

# Evaluation of recombinant *Brachyspira pilosicoli* oligopeptide-binding proteins as vaccine candidates in a mouse model of intestinal spirochaetosis

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The anaerobic intestinal spirochaete *Brachyspira pilosicoli* colonizes the large intestine of humans, and various species of animals and birds, in which it may induce a mild colitis and diarrhoea. The aim of the current study was to evaluate the use of putative oligopeptide-binding proteins of *B. pilosicoli* as vaccine components. A partial genome sequence of *B. pilosicoli* porcine strain 95/1000 was subjected to bioinformatics analysis, and six genes predicted to encode oligopeptide-binding proteins were selected. Following a PCR-based distribution study of the genes across different strains of the spirochaete, they were amplified from *B. pilosicoli* human strain WesB and cloned in *Escherichia coli*. The recombinant histidine-tagged proteins were purified and subjected to *in vitro* and *in vivo* immunogenicity analysis. Recombinant products (P-1 and P-3) from two genes that were immunogenic and recognized by sera from pigs that had recovered from *B. pilosicoli* infections were tested in a mouse model of intestinal spirochaetosis. For each recombinant protein, groups of 12 C3H/HeJ mice were vaccinated subcutaneously with 100 µg protein emulsified in Freund's incomplete adjuvant, twice with a 2 week interval. Two weeks later the vaccinated and non-vaccinated control animals were challenged orally with *B. pilosicoli* strain WesB. Both proteins induced systemic and local colonic IgG antibody responses, and, following experimental infection, the cumulative number of colonization days was significantly ( $P < 0.001$ ) less in both groups of vaccinated mice compared to the control mice. There were significantly ( $P = 0.012$ ) fewer mice colonized in the group vaccinated with P-1 than in the non-vaccinated control group. The results suggest that oligopeptide-binding proteins may have potential for use as components of vaccines for *B. pilosicoli*.

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## INTRODUCTION

*Brachyspira pilosicoli* is a weakly haemolytic anaerobic spirochaete that colonizes the large intestine of human beings, and a number of species of animals and birds, notably chickens, pigs, dogs and horses (Hampson *et al.*, 2006). In pigs the infection can lead to diarrhoea and poor growth rates (Hampson & Duhamel, 2006), and in adult chickens it is associated with wet faeces and reduced egg production (Hampson & Swayne, 2008). In humans, colonization may result in various non-specific problems, including abdominal pain, chronic diarrhoea and a failure to thrive in children (Douglas & Crucioli, 1981; Peghini *et al.*, 2000; Brooke *et al.*, 2006; Esteve *et al.*, 2006). *B.*

*pilosicoli* also has been isolated from the bloodstream of critically ill and immunocompromised patients (Fournié-Amazouz *et al.*, 1995; Trott *et al.*, 1997b; Kanavaki *et al.*, 2002; Bait-Merabet *et al.*, 2008; Zeeshan *et al.*, 2009). Although intestinal spirochaetosis (IS) caused by *B. pilosicoli* is not commonly reported in the general western population, the spirochaete is found at a high prevalence amongst people who live in conditions of poor hygiene in rural areas of developing countries (Trott *et al.*, 1997a; Margawani *et al.*, 2004), in indigenous populations such as Australian Aboriginals (Lee & Hampson, 1992), in homosexual males (Trivett-Moore *et al.*, 1998) and in people infected with human immunodeficiency virus (Käsbohrer *et al.*, 1990).

Control of IS largely relies on the use of antimicrobials, although drug resistance in *B. pilosicoli* is quite widespread (Mortimer-Jones *et al.*, 2008). Commercial vaccines for IS are not available, and an autogenous bacterin vaccine failed to protect pigs that were experimentally infected with *B.*

**Abbreviations:** HRP, horseradish peroxidase; IS, intestinal spirochaetosis.

The GenBank/EMBL/DDBJ accession numbers for the *B. pilosicoli* sequences reported in this work are GQ478427–GQ478432.

Tables of primer sequences are available as supplementary data with the online version of this paper.

*pilosicoli* (Hampson *et al.*, 2000). Since vaccines based on recombinant proteins have been developed for the related spirochaete *Brachyspira hyodysenteriae* (La *et al.*, 2004; Song *et al.*, 2009), the use of similar recombinant proteins was worth investigating for *B. pilosicoli*. Oligopeptide-binding proteins (OppA) are part of the ATP-binding cassettes (ABC) that function in the transport of proteins across the bacterial cell envelope. Different forms of these permeases may be potential virulence determinants in various bacterial species, including spirochaetes (Hiron *et al.*, 2007; Veith *et al.*, 2009; Wu *et al.*, 2007). Antibodies against OppA are found in patients with early Lyme disease (Nowalk *et al.*, 2006), and recombinant OppA has been used successfully as a vaccine to provide protection in mice experimentally infected with *Yersinia pestis* (Tanabe *et al.*, 2006). The purpose of the current study was to identify genes predicted to encode OppA-like oligopeptide-binding proteins in *B. pilosicoli*, and to test the recombinant proteins as potential vaccine candidates in a mouse model of IS.

## METHODS

**Permissions.** The infection studies were conducted with the approval of the Murdoch University Animal Ethics Committee, under permit number R2104/07.

**Spirochaete strains and cultivation.** A total of 27 well-characterized strains of *B. pilosicoli* were used in the study, including 20 from pigs and 7 from humans. Twenty-one of the strains were isolated in Australia, and two each were from the UK, the USA and Italy. The strains were obtained as frozen stocks from the culture collection held at the Reference Centre for Intestinal Spirochaetes, School of Veterinary and Biomedical Sciences, Murdoch University. They were thawed, and grown on trypticase soy agar (TSA) (Sigma-Aldrich) plates containing 5% (v/v) defibrinated ovine blood. The plates were incubated for 7–10 days at 37 °C in an anaerobic environment (94% N<sub>2</sub>, 6% CO<sub>2</sub>) generated using anaerobic GasPak Plus sachets (BBL). The purity of the cultures was examined by phase-contrast microscopy, and cells were propagated in modified Kunkle's pre-reduced anaerobic broth, containing 3.5% (v/v) fetal calf serum and 0.5% (v/v) newborn calf serum (Kunkle *et al.*, 1986).

**Genomic sequencing and *in silico* analysis.** The genome of Australian porcine *B. pilosicoli* strain 95/1000 was partially sequenced using a shotgun sequencing approach at the Australian Genome Research Facility, University of Queensland, Brisbane, Queensland, Australia, under a commercial contract, as previously described (Motro *et al.*, 2008). Phred was used for fragment assembly (Ewing *et al.*, 1998), with subsequent viewing using Consed (Gordon *et al.*, 1998). ORF identification was carried out using Glimmer and GeneMark (Lukashin & Borodovsky, 1998; Salzberg *et al.*, 1999). BLAST ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)) was used to search for similarity between the query sequences and sequences in the nucleotide database BLASTN and peptide database BLASTP (Altschul *et al.*, 1990). Pfam (Bateman *et al.*, 2002) and CDD (Marchler-Bauer *et al.*, 2003) were searched to determine the structural and functional building blocks of ORF products or protein domains. PSORTb version 2.0 (<http://www.psort.org/psortb/>) (Nakai, 2000), SignalP version 3.0 HMM (<http://www.cbs.dtu.dk/services/SignalP/>) (Bendtsen *et al.*, 2004), LipoP (<http://www.cbs.dtu.dk/services/LipoP/>) and TMPred ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)) were used to predict the subcellular localization of the ORF products. Six ORFs

predicted to encode membrane-associated oligopeptide or amino acid-binding ABC-type transporter proteins were selected for further analysis. These were given a temporary designation as ORF-1 through ORF-6, with their products given the corresponding name P-1 through P-6. The nucleotide sequences were deposited in GenBank, and the accession numbers are listed in Table 1.

**Gene distribution analysis by PCR.** Pairs of primers that annealed to internal regions of the ORFs were designed using SeqEd version 1.0.3 and the Amplify program version 1.2 (University of Wisconsin) (Supplementary Table S1 available with the online journal), and the distribution of the ORFs amongst the strains of *B. pilosicoli* was analysed by PCR. The amplification mixtures consisted of 1 × PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.6 U *Taq* DNA polymerase, 0.25 mM each dNTP (Promega), 0.5 μM of the primer set, and 50–100 ng chromosomal DNA template in a total reaction volume of 25 μl or 50 μl. The reactions were loaded into 0.2 ml thin-walled thermocycler tubes (Multiply-Pro) and Ultrapure water (Fisher Biotech) was used to make up the reaction volume. Thermocycling conditions consisted of an initial template denaturation for 5 min at 94 °C, followed by 30–35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and primer extension at 72 °C for 1–1.5 min. The final cycle had the extension time increased to 7 min to complete synthesis of all strands. The amplified products were separated by electrophoresis using 1.2% (w/v) agarose in 1 × TAE buffer (40 mM Tris/acetate, 1 mM EDTA), stained by immersion in a 1 μg ethidium bromide ml<sup>-1</sup> solution and viewed with UV light.

**Preparation of recombinant histidine-tagged proteins.** The ORFs were amplified from human *B. pilosicoli* strain WesB using the primers listed in Supplementary Table S2 (available with the online journal). The PCR was performed as for the gene distribution study, except for using 0.06 U *Pfu* DNA polymerase and 12.5 pM each primer. The amplified products were restricted with *Eco*RI and *Bam*HI (New England Biolabs), in a double digest according to the manufacturer's instructions. The restricted insert DNAs were purified using the UltraClean PCR clean-up kit and cloned into the pTrcHis A vector (Invitrogen) according to the manufacturer's instructions. Recombinant histidine-tagged proteins were expressed in *E. coli* BL21 Star (DE3) pLys (Invitrogen) grown in 2YT medium supplemented with 100 μg ampicillin ml<sup>-1</sup>, 0.5–1 mM IPTG and 0.1–1% (w/v) glucose. The recombinant His<sub>6</sub>-proteins were purified by affinity chromatography using nickel nitrilotriacetic acid metal affinity chromatography (Qiagen), conducted as described in the QIAexpressionist handbook. The purified proteins were dialysed against distilled water, and then lyophilized and resuspended in PBS (pH 7.2). Quantification of recombinant His<sub>6</sub>-proteins was carried out by loading serial dilutions of BSA and lysozyme protein standards (100, 250, 500, 1000 ng) on an SDS-PAGE gel, then acquiring gel images using a densitometer (proxPRESS proteomic imaging system; PerkinElmer Life Science). The images were analysed using the Proteome ID analyser version v 1.10 (PerkinElmer Life Science) to calculate the protein concentrations.

**Whole-cell protein preparations of *B. pilosicoli*.** Cells of *B. pilosicoli* strain WesB were centrifuged at 1000 g for 10 min at 4 °C, resuspended in cold PBS and washed three times. The cells were suspended at a concentration of 10<sup>8</sup> cells ml<sup>-1</sup> in PBS, and subjected to four cycles of freeze–thawing followed by sonication on ice for four cycles of 30 s with a 2 min pause between cycles. The sonicate was centrifuged at 10 000 g for 30 min and the supernatant was separated for use in a blot assay. The total protein concentration was quantified using the Bio-Rad Protein assay kit, according to the manufacturer's instructions.

**Preparation of mouse polyclonal antiserum against *B. pilosicoli* strain WesB.** A whole cell bacterin was prepared from *B.*

**Table 1.** Results of bioinformatics analysis of the six ORFs selected for further investigation

Temporary locus ID	GenBank accession no.	Putative identity (e value)	Signal peptide (SignalP)	TM helices (TMpred)	Lipoprotein (LipoP 1.0)	Theoretical molecular mass of product (kDa)
ORF-1	GQ478427	ABC-type oligopeptide transport system (1e-121)	No	2	Yes	60.35
ORF-2	GQ478428	ABC-type oligopeptide transport system (1e-114)	Yes	1	Yes	61.10
ORF-3	GQ478429	ABC-type oligopeptide transport system (2e-75)	Yes	2	Yes	39.40
ORF-4	GQ478430	ABC-type oligopeptide transport system (3e-73)	No	1	No	36.04
ORF-5	GQ478431	ABC-type amino acid transport system (3e-61)	Yes	1	Yes	30.52
ORF-6	GQ478432	ABC-type amino acid transport system (2e-61)	Yes	1	Yes	30.31

*pilosicoli* strain WesB, as previously described (Hampson *et al.*, 2000). A total of  $10^8$  formalinized WesB cells were emulsified in an equal volume of Freund's incomplete adjuvant (Sigma) in a total volume of 100  $\mu$ l and administered subcutaneously into five C3H/HeJ male mice of 5 weeks of age three times at 2 week intervals. Three weeks after the last inoculation, the mice were euthanized by methoxyflurane inhalation followed by cervical dislocation, blood was collected by cardiac puncture, and the serum was separated and stored at  $-20^\circ\text{C}$  until used.

**In vitro immunogenicity analysis.** The recombinant proteins were tested by Western immunoblot with the mouse polyclonal antiserum against *B. pilosicoli*, as well as swine serum obtained from the Centre for Intestinal Spirochaete Research, Murdoch University. The sera originated from healthy pigs as well as animals naturally or experimentally infected with *B. pilosicoli*.

SDS-PAGE gels were used to separate 10  $\mu$ g of each recombinant His<sub>6</sub>-protein and these were electro-transferred to nitrocellulose membranes, which were blocked with TBS-T (Tris buffered saline supplemented with 0.05% v/v Tween 20) containing 5% (w/v) skimmed milk powder, and assembled into a multi-probe apparatus. The mouse and pig sera were diluted 50–100-fold, and were added to the membranes, which were incubated for 1 h on a shaker at room temperature, and then washed three times with TBS-T. The membranes were reacted with a 1:5000 dilution of anti-pig or anti-mouse IgG-alkaline phosphate conjugate for 1 h at room temperature. The membranes were then developed with alkaline phosphatase developing buffer (Bio-Rad), after three washes with Tris buffered saline.

**In vivo immunogenicity analysis.** Thirty-five C3H/HeJ male mice of 5 weeks of age were housed in seven groups of five in the Animal House at Murdoch University. Six groups of mice were injected subcutaneously twice, at a 2 week interval, with 100  $\mu$ g of the appropriate His<sub>6</sub>-protein, resuspended in 50  $\mu$ l PBS, and emulsified in an equal volume of Freund's incomplete adjuvant in a total volume of 100  $\mu$ l. The last group of mice was left unvaccinated. Two weeks after the last inoculation, all the mice were euthanized and the blood was collected as described previously. The immunogenicity of each recombinant protein was confirmed using Western blot against whole-cell protein preparations of *B. pilosicoli* strain WesB and each recombinant His<sub>6</sub>-protein.

**Vaccine efficacy study.** Two selected recombinant proteins (P-1 and P-3) were tested as candidate vaccine antigens against IS in C3H/HeJ mice. Seventy-two female mice of 5 weeks of age were housed in six groups of twelve per cage. The mice were fed on a commercial pelleted basal mouse diet from their arrival until 10 days before the day of first infection with *B. pilosicoli*, when the diet was changed to a

balanced diet containing 63% glucose that has been reported to support infection with *B. pilosicoli* in mice (Sacco *et al.*, 1997). The mice were killed 16 days after the last day of experimental infection.

For each recombinant protein, 12 mice were used as a vaccinated/non-infected group and 12 as a vaccinated/infected group. The mice were injected subcutaneously twice at a 2 week interval with 100  $\mu$ g of the appropriate His<sub>6</sub>-P recombinant protein emulsified in Freund's incomplete adjuvant in a total volume of 100  $\mu$ l. Two control groups each of 12 mice were included, comprising non-vaccinated/infected (infection control) and non-vaccinated/non-infected (negative control) mice. Two weeks after the second vaccination, the mice in the vaccinated/non-infected groups were killed to obtain blood and intestinal samples to analyse systemic and local antibody responses to the vaccines. The mice in the vaccinated/infected groups, and the infection control group were deprived of water for 1 h, then they were infected via gastric intubation with 500  $\mu$ l of fresh motile *B. pilosicoli* strain WesB culture at mid-exponential phase growth at a density of approximately  $10^8$  cells ml<sup>-1</sup>. The inoculation procedure was repeated daily on the following 3 days. The negative control group was not infected and the mice were killed at the end of the experiment and blood and intestine samples collected.

**Faecal sampling, culture and DNA detection.** Faecal pellets (3–4) from each mouse were collected before vaccination, before infection and twice a week thereafter, starting 3 days after the last oral inoculation. Bacteriological swabs were inserted into the faeces, streaked onto selective TSA–CSV plates [TSA containing 20  $\mu$ g colistin ml<sup>-1</sup>, 400  $\mu$ g spectinomycin ml<sup>-1</sup> and 20  $\mu$ g vancomycin ml<sup>-1</sup> (the antimicrobials were supplied by Sigma), and 5% defibrinated ovine blood] (Jenkinson & Wingar, 1981), and incubated for 7–10 days at 37  $^\circ\text{C}$  in an anaerobic environment before being examined. The presence of spirochaetes was identified by a zone of weak  $\beta$ -haemolysis surrounding a low flat haze of bacterial growth. The bacterial growth was examined by phase-contrast microscopy and then resuspended in 50  $\mu$ l lysis buffer [10mM Tris-HCl, 1mM EDTA (pH 8.0)]. The suspension was mixed by vortex and then heated in a boiling water bath for 5 min. The extracts were subjected to a *B. pilosicoli*-specific PCR (La *et al.*, 2003). The products were separated by electrophoresis in a 1.2% agarose gel, stained by immersion for 30 min in 5  $\mu$ g ethidium bromide ml<sup>-1</sup> solution, and viewed with UV light.

Similar faecal samples collected pre-vaccination, post-vaccination and at post-mortem were prepared for measuring antibody content. A 500  $\mu$ l volume of cold PBS containing 5% skimmed milk and 0.5% proteinase inhibitor was added to each sample, which was incubated at 4  $^\circ\text{C}$  overnight until dissolved. After mixing by vortex the supernatant was collected by centrifugation at 16 000 g for 10 min and stored at  $-20^\circ\text{C}$  until used. Blood collected by post-mortem

heart puncture was allowed to stand overnight at 4 °C, the clot was removed and the serum was centrifuged at 2000 g for 10 min at 4 °C to pellet the debris. The sera were removed, mixed with an equal volume of 100 % glycerol and stored at -20 °C.

**Post-mortem tissue and faecal samples.** The luminal surfaces of the caecal and colon walls were rubbed with sterile bacteriological swabs, and the swabs cultured for *B. pilosicoli* as described for the faecal swabs. Contents equal to 3–4 faecal pellets were collected from the caecum and colon into 500 µl cold PBS containing 5 % skimmed milk and 0.5 % proteinase inhibitor for antibody quantification. Sections of the caecal and colon walls were excised and rinsed in cold PBS to remove digesta, and then were placed in 10 % (v/v) buffered formalin for histological examination. The fixed tissue was processed through to paraffin blocks, cut at 4 µm, and stained with haematoxylin and eosin.

**Assays for antibody responses.** After being optimized, ELISAs and Western immunoblots were used to evaluate the systemic and mucosal IgG and IgA responses against the recombinant proteins. The ELISA used 100 ng either of each recombinant protein or of a whole-cell protein preparation of *B. pilosicoli* per well in 0.1 M carbonate buffer (pH 9.6) plus blocking solution (TBS-T containing 5 % (w/v) skimmed milk powder) and mouse serum (1:200) or faecal extract (1:2). Positive ELISA samples also were analysed by Western blot using either 3000 ng recombinant protein or 5000 ng whole cell *B. pilosicoli* protein preparation as antigen. The procedure was as previously described except that goat anti-mouse IgG-HRP (horse-radish peroxidase) or IgA-HRP conjugate at 1:4000 dilution was used to detect mouse primary antibodies, and HRP substrate solution (HRP 10 mg, 16.6 ml PBS pH 7.4, 3.325 ml methanol and 10 µl hydrogen peroxide) was used to develop the colour.

**Data analysis and statistics.** Comparisons were made between the groups of mice. The ELISA values for group systemic and local IgG were calculated as means ± SDs. The statistical differences of the ELISA values between groups of mice in each experiment were analysed using one-way analysis of variance (ANOVA) (in Microsoft Office Excel). Faecal excretion of *B. pilosicoli* for each animal in each group was recorded as positive or negative to create a simple ratio of infected samples per total samples collected for each group. The ratios for each group were compared using  $\chi^2$  analysis, with pairs of observations subsequently analysed using Fischer's exact test.

## RESULTS

### Characteristics and distribution of selected ORFs

A summary of the bioinformatics analysis on the six selected ORFs is presented in Table 1. The ORFs were predicted to encode membrane-associated periplasmic proteins involved in ABC-type oligopeptide or amino acid transport. PCR analysis identified all six ORFs in 93–100 % of the 27 *B. pilosicoli* strains tested. ORF-1 and ORF-2 were not amplified from two Australian porcine strains of *B. pilosicoli*, and ORF-3 was not amplified from another Australian porcine strain. The other three ORFs were amplified from all the strains (Table 1).

### Recombinant protein expression, purification and immunogenicity

All six proteins were successfully expressed in *E. coli* BL21, and purified by affinity chromatography. The molecular

masses of the mature proteins as seen on the SDS-PAGE gels were similar to their predicted masses (Table 1). Five were strongly immunoreactive in Western immunoblots with a set of sera from convalescent pigs, while P-4 was weakly reactive. A similar pattern was seen with mouse sera raised against a bacterin preparation of *B. pilosicoli* strain WesB. Weak Western blot reactivities also were observed between the negative control sera and all the proteins except for P-4. Sera obtained by immunizing mice with the proteins were immunoreactive with their corresponding recombinant proteins, and with their specific native proteins in the whole cell preparation of *B. pilosicoli* strain WesB. Results for recombinant proteins P-1 and P-3 are shown in Fig. 1.

### Antibody responses in vaccinated and infected mice

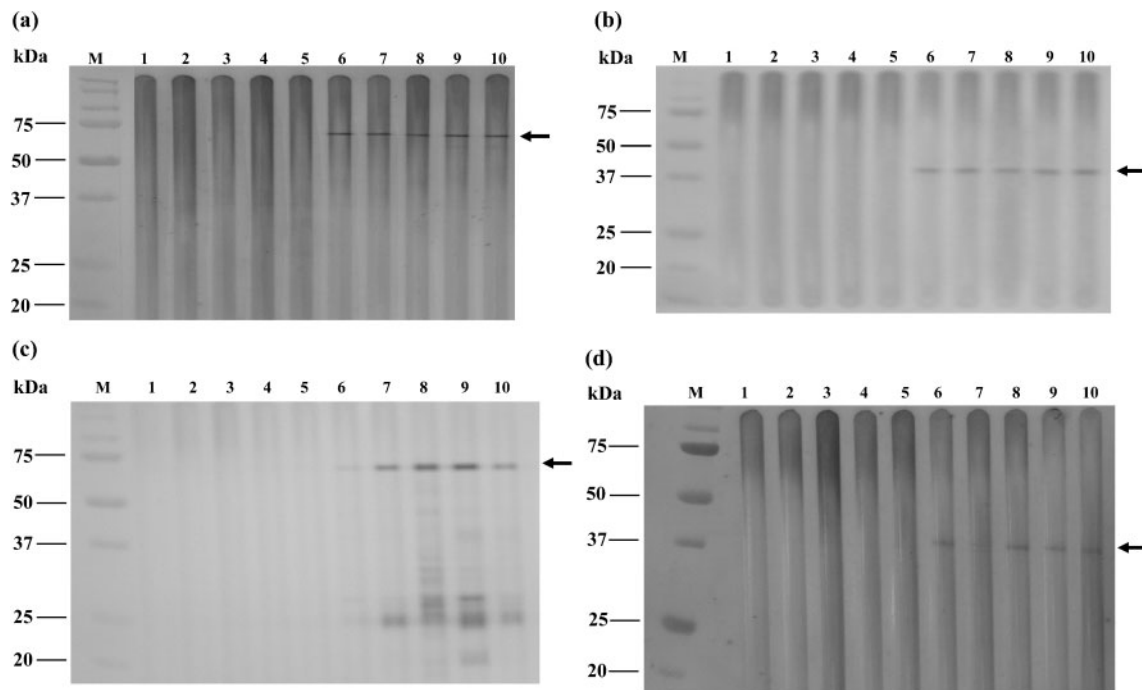
The systemic and large intestinal IgG responses of the mice immunized with P-1 and P-3 in the vaccine experiment are summarized in Table 2. Vaccinated mice had significantly more serum IgG against the corresponding proteins than did non-vaccinated mice. In contrast, infection alone did not significantly increase antibody levels to these proteins. Vaccination with P-1 but not P-3 resulted in a further significant increase in serum antibody levels in vaccinated infected mice compared to vaccinated non-infected mice. Vaccination also resulted in a significant increase in local IgG levels to the corresponding proteins in the mixed samples from the caecae and colons, although this increase was considerably greater with P-1 than with P-3. Infection also resulted in significant increases in IgG levels in the case of P-1. Infection of vaccinated mice only further significantly increased IgG levels in the case of P-3. No IgA to the recombinant proteins was detected (Table 2).

### Faecal shedding of *B. pilosicoli*

The colonization rates are summarized in Table 3. No mice were colonized pre-infection. Colonization was first observed at day 6, and was greatest at day 16, when 9 of the 12 control mice were colonized. The ratio of positive samples/total samples obtained was significantly greater ( $P < 0.001$ ) for the infection control mice compared to both groups of vaccinated mice. Significantly fewer mice vaccinated with P-1 were colonized compared to the control mice ( $P = 0.012$ ), but differences in numbers were not significant for the mice vaccinated with P-3 (Table 3).

### Clinical signs and pathological changes

The caecae and colons of the colonized mice did not show obvious gross changes compared to those of non-colonized mice. Histological examination revealed some mild abnormalities, such as an increase in the number of goblet cells and focal disruption of the epithelium in both caecal and colonic samples of the colonized mice, but no end-on attachment of spirochaetes to enterocytes was observed.



**Fig. 1.** Western immunoblots showing the reactivities of the two recombinant proteins used in the mouse vaccination experiments. Reactivity of recombinant P-1 (a) and P-3 (b) with serum from mice immunized with these proteins. (c, d) Reactivity of the same mouse sera shown in (a) and (b), respectively, with a whole cell preparation of *B. pilosicoli* strain WesB. Lanes 1–5, serum samples from non-vaccinated mice; lanes 6–10, serum samples from mice vaccinated with the respective recombinant proteins; lane M, protein mass marker. The arrows indicate the positions of the reactive proteins, where molecular masses were similar to the predicted molecular masses (Table 1).

## DISCUSSION

The mouse model of IS adapted in the current study was based on that used by Sacco *et al.* (1997), including feeding a disaccharide-rich diet and using C3H/HeJ mice. The model worked reasonably well, with 75% of the non-vaccinated control animals becoming colonized; however, end-on attachment of spirochaetes was not observed, and colonization did not induce clinical signs or consistent pathological changes. In a previous study, the same *B. pilosicoli* strain (WesB) similarly failed to attach to colonic enterocytes or cause obvious pathology in a mouse model

of IS (Jamshidian *et al.*, 2004). Despite this limitation, vaccine efficacy could be judged in relation to colonization rates in this model.

The aim of the study was to determine whether immunization with Opp-like oligopeptide-binding proteins from *B. pilosicoli* could protect from IS, based on the report that recombinant Opp has shown efficacy against *Y. pestis* infection in mice (Tanabe *et al.*, 2006). To achieve this, a small number of genes predicted to encode membrane-associated oligopeptide-binding proteins in *B. pilosicoli* were selected from an incomplete genome

**Table 2.** Means and SDs of group systemic and local large intestinal IgG levels (OD<sub>450</sub> values) against the recombinant proteins in vaccinated and non-vaccinated mice that were either not infected or infected with *B. pilosicoli*

ELISA antigen/ vaccine group	Antibody	Non-infected		Infected		P value (ANOVA)*
		Non-vaccinated	Vaccinated	Non-vaccinated	Vaccinated	
P-1	Systemic	0.198 ± 0.039 <sup>a</sup>	2.935 ± 0.153 <sup>b</sup>	0.277 ± 0.114 <sup>a</sup>	3.211 ± 0.123 <sup>c</sup>	<0.001
	Local	0.036 ± 0.004 <sup>a</sup>	1.263 ± 0.47 <sup>c</sup>	0.101 ± 0.036 <sup>b</sup>	1.434 ± 0.581 <sup>c</sup>	<0.001
P-3	Systemic	0.134 ± 0.059 <sup>a</sup>	2.195 ± 0.444 <sup>b</sup>	0.078 ± 0.012 <sup>a</sup>	2.608 ± 0.417 <sup>b</sup>	<0.001
	Local	0.040 ± 0.004 <sup>a</sup>	0.192 ± 0.089 <sup>b</sup>	0.058 ± 0.007 <sup>a</sup>	0.327 ± 0.117 <sup>c</sup>	<0.001

\*Within each row, values with a different superscript letter differ at the 5% level of significance.

**Table 3.** Number of colonized mice and cumulative group colonization results for the groups of 12 vaccinated and non-vaccinated mice orally challenged with *B. pilosicoli*

Group	Time post-infection (days)					Positive samples/ total samples*	No. of colonized mice†
	3	6	9	12	16		
P-1 vaccinated	0	1	2	2	2	7/60	2
P-3 vaccinated	0	2	3	3	4	12/60	5
Infection control	0	4	7	9	9	29/60	9

\*The ratio was significantly greater ( $P < 0.001$ ) for the infection control mice compared to both groups of vaccinated mice.

†Significantly fewer mice vaccinated with P-1 were colonized compared to the control mice ( $P = 0.012$ ). Other differences were not significant.

sequence. Clearly, in future studies, it also would be possible to use the same 'reverse vaccinology' process, starting with the genome sequence, to select and test other classes of potential vaccine candidates, such as outer-membrane lipoproteins and secreted proteins (Movahedi & Hampson, 2008). The genes that were selected in the current study were widely distributed amongst different *B. pilosicoli* strains, indicating that if the products were effective as vaccine components against one strain they probably would be effective against a wide range of strains. The corresponding recombinant proteins were all immunogenic in mice, and they were recognized by serum from naturally or experimentally infected pigs. This finding supported the likelihood that these molecules could induce an immune response with potential to protect from infection. Reactivity to P-4 was weaker than to the other proteins, and so it was excluded on that basis. Interestingly, P-4 was the only molecule that was not predicted to be a lipoprotein. P-5 and P-6 were predicted to be involved in amino acid transport rather than oligopeptide transport, so these were not selected for testing in mice. Two of the remaining three recombinant proteins were predicted to have signal peptides, suggesting that they were secretory, and one of each sort were selected for testing in the mouse model of IS.

As anticipated, the experimental vaccines induced significant increases in systemic IgG levels against the corresponding proteins. The subcutaneous route of vaccine administration induced a mucosal IgG antibody response in the large intestine, although this was only relatively high in the case of P-1. Infection alone also induced a similar local response against P-1. Infection of vaccinated mice further significantly increased systemic IgG only in the case of P-1, whereas there was an increased antibody response to P-3 but not to P-1 following infection of the vaccinated mice. The lack of a local IgA response in the infected mice was unexpected, but in part this may reflect the relatively short period that the mice were kept in the experiments.

Both vaccinated groups had significantly fewer days of colonization than did the infection control group, hence providing evidence that the vaccines were having some effect in reducing colonization. Furthermore, vaccination

with P-1, which induced the highest levels of systemic and local IgG, also significantly reduced the number of colonized mice. Further studies are now required to test these recombinant proteins as vaccine candidates in other natural hosts, such as chickens and pigs. In addition, a more detailed investigation of the structure and function of these predicted oligopeptide-binding proteins is required.

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