium* aroA CS332. The results showed that

Fig. 3. Expression of P97R1 fusion protein in COS-7 cells by Western blot. Lanes: 1, BenchMark Protein Ladder; 2, COS-7 cells transfected with pcDNA3.1-P97R1; 3, COS-7 cells transfected with pcDNA3.1 vector. Detection of the P97R1 fusion protein was carried out using a WesternBreeze Novex Chromogenic Western Blot Immunodetection kit (Invitrogen) using anti-V5-AP antibody (Invitrogen).

Table 1. Evaluation of the *in vitro* invasiveness of *S. typhimurium* aroA CS332

Results are given as the mean number of viable bacteria (c.f.u.) per well $\pm$ SEM from duplicate wells and counted by plating in duplicate.

Strain	Invasiveness
<i>S. typhimurium</i> aroA CS332	$4.8 \times 10^3 \pm 227$
<i>Salmonella</i> (pTrcHis2 vector)	$4.4 \times 10^3 \pm 107$
<i>Salmonella</i> (pcDNA3.1 vector)	$5.7 \times 10^3 \pm 175$
<i>Salmonella</i> (pTrcP97R1)	$4.1 \times 10^3 \pm 155$
<i>Salmonella</i> (pcDNA3.1-P97R1)	$6.5 \times 10^3 \pm 85$

the ability of *S. typhimurium* aroA CS332 to invade the human intestinal cell line HI-407 was not affected by the presence of either plasmid vector or recombinant plasmid (Table 1).

Analysis of antibody response by ELISA

Sera and lung washes were analysed for antigen-specific antibody responses. No serum or mucosal antibody (IgG or IgA) responses against the P97R1 fusion protein were detected in mice immunized with *Salmonella*(pTrcP97R1) or *Salmonella*(pcDNA3.1-P97R1) when compared with control groups vaccinated with PBS, *Salmonella*(pTrcHis2 vector) or *Salmonella*(pcDNA3.1 vector). All of the groups, except for the PBS control group, induced serum IgG responses against *S. typhimurium* whole-cell lysate (Table 2). Although each group was immunized with the same amount of *S. typhimurium*, the magnitude of the IgG responses among these groups varied. However, no mucosal IgA against *S. typhimurium* aroA whole-cell lysate was detected in the lung washes of any of these groups.

Measurement of cytokine levels by ELISA

Splenocytes isolated from pooled spleens from each group of five mice were stimulated with the P97R1 fusion protein and cultured in duplicate *in vitro* for 3 days. The supernatants were removed and used for determination of

Fig. 4. *In vitro* production of IFN- γ by P97R1-stimulated splenocytes. Pbs, Mice vaccinated with PBS; Trv, mice vaccinated with *Salmonella*(pTrcHis2 vector); Pcv, mice vaccinated with *Salmonella*(pcDNA3.1 vector); TrP97R1, mice vaccinated with *Salmonella*(pTrcP97R1); PcP97R1, mice vaccinated with *Salmonella*(pcDNA3.1-P97R1). Results are shown as the mean $\pm$ SEM from duplicate culture wells pooled from five mice. *, Significant difference in IFN- γ production ($P < 0.05$) compared with the control groups (Pbs, Trv and Pcv).

cytokine production using a mouse cytokine ELISA. Both the *Salmonella*(pTrcP97R1) and *Salmonella*(pcDNA3.1-P97R1) groups produced significant levels of P97R1-specific gamma interferon (IFN- γ) but not interleukin-4 when compared with the control groups (Fig. 4).

DISCUSSION

Studies of P97R1 in different strains have shown that they contain different numbers of repeats of the amino acid sequence (A/T)KP(E/V)(A/T) arranged in tandem (Wilton *et al.*, 1998). In our study, analysis of *M. hyopneumoniae* strain A518/1 revealed that P97R1 contains 11 (A/T)KP(E/V)(A/T) repeats and one VKPVA sequence. The presence of VKPVA is not common, but has also been found in strain Sue (Wilton *et al.*, 1998). Our results confirmed the variability of both the number and the composition of P97R1 repeats in *M. hyopneumoniae*.

Table 2. Estimated antibody levels against P97R1 and the antigen carrier *S. typhimurium* aroA CS332 in vaccinated versus control mice

Results are given as the mean titre $\pm$ SD from the five mice in each group.

Experimental groups	Antibody titres against P97R1		Antibody titres against <i>Salmonella</i>	
	Serum IgG	Secretory IgA	Serum IgG	Secretory IgA
PBS control	14.12 $\pm$ 2.75	5.23 $\pm$ 1.24	15.43 $\pm$ 3.21	5.77 $\pm$ 3.48
<i>Salmonella</i> (pTrcHis2 vector) (prokaryotic plasmid control)	16.64 $\pm$ 3.04	6.43 $\pm$ 1.45	583.18 $\pm$ 357.23	7.57 $\pm$ 4.92
<i>Salmonella</i> (pcDNA3.1 vector) (eukaryotic plasmid control)	15.87 $\pm$ 3.10	5.87 $\pm$ 2.11	1412.56 $\pm$ 935.62	7.92 $\pm$ 3.67
<i>Salmonella</i> (pTrcP97R1) (vaccine)	18.52 $\pm$ 4.56	5.38 $\pm$ 2.46	627.58 $\pm$ 453.30	6.67 $\pm$ 3.23
<i>Salmonella</i> (pcDNA3.1-P97R1) (vaccine)	17.16 $\pm$ 4.88	7.65 $\pm$ 3.28	168.67 $\pm$ 23.10	7.48 $\pm$ 3.53

In this present study, the immunogenicity of *S. typhimurium* harbouring P97R1 under the control of a eukaryotic or prokaryotic expression system was investigated in mice. Serum and local immune responses were compared between *Salmonella*(pTrcP97R1) and *Salmonella*(pcDNA3.1-P97R1). Serum ELISA results showed that neither *Salmonella*-(pTrcP97R1) nor *Salmonella*(pcDNA3.1-P97R1) induced measurable P97R1-specific IgG, IgM or IgA antibodies compared with the control groups. Similarly, no IgG or IgA against P97R1 was detected in the lung washes of mice vaccinated with either *Salmonella*(pTrcHis2-P97R1) or *Salmonella*(pcDNA3.1-P97R1). In contrast, a previous study (Fagan *et al.*, 1997) reported that mice immunized orally with *S. typhimurium aroA* strain SL3261 expressing the *M. hyopneumoniae* NrdF fragment produced significant NrdF-specific IgA in the lungs, but failed to elicit significant levels of IgG, IgM or IgA in the serum. The failure to induce serum antigen-specific antibody responses in both studies is unclear, but possibly is due to the fact that the nature of the antigen may have had an impact on the type of immune response, as suggested by Boyle *et al.* (1997). However, the failure to induce P97R1-specific mucosal IgA in our study may have been due to the use of *S. typhimurium aroA* strain CS332 rather than strain SL3261. In all of the groups vaccinated with *S. typhimurium aroA* CS332, only serum IgG against *S. typhimurium* was detected and no lung IgA was found. This observation is supported indirectly by previous studies (Mukkur & Walker, 1992; Mukkur *et al.*, 1995) in which mice and sheep vaccinated orally with strain CS332 did not induce detectable mucosal IgA in intestinal washes, despite the fact that strain CS332 was invasive *in vivo*, as judged by the production of acute enteritis of jejunum and ileum accompanied by multiple focal hepatitis, albeit low grade, although the latter was not observed in sheep (Begg, 1990). To ensure that *S. typhimurium aroA* CS332 was invasive in this study, an *in vitro* invasion assay was performed, which showed that *S. typhimurium* invaded intestinal HI-407 cells relatively efficiently and that there was no significant difference in invasiveness between *S. typhimurium* and *S. typhimurium* carrying heterologous plasmids. Thus it was apparent that other factors may have contributed to the variability in immunogenicity in mucosal vaccination models. The animal and the strain used have been shown to affect the extent and quality of the immune response (Begg, 1990; Dunstan *et al.*, 1998; Soo *et al.*, 1998; Valentine *et al.*, 1998). For example, it has been shown that *Ity^F* mice vaccinated orally with *S. typhimurium* expressing *Leishmania* gp63 antigen induce Th1 responses, whereas *Ity^B* mice induce Th2 responses when immunized with the same vaccine (Soo *et al.*, 1998). The gene selected for insertional mutagenesis of the bacterial live vector has also been shown to influence the extent and quality of the immune response. Studies comparing three *S. typhimurium* MudJ *lacZ*-inactivated mutants (CL288, CL401 and CL554) and a prototype *aroA* mutant showed that the former mutant strains elicited higher serum IgG and/or mucosal IgA titres than the prototype *aroA* mutant strain (Valentine *et al.*, 1998), presumably because they were able to persist in

the murine tissues for longer periods than the *aroA* mutant strain.

Another possible explanation for the poor induction of local mucosal IgA by mucosal immunization could be the observation that cells presenting the encoded antigen after DNA transfer or vaccine carrier have been found to persist mainly in spleen and lymph nodes for a considerable length of time. This may be one reason for the immune response being Th1-oriented, with very little local antibody production (Darji *et al.*, 2000; Dunstan *et al.*, 1998; Reinhardt *et al.*, 2001; Roberts *et al.*, 1998; Woo *et al.*, 2001; Zhang *et al.*, 2001). Similarly, in our study, although *Salmonella*(pTrcHis2-P97R1) and *Salmonella*(pcDNA3.1-P97R1) did not induce P97R1-specific serum IgG or mucosal IgA, both groups induced significant P97R1-specific IFN- γ production compared with the control groups. This finding is supported by other studies in which *Salmonella*-based DNA vaccines or *Salmonella* expressing heterologous antigens were reported to induce a predominately Th1 response (Darji *et al.*, 2000; Dunstan *et al.*, 1998; Reinhardt *et al.*, 2001; Roberts *et al.*, 1998; Woo *et al.*, 2001; Zheng *et al.*, 2001). *M. hyopneumoniae* potential antigens NrdF and P97 used as vaccines have also been shown to induce a presumed antigen-specific Th1 response, although only in pigs, considered to be responsible for higher mean daily weight gains and reduced lung lesion scores (Fagan *et al.*, 2001; Shimoji *et al.*, 2003). In contrast, pigs vaccinated intramuscularly with the P97 fusion protein induced a strong P97-specific serum antibody response, but failed to protect pigs against *M. hyopneumoniae* challenge (King *et al.*, 1997). In fact, serum antibodies induced by commercial *M. hyopneumoniae* vaccines do not correlate with protection against *M. hyopneumoniae* challenge (Maes *et al.*, 1999; Pallares *et al.*, 2000; Thacker *et al.*, 1998). Thus it has been suggested that serum antibodies may not play a protective role in PEP; instead cell-mediated immune responses may be important in mediating protection against PEP (Darji *et al.*, 1997; Shimoji *et al.*, 2003; Thacker *et al.*, 2000).

In our investigation, we showed that P97R1 adhesin presented to the mouse as either a bacterium-based DNA vaccine or a bacterium expressing the heterologous P97R1 antigen induced P97R1-specific Th1 responses in mice but without any anti-P97R1 mucosal antibody responses. Whether the failure of *S. typhimurium aroA* CS332 to induce mucosal antibody responses in this investigation was associated with the characteristics of the insertional mutation in the antigen-carrier *S. typhimurium* strain warrants further investigation.

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