Identification and cloning of the gene encoding BmpC: an outer-membrane lipoprotein associated with *Brachyspira pilosicoli* membrane vesicles

Darren J. Trott,[1] David P. Alt,[3] Richard L. Zuerner,[3] Dieter M. Bulach,[2] Michael J. Wannemuehler,[6] Judi Stasko,[4] Kirsty M. Townsend[1] and Thaddeus B. Stanton[5]

Correspondence
Darren J. Trott
d.trott@mailbox.uq.edu.au

[1]School of Veterinary Science, The University of Queensland, St Lucia, Queensland, Australia

[2]Bacterial Pathogenesis Research Group, Department of Microbiology, Monash University, Victoria, Australia

[3,4,5]Bacterial Diseases of Livestock Research[3], Microscopy Services[4], Pre-Harvest Food Safety Research[5], National Animal Disease Center, Ames, IA, USA

[6]Veterinary Medical Research Institute, Iowa State University, Ames, IA, USA

Received 9 September 2003
Revised 11 November 2003
Accepted 15 December 2003

The intestinal spirochaete *Brachyspira pilosicoli* causes colitis in a wide variety of host species. Little is known about the structure or protein constituents of the *B. pilosicoli* outer membrane (OM). To identify surface-exposed proteins in this species, membrane vesicles were isolated from *B. pilosicoli* strain 95-1000 cells by osmotic lysis in dH_2O followed by isopycnic centrifugation in sucrose density gradients. The membrane vesicles were separated into a high-density fraction (HDMV; $\rho = 1{\cdot}18$ g cm^{-3}) and a low-density fraction (LDMV; $\rho = 1{\cdot}12$ g cm^{-3}). Both fractions were free of flagella and soluble protein contamination. LDMV contained predominantly OM markers (lipo-oligosaccharide and a 29 kDa *B. pilosicoli* OM protein) and was used as a source of antigens to produce mAbs. Five *B. pilosicoli*-specific mAbs reacting with proteins with molecular masses of 23, 24, 35, 61 and 79 kDa were characterized. The 23 kDa protein was only partially soluble in Triton X-114, whereas the 24 and 35 kDa proteins were enriched in the detergent phase, implying that they were integral membrane proteins or lipoproteins. All three proteins were localized to the *B. pilosicoli* OM by immunogold labelling using specific mAbs. The gene encoding the abundant, surface-exposed 23 kDa protein was identified by screening a *B. pilosicoli* 95-1000 genome library with the mAb and was expressed in *Escherichia coli*. Sequence analysis showed that it encoded a unique lipoprotein, designated BmpC. Recombinant BmpC partitioned predominantly in the OM fraction of *E. coli* strain SOLR. The mAb to BmpC was used to screen a collection of 13 genetically heterogeneous strains of *B. pilosicoli* isolated from five different host species. Interestingly, only strain 95-1000 was reactive with the mAb, indicating that either the surface-exposed epitope on BmpC is variable between strains or that the protein is restricted in its distribution within *B. pilosicoli*.

INTRODUCTION

Brachyspira pilosicoli is the agent of porcine intestinal spirochaetosis (also known as porcine colonic spirochaetosis), a production-limiting disease of swine characterized by non-bloody, mucus-containing diarrhoea, poor feed conversion and depressed growth rates (Hampson & Trott, 1999; Ochiai *et al.*, 1997; Stanton, 2002; Thomson *et al.*, 1998; Trott *et al.*, 1996d). *B. pilosicoli* infection also results in delayed onset of egg production and pasty, wet faeces in poultry (Stephens & Hampson, 2002; Trampel *et al.*, 1994). This spirochaete has been isolated from a wide range of other monogastric hosts, including dogs (Duhamel *et al.*, 1998), monkeys (Duhamel, 2001), game birds (Trott *et al.*, 1996c) water birds (Oxberry *et al.*, 1997) and humans (Lee & Hampson, 1994). In humans, a high incidence of *B. pilosicoli* infection

Abbreviations: DIG-AMP, digoxigenin-labelled ampicillin; HDMV, high-density membrane vesicles; IM, inner membrane; LDMV, low-density membrane vesicles; LOS, lipo-oligosaccharide; OM(P), outer membrane (protein); PBP, penicillin-binding protein; SP, soluble protein; SSR, short sequence repetitive element; TM, total membrane.

The GenBank accession numbers for the sequences reported in this paper are AY363613, AY363614 and AY376355.

($\sim$30 %) has been reported in individuals in developing communities (Trott *et al.*, 1997) and in AIDS patients and homosexual males in more affluent societies (Trivett-Moore *et al.*, 1998). Rates of colonization amongst other groups of individuals are extremely low. Day-old SPF chicks (Muniappa *et al.*, 1996; Trott *et al.*, 1995), adult chickens (Stephens & Hampson, 2002), newly weaned pigs (Duhamel, 1996; Thomson *et al.*, 1997; Trott *et al.*, 1996a) and laboratory mice (Sacco *et al.*, 1997) have been used to demonstrate the pathogenic capability of *B. pilosicoli* strains isolated from both humans and animals; however, the mechanisms whereby *B. pilosicoli* infection results in colitis and mild diarrhoea are currently unknown.

A characteristic feature of both natural and experimental *B. pilosicoli* infections is the attachment of large numbers of spirochaetes by one end to the epithelium of the caecum and large intestine, forming a false brush border (Hampson & Trott, 1999). *B. pilosicoli* also localizes within intestinal crypts and can invade the lamina propria (Duhamel, 2001; Muniappa *et al.*, 1996). The *B. pilosicoli* outer membrane (OM) is the interface between the bacterium and the host. By analogy with other pathogens, surface-exposed proteins of *B. pilosicoli* should have a significant role in colonization, intimate attachment and pathogenesis. However, little is known about the OM structure of *B. pilosicoli* and very few OM proteins (OMPs) have been identified for *Brachyspira*. For *B. pilosicoli*, a putative 29 kDa pyruvate oxidoreductase (Rayment *et al.*, 1998) and a 36 kDa glucose/galactose-transport/chemoreceptor have been identified (Zhang *et al.*, 2000), whereas in *Brachyspira hyodysenteriae*, the agent of swine dysentery, identified OMPs include SmpA (Thomas & Sellwood, 1993), and the Vsp (Gabe *et al.*, 1998; McCaman *et al.*, 1999; McCaman *et al.*, 2003) and BmpB/Blp families of paralogous genes (Cullen *et al.*, 2003; Lee *et al.*, 2000).

Isolating and characterizing surface-exposed *B. pilosicoli* proteins and investigating their interactions with the host first requires a technique for OM enrichment. However, selective removal of the spirochaete OM is hampered by its labile nature and by the periplasmic location of the flagella which contaminate OM preparations obtained using techniques reliably applied to other non-spirochaete bacteria. Solubilization of *Brachyspira* spp. OMPs in detergents selectively releases the periplasmic flagella or causes cell lysis and release of cytoplasmic proteins (Chatfield *et al.*, 1988; Gabe *et al.*, 1995; Joens *et al.*, 1993; Trott *et al.*, 2001). The detergent Triton X-114 has been used to selectively release spirochaete OMPs (Cunningham *et al.*, 1988a; Cunningham *et al.*, 1988b; Lee & Hampson, 1995; Lee & Hampson, 1996; Tenaya *et al.*, 1998; Thomas *et al.*, 1992; Zuerner *et al.*, 1991). However, due to the labile nature of the *Brachyspira* OM, it is possible that proteins anchored to the inner membrane (IM), could also be solubilized in the detergent phase (Tenaya *et al.*, 1998; Trott *et al.*, 2001). Membrane vesicle fractionation may offer an advantage over detergent solubilization as it maintains proteins in their location within the OM. Membrane vesicle fractions have been obtained from *B. hyodysenteriae* by French pressure cell disruption and density gradient ultracentrifugation (Plaza *et al.*, 1997). The *B. hyodysenteriae* OM has a very low density ($\rho = 1{\cdot}10$ g cm^{-3}) compared to the IM ($\rho = 1{\cdot}16$ g cm^{-3}) and is unusual in containing cholesterol as a major lipid bilayer constituent. The presence of cholesterol may explain the extreme fragility of the *Brachyspira* OM and the difficulty previous researchers have experienced in isolating OMPs free of contamination from other cellular components (Plaza *et al.*, 1997).

The goal of this study was to identify and characterize *B. pilosicoli* OMPs and clone their genes. Membrane vesicles generated by osmotic lysis of whole cells were purified by isopycnic gradient ultracentrifugation. An OM-rich fraction was used as antigen to obtain mouse mAbs that were then used to identify selected proteins in *B. pilosicoli* strain 95-1000, including a 23 kDa protein that was found in abundance on the OM surface. The gene encoding this protein was isolated, sequenced and expressed in *Escherichia coli*. Analysis of the sequence implies that this protein, designated BmpC, is a lipoprotein.

METHODS

Bacterial strains and culture conditions. *Brachyspira pilosicoli* strain 95-1000 was obtained from Professor David Hampson, School of Veterinary and Biomedical Science, Murdoch University, Western Australia. Strain 95-1000 was originally cultured from the colonic scrapings of a grower pig with porcine intestinal spirochaetosis and is pathogenic, producing polar attachment in experimentally infected swine (Trott *et al.*, 1996a). Spirochaetes were cultivated in stirred, pre-reduced medium under an atmosphere of 99 % N_2/1 % O_2 as described previously (Stanton & Lebo, 1988; Trott *et al.*, 1996b).

Preparation of *B. pilosicoli* total membrane (TM) and soluble protein (SP) extracts. Unless indicated, all procedures were performed at 4 °C. TM extract consisting of OM, IM and flagella, and SP extract were prepared from a 1 l culture of *B. pilosicoli* 95-1000 cells in exponential growth phase ($OD_{620} = 0{\cdot}8$, approximately 5×10^8 cells ml^{-1} by direct counts). The cells were harvested by centrifugation (4000 ***g***, 8 min), washed in buffer I (20 mM HEPES, 50 mM NaCl, pH 7·6) and resuspended in 15 ml buffer I containing 10 % sucrose, 2 mM EDTA, 0·00425 % PMSF and 20 µl each of DNase type I and RNase type A. The cells were passed twice through a French pressure cell at 15 000 p.s.i., centrifuged at 10 000 ***g*** for 20 min and the supernatant was collected and centrifuged at 100 000 ***g*** for 1 h. The supernatant (SP extract) was removed and concentrated tenfold in a Microcon 10 concentrator whilst the pellet (TM extract) was washed twice and resuspended in 500 µl buffer I. Both fractions were stored at −70 °C.

Isolation of *B. pilosicoli* membrane vesicles. A 4 l culture of *B. pilosicoli* 95-1000 was grown at 39 °C to an OD_{620} of 0·8 and incubated on ice overnight. Unless otherwise stated, all procedures were performed at 4 °C. The cells were harvested by centrifugation (4000 ***g***, 8 min), washed in buffer I and resuspended in buffer I containing 1 µM octadecyl rhodamine B chloride (Molecular Probes) and 0·00425 % PMSF. The cells were then harvested by centrifugation (4000 ***g***, 8 min) and resuspended in sterile dH_2O (70 ml g^{-1} wet wt). The cell suspension was mixed at room temperature for 2 h on a Nuova stir plate set at speed 1 using a 4 × 0·8 cm magnetic stir bar. Whole cells and protoplasmic cylinders were removed by centrifugation at 8000 ***g*** for 20 min and 10 000 ***g*** for 20 min, respectively, and the supernatant was then centrifuged at 100 000 ***g*** for 1 h to sediment the membrane vesicles. Membrane vesicles were resuspended in 10 ml buffer I containing 10 % sucrose and 0·00425 % PMSF. The membrane vesicle suspensions (5 ml) were layered onto a (w/w) sucrose gradient made up in buffer I containing 5 ml 21 % sucrose, 16 ml 35 % sucrose and 11 ml 45 % sucrose and subjected to ultracentrifugation using a Beckman SW28 rotor at 100 000 ***g*** for 16 h. High and low-density membrane vesicle fractions (HDMV and LDMV, respectively) were harvested from the side of the tube using a 21G needle and syringe and sedimented by centrifugation at 100 000 ***g*** for 1 h. HDMV and LDMV fractions were resuspended in buffer I containing 10 % sucrose and further purified by isopycnic centrifugation (1 ml 33 % sucrose, 3·5 ml 38 % sucrose and 1 ml 43 % sucrose for HDMV, and 1 ml 21 % sucrose, 2·5 ml 33 % sucrose and 1 ml 38 % sucrose for LDMV) using a Beckman SW55 rotor at 100 000 ***g*** for 16 h. The membrane vesicle fractions (the top layer from the LDMV gradient and the bottom layer from the HDMV gradient) were collected by needle aspiration, centrifuged at 150 000 ***g*** for 3 h, resuspended in buffer I and stored at −70 °C for further analysis. In separate experiments, membrane vesicle fractions (1 ml) were collected from the bottom of each gradient using a Beckman gradient fractionator. The density and protein concentration of the fractions were determined using a Bausch and Lomb refractometer and by measuring A_{280} in a Beckman DU 640 spectrophotometer, respectively.

Triton X-114 extraction. Triton X-114 extraction and phase-partitioning of membrane proteins was performed using *B. pilosicoli* 95-1000 cells grown to an OD_{620} of 0·8 as described by Cunningham *et al.* (1988b).

Transmission electron microscopy. Membrane vesicles or cells were diluted 1 : 5 in dH_2O and 10 µl aliquots were negatively stained with an equal volume of 2·5 % phosphotungstic acid (pH 7) (Trott *et al.*, 1996d). For cross sections, membranes or cells were fixed in 2 % paraformaldehyde/0·05 % glutaraldehyde for 1 h at 4 °C, harvested by centrifugation at 14 000 ***g*** for 20 min, washed twice in cacodylate buffer (pH 7·4) and resuspended in 100 µl warm (45 °C) cacodylate buffer containing 2 % agarose. The agar pieces were dehydrated in an ethanol series, embedded in Epon and ultrathin sections were cut and transferred onto 400 mesh grids. Grids were stained with uranyl acetate and lead citrate and examined at 80 kV by using a Phillips model 410 transmission electron microscope.

SDS-PAGE and Western blotting. Protein concentrations were determined using the modified Lowry assay (Markwell *et al.*, 1978). Protein preparations (5 or 10 µg) were separated by SDS-PAGE in precast 4–20 % acrylamide gradient gels with a Mini-Protean II gel electrophoresis apparatus (Bio-Rad) using standard techniques (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes with a Transblot electrophoretic transfer cell (Bio-Rad) (Towbin *et al.*, 1979). Immunodetection of proteins in Western blots was performed using the Enhanced Chemiluminescence System according to the manufacturer's recommendations (Amersham Pharmacia Biotech).

Assays for cellular and membrane markers. The composition of the various fractions of *B. pilosicoli* was investigated by assaying for specific proteins and cellular components with known cellular locations. NADH oxidase, a cytoplasmic marker was detected on Western immunoblots by using polyclonal gnotobiotic pig antisera raised against the partially purified 48 kDa NOX protein of *B. hyodysenteriae* (Stanton & Jensen, 1993). FlaA1, a flagellar sheath protein, was detected using rabbit antisera raised against the 44 kDa *B. hyodysenteriae* protein (Li *et al.*, 1993). The OM markers used were a 29 kDa *B. pilosicoli* OMP, detected using a mouse mAb (Lee & Hampson, 1995), and lipo-oligosaccharide (LOS). LOS was demonstrated by incubating membrane and SP extracts (75 µg) with proteinase K (2 mg ml^{-1}) at 55 °C for 2 h. The extracts were then resolved on a 14 % acrylamide, 9 M urea separating gel containing a bilayered stacking gel and LPS was detected by silver staining (Inzana & Apicella, 1999; Tsai & Frasch, 1982). Detection of penicillin-binding proteins (PBPs) (IM marker) was performed as described by Weigel *et al.* (1994). The FlaA1 polyclonal antisera and the 29 kDa OMP mAb were kindly provided by Professor Mario Jacques, Université de Montréal, St Hyacinthe, Quebec, Canada, and Professor David Hampson, School of Veterinary and Biomedical Science, Murdoch University, Western Australia, respectively.

mAb production. BALB/c mice were immunized by the intraperitoneal route at days 0 and 15 and by the intravenous route on day 24 with LDMV (15–25 µg protein) mixed 70 : 30 with TiterMax adjuvant (CytRx Corp.). Spleen cells were harvested on day 40 and fused with SP 2/0 mouse myeloma cells using polyethylene glycol. The fusion mixture was distributed into 96-well plates and resulting hybridomas were grown in Dulbecco's Modified Eagle Medium supplemented with 15 % (v/v) fetal calf serum. Hybridomas were screened for antibody production by dot-blot analysis using 50 ng TM from *B. pilosicoli* 95-1000 as antigen. Hybridomas with high antibody titres were then screened by slot-blot analysis against *B. pilosicoli* TM proteins with and without proteinase K treatment to remove those that bound to *B. pilosicoli* LOS determinants. Whole-cell extracts of the type strains of *B. hyodysenteriae*, '*Brachyspira intermedia*', *Brachyspira innocens*, '*Brachyspira murdochii*' and *Brachyspira alvinipulli* were then used to further select hybridomas. Five hybridomas producing mAbs (4F2, 2G3, 1G2, 2H3 and 2E10) that reacted with *B. pilosicoli* membrane protein epitopes, but not with whole-cell extracts derived from the type strains of other species of *Brachyspira*, were identified, cloned and concentrated using the Cellmax Hollow Fibre System (Cellco Inc.). Purified mAbs were tested for reactivity in Western blots of whole-cell protein profiles using 13 genetically diverse *B. pilosicoli* strains isolated from different host species. These included five isolates from pigs (95-1000, P43/6/78^T, Will3D4, Win3 and V883), three isolates from humans (WesB, V1H78, Rosie 2299), one isolate from a dog (V1D1) and four isolates from avian species (QU-1, 13316, 92-S76 and R4). The antibody isotype of each mAb was determined using an ELISA-based mouse monoclonal isotype kit (Bio-Rad) according to the manufacturer's instructions.

Immunogold labelling. Cells from 1 ml *B. pilosicoli* 95-1000 culture were harvested by centrifugation, washed in 10 mM Tris/150 mM NaCl (pH 7·4) and resuspended in 200 µl TBS containing 2 % bovine serum albumin. A 200 µl volume of the appropriate mAb was added and the cells were gently mixed and incubated at 37 °C for 30 min in a Coy anaerobic chamber. The cells were pelleted (4000 ***g***, 7 min), washed twice and resuspended in 100 µl TBS containing 1 % BSA. They were then absorbed onto carbon-coated grids for 5 min and incubated in a 1 : 10 solution of goat anti-mouse colloidal gold conjugate (BC-GAR-30; EBSciences) in TBS containing 1 % BSA. The grids were washed three times in 25 mM Tris, pH 7·2, twice in dH_2O and stained with 2 % phosphotungstic acid before being examined with a Phillips model 410 transmission electron microscope operating at 80 kV.

Cloning and sequencing. A genomic library of strain 95-1000 was prepared in λ ZAP II (Stratagene). The library was screened for phage plaques binding the 2E10 mAb using the *pico*Blue immunoscreening kit (Stratagene). Two positive clones (p151 and p421) were isolated and the inserts were excised from λ ZAP II recombinant phage and subcloned into the pBluescript SK^- phagemid vector according to the manufacturer's instructions. Plasmid preparations of these clones were then purified by CsCl centrifugation. The insert sequences were obtained by PCR amplification by using both commercially available primers (T3 and T7-1) and synthesized

Table 1. Oligonucleotide primers used for amplification and sequencing of inserts p151 and p421 containing *B. pilosicoli bmpC*

Primer	Sequence (5′–3′)
T3	AT TAA CCC TCA CTA AAG
T7-1	AA TAC GAC TCA CTA TAG
1513	AGA AGG CGA AGC TAT ACC AA
151+	TTC AGA TGA AGG CAG TAT TG
421+	ATC AGC CTA ATA CTG CAA CT
421u	TTG CCA GAT ATA ATT GTC GT
BPCN01	CTG TAG TTC CAC CTC CAA TA
BPCN02	TTA TAT CCA TCG TAT ACA CT

oligonucleotide primers based on the 3′-OH end of the upstream insert sequences (Table 1). In addition the contiguous sequence was amplified directly from *B. pilosicoli* 95-1000 whole cells by PCR and sequenced for comparison with p421 and p151. Sequencing was done using standardized dye-termination sequencing reactions separated on ABI PRISM model 377 DNA sequencers at the DNA Sequencing Facility at Iowa State University (Ames, IA, USA). Sequence data were compiled and analysed using Sequencher Version 4.05 (Gene Codes Corp.). The deduced hypothetical ORF was used to search for homology against the GenBank nucleotide database and the deduced amino acid sequence was compared with the SWISS-PROT protein database. Sequence data for the cloned inserts p151 and p421 and the contiguous sequence amplified from *B. pilosicoli* 95-1000 were submitted to GenBank and were assigned the accession numbers AY363613, AY363614 and AY376355, respectively.

Expression of BmpC in *E. coli*. *E. coli* strain SOLR (Stratagene) containing either recombinant phagemid p151 or a non-reactive phagemid, p371, were grown overnight with shaking at 37 °C in 50 ml LB broth containing ampicillin (50 $\mu g\ ml^{-1}$) and 1 mM IPTG. Cells were pelleted and used either as whole-cell preparations or were separated into periplasmic, soluble, cytoplasmic membrane and OM fractions (Oliver & Beckwith, 1982) for analysis by SDS-PAGE and Western blotting. Bands that were immunoreactive with mAb 2E10 were identified by incubation with HRPO-conjugated goat anti-mouse antibodies and visualized using 4-chloro-1-naphthol colour reagent. For sequence analysis of the N terminus of the expressed protein, OM fractions of *E. coli* containing p151 were resolved on a Tris/Tricine 4 % stacking/15 % separating SDS-PAGE gel and blotted onto a PVDF membrane. The expressed protein band identified in *E. coli* SOLR containing p151 was excised from the Coomassie-blue-stained PVDF membrane and submitted to the Protein Facility of Iowa State University (Ames, IA, USA) for N-terminal amino acid sequencing by Edman degradation and HPLC analysis (Matsudaira, 1987).

RESULTS

Osmotic lysis of *B. pilosicoli* 95-1000

Membrane fractions from *B. pilosicoli* cells were initially prepared by the method of Plaza *et al.* (1997). Analysis of the fractions obtained by this technique using known *B. pilosicoli* cellular markers demonstrated that the membrane fractions were free of flagella and the cytoplasmic NOX protein. However, there was no difference between the fractions in the composition of key IM and OM markers and their SDS-PAGE profiles were very similar.

Our strategy for the isolation of OMPs was based on electron microscopy observations that resuspension of *B. pilosicoli* cells in dH_2O caused lysis of the OM, whilst maintaining the integrity of the protoplasmic cylinder (S. Humphrey, personal communication). We therefore developed a membrane vesicle enrichment technique based on osmotic lysis in dH_2O. Following 2 h of gentle stirring, examination by phase-contrast microscopy showed that the majority of cells had a reduced cell diameter, suggesting that their OM had been removed. However, a proportion of the cells (< 20 %) still retained their original cell diameter or had lost their helical shape and formed spherical bodies. As determined by electron microscopy, the cells with reduced cell diameter

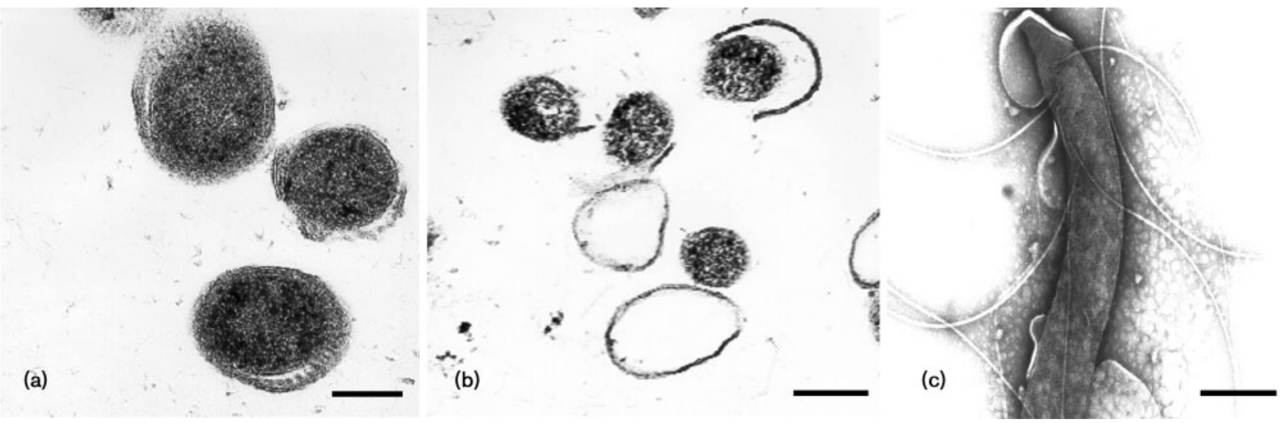

Fig. 1. Transmission electron micrographs of osmotic-induced lysis of the *B. pilosicoli* 95-1000 OM following mixing in dH_2O. (a) Ultrathin section of *B. pilosicoli* 95-1000 cells prior to osmotic lysis. Bar, 0·15 μm. (b) Ultrathin section of *B. pilosicoli* cells following 2 h of gentle mixing in dH_2O. Bar, 0·3 μm. (c) Negatively stained *B. pilosicoli* protoplasmic cylinder following 2 h of gentle mixing in dH_2O. The periplasmic flagella have unwound from the protoplasmic cylinder, but are still attached by their insertion discs. Bar, 0·3 μm.

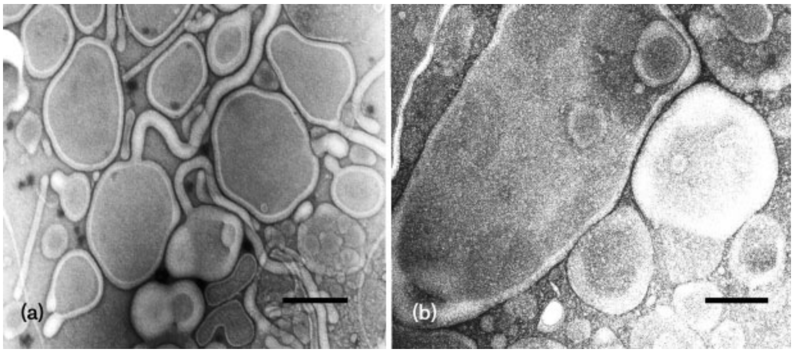

Fig. 2. Transmission electron micrographs of negatively stained *B. pilosicoli* 95-1000 membrane vesicles obtained by osmotic lysis and isopycnic centrifugation. (a) HDMV, $\rho = 1{\cdot}18$ g cm^{-3}. Bar, 0·3 μm. (b) LDMV, $\rho = 1{\cdot}12$ g cm^{-3}. Bar, 0·2 μm.

consisted of protoplasmic cylinders that had lost their OM (Fig. 1a, b). The flagella had unwound from the protoplasmic cylinder but were still attached to the terminal ends of the cell (Fig. 1c).

Harvested membranes were fractionated by sucrose density gradient ultracentrifugation. Broken flagella and remnant protoplasmic cylinders could be separated from membrane vesicles based on their higher density. As determined by electron microscopy, these cellular components formed a pellet ($\rho > 1{\cdot}20$ g cm^{-3}) at the bottom of the tube. The HDMV and LDMV bands were visualized by staining with octadecyl rhodamine B chloride and were harvested by needle aspiration. Examination of both negatively stained and thin sections of HDMV (Fig. 2a) and LDMV (Fig. 2b) by electron microscopy showed that both fractions consisted of unilamellar membrane vesicles. A second round of sucrose density ultracentrifugation was used to further purify the HDMV and LDMV fractions. The respective densities were 1·18 and 1·12 g cm^{-3}.

Assay of membrane vesicle fractions for cellular and membrane markers

The protein electrophoresis profiles of the HDMV and LDMV fractions differed considerably (Fig. 3). The HDMV fraction contained prominent 23, 45 and 55 kDa proteins (Fig. 3, lane 2), which were present, but reduced in the LDMV fraction (Fig. 3, lane 3). Known cellular markers were used to further characterize the composition of the fractions.

NADH oxidase (48 kDa) was detected in the SP fraction and was absent from the TM, HDMV and LDMV fractions (Fig. 4a). Similarly, a 44 kDa flagella sheath protein was only identified in the TM fraction (Fig. 4b). Non-specific binding was not observed in immunoblots probed with polyclonal antibodies to either NADH oxidase or FlaA1. These data indicate that the *B. pilosicoli* membrane vesicle fractions obtained by osmotic lysis and isopycnic centrifugation were free of both cytoplasmic and flagella contamination.

When probed with an mAb to the surface-exposed 29 kDa *B. pilosicoli* protein (Fig. 4c) the LDMV showed a higher concentration of the OM-associated protein compared to the HDMV. A similar profile was obtained when the proteinase-K-treated membrane vesicle fractions were silver-stained for LOS (Fig. 4d). PBPs are known to be present in spirochaete IMs (Radolf *et al.*, 1989) and can be detected by using a digoxigenin-ampicillin (DIG-AMP) assay (Weigel *et al.*, 1994). At least five major PBPs were observed in *B. pilosicoli* 95-1000 TM preparations, having molecular masses of 21, 37, 48, 74 and 83 kDa (Fig. 4e, lane 1). Binding of DIG-AMP was completely inhibited by unlabelled ampicillin, indicating the specificity of this assay for PBPs. PBPs were detected in the HDMV fraction at reduced levels compared to the TM fraction, although there was significant enrichment of the 21 kDa protein (Fig. 4e, lane 2). The proportion of PBPs was markedly reduced in LDMV compared to the other two fractions (Fig. 4e, lane 3).

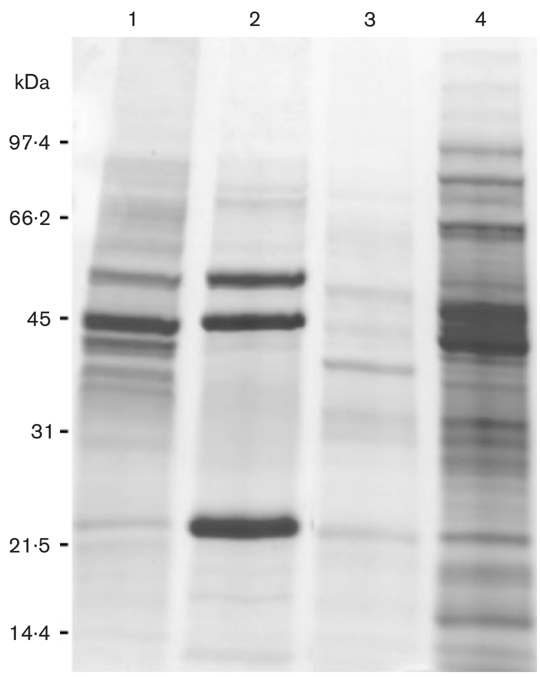

Fig. 3. SDS-PAGE profiles of *B. pilosicoli* 95-1000 membrane vesicle fractions obtained by osmotic lysis and sucrose density ultracentrifugation. Lanes 1, TM fraction; 2, HDMV; 3, LDMV; 4, SP fraction. Lanes were loaded with 10 μg protein. The relative mobilities of molecular mass markers are indicated on the left.

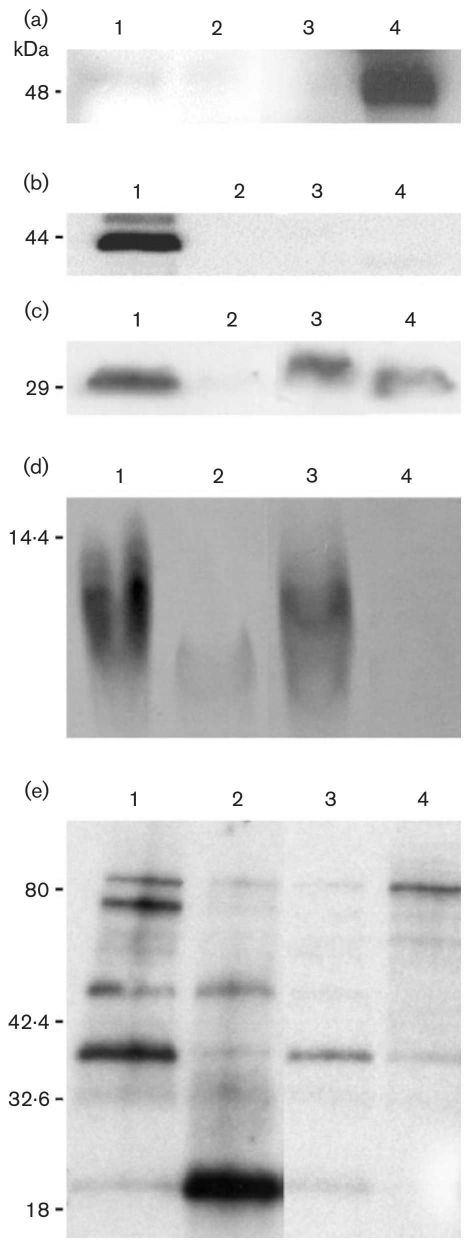

Fig. 4. Analysis of *B. pilosicoli* 95-1000 membrane vesicle fractions obtained by osmotic lysis and sucrose density ultracentrifugation for specific cellular and membrane markers. (a) SP marker (Western blot probed with polyclonal antibody against the 48 kDa *B. hyodysenteriae* NADH oxidase). (b) Flagella protein marker (Western blot probed with polyclonal antibody against the 44 kDa *B. hyodysenteriae* flagella sheath protein). (c) OM marker 1 (Western blot probed with mAb against a 29 kDa *B. pilosicoli* OMP). (d) OM marker 2 (Silver stained SDS-PAGE gel showing the lipid A portion of *B. pilosicoli* LOS). (e) DIG-AMP PBP assay. Lanes: 1, TM fraction; 2, HDMV; 3, LDMV; 4, SP fraction. Lanes were loaded with 5 μg protein, except for (d) where each lane was loaded with 75 μg protein. The relative mobilities of molecular mass markers are indicated on the left.

In overview, the LDMV fraction was free of flagella and cytoplasmic components and contained OM markers. IM components were present, but were substantially reduced when compared to the TM fraction. On the basis of these marker assays, LDMV represented enriched OM vesicles and proteins identified in this fraction were likely to be OM-associated.

Production of mAbs using LDMV as a source of antigen

To further characterize *B. pilosicoli*-specific OM-associated proteins, the LDMV fraction was used to immunize mice for mAb production. The mAbs were screened and comprised three groups. The first represented mAbs that reacted with proteinase-K-resistant material, most probably LPS. The second group of mAbs cross-reacted with proteins from other *Brachyspira* species. A third group of five mAbs reacted with *B. pilosicoli*-specific proteinase-K-sensitive proteins of 23, 24, 35, 61 and 79 kDa, based on electrophoretic migration. Three of these mAbs were of the IgG_1 subclass, one of the IgG_{2b} subclass and one of the IgA class. mAbs 1G2 and 2G3 (directed against the 35 and 61 kDa proteins, respectively) reacted with all 13 *B. pilosicoli* strains tested, whereas mAb 2E10, directed against the 23 kDa protein, only reacted with *B. pilosicoli* 95-1000 (Table 2).

Each mAb was used to probe TM, HDMV, LDMV and SP fractions to determine the distribution of the proteins in these fractions. In addition, to identify which of the mAbs were likely to bind to integral membrane proteins, the mAbs were also used to probe insoluble, detergent-phase and aqueous-phase Triton X-114 extracts of *B. pilosicoli* 95-1000 whole cells (Fig. 5). The proteins identified by the mAbs showed differences in their pattern of distribution in the membrane vesicle and Triton X-114 fractions. The 23 and 61 kDa proteins were distributed in TM, HDMV and

Table 2. Characteristics of proteins reacting with *B. pilosicoli*-specific mAbs

mAb	Size (kDa)	Antibody subclass	TX-114 Phase partitioning	Reactive *B. pilosicoli* strains*
4F2	79	IgG_1	−	9/13
2G3	61	IgA	−	13/13
1G2	35	IgG_1	+†	13/13
2H3	24	IgG_1	+†	12/13
2E10	23	IgG_{2b}	−	1/13

*mAbs were tested against 13 genetically diverse *B. pilosicoli* strains isolated from different host species, including five isolates from pigs (95-1000, P43/6/78^T, Will3D4, Win3 and V883), three isolates from humans (WesB, V1H78, Rosie 2299), one isolate from a dog (V1D1) and four isolates from avian species (QU-1, 13316, 92-S76 and R4).
†Indicates protein was enriched in the Triton X-114 detergent phase compared to the insoluble phase, suggesting that it is an integral OMP.

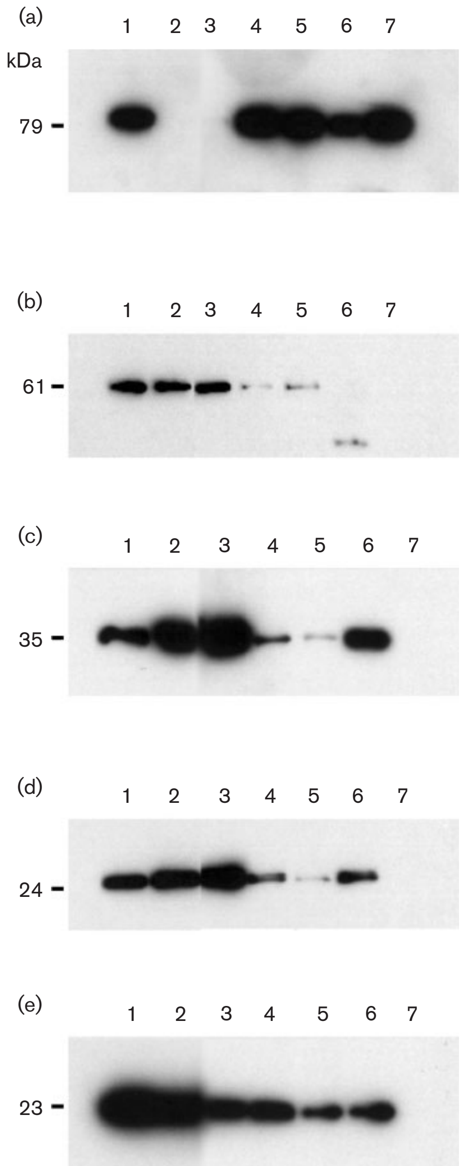

Fig. 5. Probing of *Brachyspira pilosicoli* 95-1000 membrane vesicle fractions and Triton X-114 extracts with *B. pilosicoli* membrane-protein specific mAbs obtained by immunizing mice with LDMV. (a) 4F2; (b) 2G3; (c) 1G2; (d) 2H3; (e) 2E10. Lanes: 1, TM fraction; 2, HDMV; 3, LDMV; 4, SP fraction; 5, Triton X-114 insoluble material; 6, Triton X-114 detergent phase; 7, Triton X-114 aqueous phase. Lanes were loaded with 5 μg protein. The relative mobilities of molecular mass markers are indicated on the left.

LDMV membrane vesicle fractions, but comparatively reduced in the Triton X-114 detergent fractions. Furthermore, there was no difference in the pattern of distribution between the detergent and insoluble phases, suggesting that both proteins were relatively insoluble in Triton X-114. Triton X-114 detergent extraction appeared to result in cleavage of the 61 kDa protein to a smaller product. In contrast, the 24 and 35 kDa proteins were enriched in both the LDMV fraction and the Triton X-114 detergent phase, but not in the aqueous and insoluble Triton X-114 phases. This suggests the possibility that both are integral OMPs or lipoproteins anchored to the OM. The mAb reacted with a 79 kDa protein that was present in both TM and SP fractions. However, this protein could not be detected in the HDMV fraction and was present in only trace amounts in the LDMV fraction, suggesting that it had either undergone proteolysis or had become detached from the membrane during osmotic lysis and isopycnic ultracentrifugation. However, it may be conceivable that the 79 kDa protein is a periplasmic protein that has contaminated the membrane fractions.

Immunogold labelling of *B. pilosicoli* whole cells

Immunogold labelling of negatively stained whole cells with mAb 2E10 (directed against the 23 kDa protein) identified an antigen that was present in abundance on the outer surface of *B. pilosicoli* 95-1000 cells (Fig. 6a). Immunogold labelling of *B. pilosicoli* cells that had been subjected to osmotic lysis and had lost their OM showed that the protein did not appear to be associated with the protoplasmic cylinder (Fig. 6b). Localization of the protein to the *B. pilosicoli* 95-1000 OM was also confirmed by immunogold labelling of fixed-cell cross-sections (data not shown). Immunogold labelling with mAbs 1G2 and 2H3 also identified proteins that were surface-associated, but not as abundant as the 23 kDa protein identified by 2E10 (data not shown). Immunogold labelling of both whole cells and protoplasmic cylinders with mAbs 4F2 and 2G3 did not conclusively localize the 79 kDa or the 61 kDa proteins to either the OM or the IM.

Identification, cloning and sequence analysis of BmpC

The 23 kDa protein was an abundant constituent of the *B. pilosicoli* OM. Therefore, to facilitate identification and sequencing of the gene encoding the 23 kDa protein, mAb 2E10 was used to screen a *B. pilosicoli* genomic library in λ ZAP II. Two recombinant phagemids (p151 and p421) containing DNA-encoding proteins that were reactive with mAb 2E10 and a third phagemid containing an unreactive insert (p371) were selected and were found to be stable in *E. coli* SOLR cells. In whole-cell lysates of *E. coli* SOLR cells containing p151 or p421, mAb 2E10 reacted strongly with a protein of approximately 28 kDa and weakly with a band of 48 kDa (Fig. 7, lane 8). Furthermore, the 28 kDa band was enriched in the *E. coli* OM fraction but was only present in trace amounts in the periplasmic fraction compared to the soluble and cytoplasmic membrane fractions. In contrast, the 48 kDa band was reduced in the cytoplasmic membrane fraction compared with the other fractions. *E. coli* SOLR containing p371 was consistently unreactive with mAb 2E10, confirming that the reactivity was associated with the DNA insert and not the carrier strain (Fig. 7, lane 7).

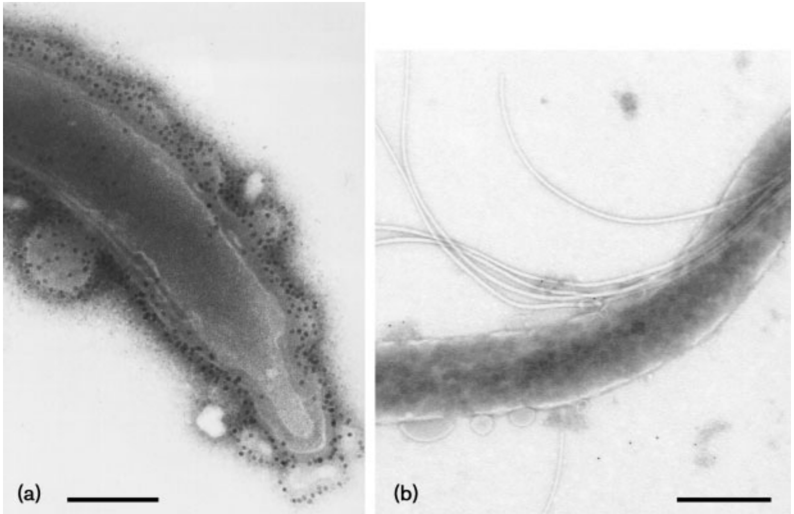

Fig. 6. (a) Immunogold labelling of an intact *B. pilosicoli* 95-1000 cell with mAb 2E10, raised against a 23 kDa *B. pilosicoli* membrane protein. The protein is present in abundance on the outer surface of the cell. Bar, 0·2 μm. (b) Following removal of the *B. pilosicoli* OM by osmotic lysis, there is no immunogold labelling of the cell wall/cytoplasmic membrane complex. Bar, 0·25 μm.

The cloned inserts carried on p151 and p421 were determined to be 3·7 and 4·0 kb respectively. Analysis of the 4·9 kb contiguous sequence determined from these cloned inserts identified four ORFs (Fig. 8). Sequence comparisons with the SWISS-PROT database identified an aldehyde oxidoreductase (54 % sequence identity over 392 aa with *Enterococcus faecalis* aldehyde oxidoreductase), a putative synthase (45 % sequence identity over 271 aa with an *E. coli* putative synthase) and a protein provisionally designated Alp (ankyrin-like protein) that contained multiple ankyrin repeats. A 561 bp unidentified ORF showed no significant homology with any protein in the database. To further associate one of these four ORFs with the 23 kDa protein, an N-terminal amino acid sequence was determined from the recombinant 28 kDa protein expressed in *E. coli* SOLR. An N-terminal sequence of 12 residues (MNKKILSIFVMV) was obtained, enabling the definitive identification of the protein reacting with mAb 2E10 as the 561 bp unidentified ORF. The deduced amino acid sequence of this protein would encode a 166 aa polypeptide with a predicted size of 20 kDa and a 21 aa signal peptide (MNKKILSIFVMVMALSLLSIS).

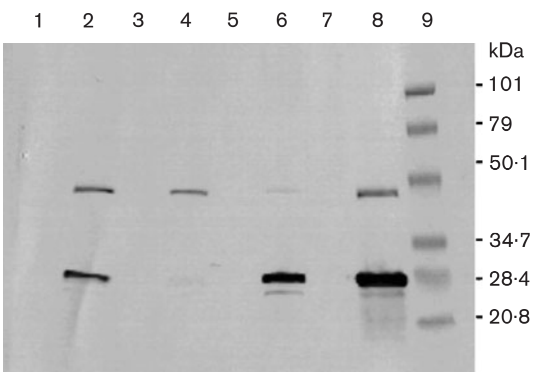

Fig. 7. Immunoblot of *E. coli* cell fractions containing plasmid p151 or p371. Fractions containing p371 (lanes 1, 3, 5, 7) and p151 (lanes 2, 4, 6, 8) were electrophoresed, transferred to PVDF and probed with mAb 2E10. Periplasmic fraction (lanes 1 and 2), soluble fraction (lanes 3 and 4), cytoplasmic membrane fraction (lanes 5 and 6) and OM fraction (lanes 7 and 8) were examined. Prestained molecular mass markers (lane 9) are indicated on the right.

A series of 7 bp short sequence repetitive elements (SSRs) with a consensus sequence of 5′-AATCAGC-3′ was identified in the intergenic region upstream of the 561 bp unidentified ORF, terminating at 237 bp prior to the start codon. Sixteen copies were present in p421, whereas 17 copies were present in p151. The contiguous sequence was also amplified and sequenced by PCR from *B. pilosicoli* 95-1000 to determine how many copies of the SSR were present in the genomic DNA. Surprisingly, only eight copies were identified, indicating the possibility of replication errors due to slipped strand mispairing. Putative −35 (ATAAACA) and −10 (TATAAT) promoter regions and a

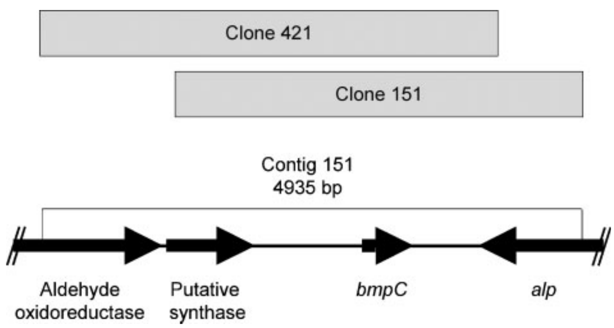

Fig. 8. Organization of the 4·9 kb *B. pilosicoli* contiguous sequence obtained from cloned inserts p151 and p421. The location and orientation of the four genes identified in the sequence, including *bmpC*, are indicated with a single-headed arrow.

ribosome-binding site (AGGAG) were identified upstream of the 561 bp ORF. The hydrophobic precursor signal peptide shared an identical putative spirochaete lipoprotein signal peptidase II recognition site (SISC) with BlpG, one of four paralogous OM lipoproteins recently identified in *B. hyodysenteriae* (Cullen *et al.*, 2003; Haake, 2000). The +2 and +3 amino acids of the mature protein were both Asn (N) residues, an uncharged amino acid identified in the +2 and/or +3 positions of the other identified *Brachyspira* OM lipoproteins (Cullen *et al.*, 2003; Lee *et al.*, 2000; Thomas & Sellwood, 1993;). The Kyte–Doolittle hydropathy plot predicted that apart from the hydrophobic precursor region, the protein contained no transmembrane domains. These analyses together with the results of immunogold labelling of *B. pilosicoli* whole cells suggest that the ORF is expressed as a lipoprotein on the *B. pilosicoli* OM, tethered to the membrane by a lipid moiety attached to the Cys at amino acid residue 22. Acylation of the mature protein may explain the apparent difference in size based on its deduced amino acid sequence with the size estimation based on its migration in SDS-PAGE gels. In accordance with the previous nomenclature adopted for *Brachyspira* membrane proteins (Lee *et al.*, 2000; Thomas & Sellwood, 1993), we propose that this unique *B. pilosicoli* OM lipoprotein be designated BmpC (*Brachyspira* membrane protein C) and that the gene encoding the protein be designated *bmpC*. Although the BmpC sequence was unique and did not share close homology with any other identified protein, 24 of the first 32 aa (75 %) were identical to *B. hyodysenteriae* SmpA. Notably SmpA and BmpC both possess signal peptides that are 21 aa in length and share an identical −1 to +4 amino acid sequence of SCNNK (Thomas & Sellwood, 1993).

DISCUSSION

Identifying *B. pilosicoli* OMPs expressed during infection is a prerequisite for the analysis of the parasite–host interaction at a molecular level. A necessary starting point is to isolate these proteins relatively free of contamination from the other cellular components. However, isolating the OM of *B. pilosicoli* by adopting previously published non-detergent based techniques for spirochaetes was found to be problematic (Blanco *et al.*, 1994; Bledsoe *et al.*, 1994; Plaza *et al.*, 1997; Radolf *et al.*, 1995a; Radolf *et al.*, 1995b; Skare *et al.*, 1995). Haake & Matsunaga, (2002) also experienced similar difficulties in obtaining *Leptospira* OM vesicles in sufficient quantities for characterization without concomitant contamination by IM components and eventually obtained relatively pure OM vesicles by gently mixing leptospiral whole cells in a hypertonic alkaline buffer containing Tris, NaCl and EDTA followed by isopycnic centrifugation. The inability to apply the same OM isolation techniques to different genera of spirochaetes demonstrates key fundamental differences in OM composition and function between *Treponema*, *Borrelia*, *Leptospira* and *Brachyspira*. In particular, *Brachyspira* OMs appear to be unusual in that they contain a significant proportion of sterols (cholesterol and cholestanol) and have a relatively low density (Plaza *et al.*, 1997; Stanton, 1987; Trott *et al.*, 2001). These unique OM characteristics may result in the *Brachyspira* OM being more susceptible to osmotic stress than other pathogenic spirochaetes, manifested by the rapid loss of *Brachyspira* OM integrity in low ionic strength buffers (S. Humphrey & D. J. Trott, unpublished data).

This paper describes a novel OM enrichment method that takes advantage of the observation that *B. pilosicoli* cells rapidly lose their OMs whilst maintaining the integrity of the protoplasmic cylinder when suspended in dH_2O. *B. pilosicoli* 95-1000 membrane vesicles were separated into LDMV and HDMV fractions with respective densities of 1·12 and 1·18 g cm^{-3} and the fractions were shown to be free of flagella and cytoplasmic protein contamination. The low density ($\rho = 1{\cdot}12$ g cm^{-3}), the presence of two OM markers (LOS and a 29 kDa OMP) and the relative absence of the IM marker (PBPs) in LDMV, suggested that this fraction represented an enrichment of OM vesicles. LDMV was therefore used to develop key immunologic reagents for further investigation of *B. pilosicoli* OMP constituents.

We identified five *B. pilosicoli*-specific mAbs that reacted with proteins in the LDMV fraction. Comparison of the distribution of these proteins in the other membrane fractions and Triton X-114 extracts also demonstrated key differences. The 24 and 35 kDa proteins were enriched in the LDMV fraction compared with the TM and HDMV fractions and both these proteins were enriched in the Triton X-114 detergent phase compared to the insoluble phase, confirming that they are likely to be integral membrane proteins or lipoproteins anchored to the OM. Immunogold labelling results were consistent with the surface location of these proteins.

BmpC, identified by mAb 2E10, was one of three major membrane-associated proteins identified by SDS-PAGE in the TM, HDMV and LDMV fractions. However, in contrast to the 24 and 35 kDa proteins, BmpC did not show selective partitioning into the Triton X-114 detergent phase, suggesting that it was either present on both the OM and the IM of *B. pilosicoli* or that the protein was poorly soluble in this non-ionic detergent. Haake & Matsunaga (2002) have previously demonstrated the poor solubility of a *Leptospira* OM porin (OmpL1) in Triton X-114. In addition, immunogold labelling confirmed that BmpC was surface-exposed on the *B. pilosicoli* 95-1000 OM surface. Immunogold labelling was not apparent on protoplasmic cylinders stripped of their OM by osmotic lysis in dH_2O.

Cell fractionation showed that recombinant BmpC was expressed predominantly in OM and CM fractions of *E. coli* strain SOLR without any apparent deleterious effects on the host strain. However, there was a noticeable size difference in the migration of recombinant and native BmpC during SDS-PAGE. It would seem that the majority of BmpC is not expressed on the OM of *E. coli* in its mature lipoprotein form, given that expression of the mature lipoprotein together with the N-terminal precursor peptide sequence (MNKKILSIFVMVMALSLLSIS) would yield a protein with a predicted molecular size approaching 28 kDa, which is close to the size of the recombinant protein as determined by SDS-PAGE. In addition, the N-terminal amino acid sequence obtained for recombinant BmpC matched the first 12 aa of the peptide leader sequence. No other proteins in *E. coli* SOLR containing p151 showed reactivity with anti-BmpC mAb, except an additional band of approximately 48 kDa that also suggests inefficient processing or post-translational modification of BmpC in *E. coli*, such as the formation of a dimer.

BmpC and *B. hyodysenteriae* SmpA share highly similar 21 aa signal peptide sequences. However, when expressed in *E. coli*, recombinant SmpA predominantly partitioned into the OM as both uncleaved prolipoprotein and fully processed, mature lipoprotein forms, with the amount of each form regulated by the stage of the growth cycle (Thomas & Sellwood, 1993). The differences demonstrated between recombinant BmpC and SmpA highlight the fundamental difficulties associated with the expression of *Brachyspira* lipoproteins in *E. coli*. Typically, to obtain stable expression of spirochaetal lipoproteins in *E. coli* in their lipidated form, it is necessary to modify the N-terminal sequence to facilitate appropriate processing, as reported for the pDUMP plasmid vector (Cullen *et al.*, 2002).

Analysis of the amino acid sequence of BmpC shows that it has some features in common with *B. hyodysenteriae* SmpA and the BmpB/BlpA family of paralogous lipoprotein genes (Cullen *et al.*, 2003; Lee *et al.*, 2000; Thomas & Sellwood, 1993). Most notably, the signal peptides of SmpA and BmpC showed significant homology and BlpG and BmpC possessed identical atypical signal peptidase II recognition sites of SISC. In *Leptospira interogans*, it is hypothesized that the sorting of leptospiral lipoproteins is governed by mechanisms similar to *E. coli*, in that the +2 and +3 amino acids of the mature, lipidated protein appear to be critical in determining the membrane location. A negatively charged amino acid in the +2 or +3 position targets the lipoprotein to the IM, whereas positively charged amino acids target the OM and neutral amino acids suggest the protein is expressed in both locations (Haake & Matsunaga, 2002; Cullen *et al.*, 2003). The +2 and +3 positions of *B. pilosicoli* BmpC are both uncharged Asn molecules, whereas in *B. hyodysenteriae* SmpA and BmpB/BlpA they are Gly and Asn (Cullen *et al.*, 2003; Lee *et al.*, 2000). By this definition, SmpA, BmpB/BlpA and BmpC would localize to both the OM and IM. However, all three proteins appear to be expressed exclusively on the *Brachyspira* OM. The lipoprotein membrane targeting system identified in *Leptospira* does not appear to be universal amongst the spirochaetes and it seems more likely that the mechanisms governing the trafficking and localization of membrane-anchored lipoproteins are different between *Leptospira* and *Brachyspira*.

Because BmpC is abundant on the *B. pilosicoli* 95-1000 cell surface, we considered it worthy of further analysis, since surface-exposed proteins are likely to mediate interactions between a bacterium and its host environment. A number of interesting features associated with BmpC warrant further investigation.

First, when tested against 13 genetically heterogeneous *B. pilosicoli* isolates obtained from four different host species, mAb 2E10 was only reactive with strain 95-1000 (Table 2). This possibly suggests that BmpC is unique to strain 95-1000. Other explanations, however, are that surface-exposed epitopes of the protein vary in sequence, specifically the target region of the BmpC-specific mAb or BmpC is expressed differently in other strains. These characteristics are likely to be important for *B. pilosicoli* to survive immune surveillance in its mammalian host. Variability in surface-exposed regions of OMPs has been previously demonstrated in *B. hyodysenteriae* and has been suggested as a potential mechanism for chronic infection and evasion of the immune response (Cullen *et al.*, 2003; Gabe *et al.*, 1998; McCaman *et al.*, 1999).

Second, *bmpC* is located downstream from a series of 7 bp SSRs. Variation in the number of repeats was demonstrated between both phagemids containing *bmpC* and the contiguous sequence amplified from *B. pilosicoli* 95-1000. These variations demonstrate the occurrence of slipped strand mispairing. Slipped strand mispairing induced by intragenic SSRs or those located between the −35 and −10 promoter regions, is often used by pathogenic bacteria such as *Neisseria meningitidis* and *Haemophilus influenzae* as a mechanism for generating variability in surface-exposed proteins (van Belkum *et al.*, 1998). SSRs of 7 bp in length are highly unusual in prokaryotes, but the fact that they are located upstream of the promoter region in *B. pilosicoli* may indicate that they have no effect on the expression of *bmpC*. It also may be possible that the same SSR is located in other regions of the *B. pilosicoli* chromosome, having an as yet unknown regulatory function.

Finally, *B. pilosicoli bmpC* is located immediately upstream of a gene (provisionally designated *alp*) encoding a protein containing multiple ankyrin repeats. Ankyrins are spectrin-binding structural proteins in red blood cells that bridge the exoskeleton to the cytoplasmic plasma membrane surface. In bacteria, many ankyrin-like proteins have been identified and they are normally located near genes involved in nutrient uptake or tolerance to adverse environmental conditions. For example, AnkA, an ankyrin-like protein of *Ehrlichia phagocytophila* may play a role in altering host-cell gene expression (Caturegli *et al.*, 2000), whereas AnkB, identified in *Pseudomonas aeruginosa*, is involved in the protective response to oxidative stress induced by hydrogen peroxide (Howell *et al.*, 2000). Notably, ankyrin-binding proteins have also been implicated in host-cell interactions by pathogenic organisms, including *Treponema pallidum* (Weinstock *et al.*, 1998). The *Coxiella burnetii* genome contains 13 proteins with ankyrin repeats. In the absence of other genes encoding typical structures for adhesion in the *C. burnetii* genome, ankyrin-like proteins may serve a function in attachment to the host-cell extracellular matrix prior to internalization (Seshadri *et al.*, 2003).

The current study illustrates the effectiveness of isolating and identifying *B. pilosicoli* OMPs through membrane vesicle fractionation and production of *B. pilosicoli*-specific mAbs in the absence of a genomic database. Identification, cloning and sequencing of the genes encoding the four remaining membrane-associated proteins will facilitate further understanding of the *Brachyspira* OM and its unique host interactions.

ACKNOWLEDGEMENTS

We thank Karen Halloum and Loren Jones for expert technical assistance. We also thank Dr Michael V. Norgard for the initial gift of 500 μl DIG-AMP, Dr Mario Jacques for providing the FlaA1 antisera and Dr David Hampson for providing *B. pilosicoli* strains and mAb to the 29 kDa OMP. The excellent advice of Sam Humphrey, Dr Justin Radolf and Dr Frank Gherardini is greatly appreciated and we especially thank Dr Paul Cullen for critical review of the manuscript.

REFERENCES

Blanco, D. R., Reimann, K., Skare, J., Champion, C. I., Foley, D., Exner, M. M., Hancock, R. E. W., Miller, J. N. & Lovett, M. A. (1994). Isolation of the outer membranes from *Treponema pallidum* and *Treponema vincentii*. *J Bacteriol* **176**, 6088–6099.

Bledsoe, H. A., Carroll, J. A., Whelchel, T. R., Farmer, M. A., Dorward, D. W. & Gherardini, F. C. (1994). Isolation and partial characterization of *Borrelia burgdorferi* inner and outer membranes by using isopycnic centrifugation. *J Bacteriol* **176**, 7447–7455.

Caturegli, P., Asanovich, K. M., Walls, J. J., Bakken, J. S., Madigan, J. E., Popov, V. L. & Dumler, J. S. (2000). *ankA*: an *Ehrlichia phagocytophila* group gene encoding a cytoplasmic protein antigen with ankyrin repeats. *Infect Immun* **68**, 5277–5283.

Chatfield, S. N., Fernie, D. S., Beesley, J., Penn, C. & Dougan, G. (1988). Characterization of the cell envelope of *Treponema hyodysenteriae*. *FEMS Microbiol Lett* **55**, 303–308.

Cullen, P. A., Lo, M., Bulach, D. M., Cordwell, S. J. & Adler, B. (2002). Construction and evaluation of a plasmid vector for the expression of recombinant lipoproteins in *Escherichia coli*. *Plasmid* **49**, 18–29.

Cullen, P. A., Coutts, S. A. J., Cordwell, S. J., Bulach, D. M. & Adler, B. (2003). Characterization of a locus encoding four paralogous outer membrane lipoproteins of *Brachyspira hyodysenteriae*. *Microbes Infect* **5**, 275–283.

Cunningham, T. M., Walker, E. M., Miller, J. N. & Lovett, M. A. (1988a). Selective release of the *Treponema pallidum* outer membrane and associated polypeptides with Triton X-114. *J Bacteriol* **170**, 5789–5796.

Cunningham, T. M., Thomas, D. D., Thompson, S. D., Miller, J. N. & Lovett, M. A. (1988b). Identification of *Borrelia burgdorferi* surface components by Triton X-114 phase partitioning. *Ann N Y Acad Sci* **539**, 376–378.

Duhamel, G. E. (1996). Porcine colonic spirochaetosis caused by *Serpulina pilosicoli*. In *Enteric Diseases, Misset Pigs*, pp. 10–13. Edited by A. Evans. Chaiwan, Hong Kong: Shiny International.

Duhamel, G. (2001). Comparative pathology and pathogenesis of naturally acquired and experimentally induced colonic spirochaetosis. *Anim Health Res Rev* **2**, 3–17.

Duhamel, G., Trott, D., Muniappa, N., Mathiesen, M., Tarasiuk, K., Lee, J. & Hampson, D. (1998). Canine intestinal spirochaetes consist of *Serpulina pilosicoli* and a newly identified group provisionally designated *Serpulina canis* sp. nov. *J Clin Microbiol* **36**, 2264–2270.

Gabe, J. D., Chang, R. J., Slomiany, R., Andrews, W. H. & McCaman, M. T. (1995). Isolation of extracytoplasmic proteins from *Serpulina hyodysenteriae* B204 and molecular cloning of the *flaB1* gene encoding a 38-kilodalton flagella protein. *Infect Immun* **63**, 142–148.

Gabe, J. D., Dragon, E., Chang, R. J. & McCaman, M. T. (1998). Identification of a linked set of genes in *Serpulina hyodysenteriae* (B204) predicted to encode closely related 39-kilodalton extracytoplasmic proteins. *J Bacteriol* **180**, 444–448.

Haake, D. A. (2000). Spirochaetal lipoproteins and pathogenesis. *Microbiology* **146**, 1491–1504.

Haake, D. A. & Matsunaga, J. (2002). Characterization of the Leptospiral outer membrane and description of three novel Leptospiral membrane proteins. *Infect Immun* **70**, 4936–4945.

Hampson, D. & Trott, D. J. (1999). Spirochetal diarrhoea/porcine intestinal spirochetosis. In *Diseases of Swine*, pp. 553–562. Edited by B. Straw, S. D'Allaire, W. Mengling & D. J. Taylor. Ames: Iowa State University Press.

Howell, M. L., Alsabbagh, E., Ma, J. F. & 10 other authors (2000). AnkB, a periplasmic ankyrin-like protein in *Pseudomonas aeruginosa*, is required for optimal catalase B (KatB) activity and resistance to hydrogen peroxide. *J Bacteriol* **182**, 4545–4556.

Inzana, T. & Apicella, M. (1999). Use of a bilayer stacking gel to improve resolution of lipopolysaccharides and lipooligosaccharides in polyacrylamide gels. *Electrophoresis* **20**, 462–465.

Joens, L. A., Marquez, R. M. & Halter, M. (1993). Comparison of outer membrane fractions of *Serpulina* (*Treponema*) *hyodysenteriae*. *Vet Microbiol* **35**, 119–132.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lee, J. I. & Hampson, D. J. (1994). Genetic characterisation of intestinal spirochaetes and their association with disease. *J Med Microbiol* **40**, 365–371.

Lee, B. J. & Hampson, D. J. (1995). A monoclonal antibody reacting with the cell envelope of spirochaetes isolated from cases of intestinal spirochaetosis in pigs and humans. *FEMS Microbiol Lett* **131**, 179–184.

Lee, B. J. & Hampson, D. J. (1996). Production and characterisation of a monoclonal antibody to *Serpulina hyodysenteriae*. *FEMS Microbiol Lett* **136**, 193–197.

Lee, B. J., La, T., Mikosza, A. S. J. & Hampson, D. J. (2000). Identification of the gene encoding BmpB, a 30 kDa outer envelope lipoprotein of *Brachyspira* (*Serpulina*) *hyodysenteriae*, and immunogenicity of recombinant BmpB in mice and pigs. *Vet Microbiol* **76**, 245–257.

Li, Z., Dumas, F., Dubreuil, D. & Jacques, M. (1993). A species-specific periplasmic flagellae protein of *Serpulina* (*Treponema*) *hyodysenteriae*. *Infect Immun* **175**, 8000–8007.

Markwell, M., Haas, S., Bieber, L. & Tolbert, N. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**, 206–210.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.

McCaman, M., Auer, K., Foley, W. & Gabe, J. (1999). Sequence characterization of two new members of a multi-gene family in *Serpulina hyodysenteriae* (B204) with homology to a 39 kDa surface-exposed protein: *vspC* and *D*. *Vet Microbiol* **68**, 273–283.

McCaman, M. T., Auer, K., Foley, W. & Gabe, J. D. (2003). *Brachyspira hyodysenteriae* contains eight linked gene copies related to an expressed 39 kDa surface protein. *Microbes Infect* **5**, 1–6.

Muniappa, N., Duhamel, G. E., Mathiesen, M. R. & Bargar, T. W. (1996). Light microscopic and ultrastructural changes in the ceca of chicks inoculated with human and canine *Serpulina pilosicoli*. *Vet Pathol* **33**, 542–550.

Ochiai, S., Adachi, Y. & Mori, K. (1997). Unification of the genera *Serpulina* and *Brachyspira* and proposals of *Brachyspira hyodysenteriae* comb. nov., *Brachyspira innocens* comb. nov. and *Brachyspira pilosicoli* comb. nov. *Microbiol Immunobiol* **41**, 445–452.

Oliver, D. B. & Beckwith, J. (1982). Regulation of a membrane component required for protein secretion in *Escherichia coli*. *Cell* **30**, 311–319.

Oxberry, S. L., Trott, D. J. & Hampson, D. J. (1997). *Serpulina pilosicoli* water and water birds: potential sources of infection for humans and other animals. *Epidemiol Infect* **121**, 219–225.

Plaza, H., Whelchel, T. R., Garcynski, S. F., Howerth, E. W. & Gherardini, F. C. (1997). Purified outer membranes of *Serpulina hyodysenteriae* contain cholesterol. *J Bacteriol* **179**, 5414–5421.

Radolf, J., Moomaw, C., Slaughter, C. & Norgard, M. (1989). Penicillin binding proteins and peptidoglycan of *Treponema pallidum* subsp. *pallidum*. *Infect Immun* **57**, 1248–1254.

Radolf, J. D., Goldberg, M. S., Bourell, K., Baker, S. I., Jones, J. D. & Norgard, M. V. (1995a). Characterization of outer membranes isolated from *Borrelia burgdorferi*, the Lyme disease spirochete. *Infect Immun* **63**, 2154–2163.

Radolf, J. D., Robinson, E. J., Bourell, K. W., Akins, D. R., Porcella, S. F., Weigel, L. M., Jones, J. D. & Norgard, M. V. (1995b). Characterization of outer membranes isolated from *Treponema pallidum*, the syphilis spirochete. *Infect Immun* **63**, 4244–4252.

Rayment, S., Lee, B., Hampson, D. & Livesley, M. (1998). Identification of a gene encoding a putative pyruvate oxidoreductase in *Serpulina pilosicoli*. *FEMS Microbiol Lett* **166**, 121–126.

Sacco, R. E., Trampel, D. W. & Wannemueler, M. J. (1997). Experimental infection of mice with avian, porcine or human isolates of *Serpulina pilosicoli*. *Infect Immun* **65**, 5349–5353.

Seshadri, R., Paulsen, I. T., Eisen, J. A. & 21 other authors (2003). Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc Natl Acad Sci U S A* **100**, 5455–5460.

Skare, J. T., Shang, E. S., Foley, D. M. & 7 other authors (1995). Virulent strain associated outer membrane proteins of *Borrelia burgdorferi*. *J Clin Invest* **96**, 2380–2392.

Stanton, T. B. (1987). Cholesterol metabolism by *Treponema hyodysenteriae*. *Infect Immun* **55**, 309–313.

Stanton, T. B. (2002). The genus *Brachyspira*. In *The Prokaryotes*. Edited by M. Dworkin. New York: Springer.

Stanton, T. B. & Jensen, N. S. (1993). Purification and characterization of NADH oxidase from *Serpulina* (*Treponema*) *hyodysenteriae*. *J Bacteriol* **175**, 2980–2987.

Stanton, T. B. & Lebo, D. F. (1988). *Treponema hyodysenteriae* growth under various culture conditions. *Vet Microbiol* **18**, 177–190.

Stephens, C. & Hampson, D. J. (2002). Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira* (*Serpulina*) *pilosicoli* causes reduced egg production. *Avian Pathol* **31**, 169–175.

Tenaya, I. W. M., Penhale, W. J. & Hampson, D. J. (1998). Preparation of diagnostic polyclonal and monoclonal antibodies against outer envelope proteins of *Serpulina pilosicoli*. *J Med Microbiol* **47**, 317–324.

Thomas, W. & Sellwood, R. (1993). Molecular cloning, expression, and DNA sequence analysis of the gene that encodes the 16-kilodalton outer membrane lipoprotein of *Serpulina hyodysenteriae*. *Infect Immun* **61**, 1136–1140.

Thomas, W., Sellwood, R. & Lysons, R. J. (1992). A 16-kilodalton lipoprotein of the outer membrane of *Serpulina* (*Treponema*) *hyodysenteriae*. *Infect Immun* **60**, 3111–3116.

Thomson, J. R., Smith, W. J., Murray, B. P. & McOrist, S. (1997). Pathogenicity of three strains of *Serpulina pilosicoli* in pigs with a naturally acquired intestinal flora. *Infect Immun* **65**, 3693–3700.

Thomson, J. R., Smith, W. J. & Murray, B. P. (1998). Investigations into field cases of porcine colitis with particular reference to infection with *Serpulina pilosicoli*. *Vet Rec* **142**, 235–239.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* **76**, 4350–4354.

Trampel, D. W., Jensen, N. S. & Hoffman, L. J. (1994). Cecal spirochetosis in commercial laying hens. *Avian Dis* **38**, 895–898.

Trivett-Moore, N. L., Gilbert, G. L., Law, C., Trott, D. J. & Hampson, D. J. (1998). Isolation of *Serpulina pilosicoli* from rectal biopsies showing evidence of intestinal spirochetosis. *J Clin Microbiol* **36**, 261–265.

Trott, D. J., McLaren, A. J. & Hampson, D. J. (1995). Pathogenicity of human and porcine intestinal spirochaetes in day-old specific pathogen free chicks: an animal model of intestinal spirochetosis. *Infect Immun* **63**, 3705–3710.

Trott, D. J., Huxtable, C. R. & Hampson, D. J. (1996a). Infection of newly weaned pigs with human and porcine strains of *Serpulina pilosicoli*. *Infect Immun* **64**, 4648–4654.

Trott, D. J., Stanton, T. A., Jensen, N. S. & Hampson, D. J. (1996b). Phenotypic characteristics of *Serpulina pilosicoli* the agent of intestinal spirochaetosis. *FEMS Microbiol Lett* **142**, 209–214.

Trott, D. J., Atyeo, R. F., Lee, J. I., Swayne, D. A., Stoutenburg, J. W. & Hampson, D. J. (1996c). Genetic relatedness amongst intestinal spirochaetes isolated from rats and birds. *Lett Appl Microbiol* **23**, 431–436.

Trott, D. J., Stanton, T. B., Jensen, N. S., Duhamel, G. E., Johnson, J. L. & Hampson, D. J. (1996d). *Serpulina pilosicoli* sp. nov. the agent of porcine intestinal spirochetosis. *Int J Syst Bacteriol* **46**, 206–215.

Trott, D. J., Combs, B. G., Mikosza, A. S. J., Oxberry, S. L., Passey, M., Taime, J., Sehuko, R., Alpers, M. P. & Hampson, D. J. (1997). The prevalence of *Serpulina pilosicoli* in humans and domestic animals in the Eastern Highlands of Papua New Guinea. *Epidemiol Infect* **119**, 369–379.

Trott, D. J., Alt, D. P., Zuerner, R. L., Wannemuehler, M. J. & Stanton, T. B. (2001). The search for *Brachyspira* proteins that interact with the host. *Anim Health Res Rev* **2**, 19–30.

Tsai, C. & Frasch, C. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**, 115–119.

van Belkum, A., Scherer, S., van Alphen, L. & Verbrugh, H. (1998). Short sequence DNA repeats in prokaryotic genomes. *Microbiol Mol Biol Rev* **62**, 275–293.

Weigel, L., Belisle, J., Radolf, J. & Norgard, M. (1994). Digoxigenin-ampicillin conjugate for detection of penicillin-binding proteins by chemiluminescence. *Antimicrob Agents Chemother* **38**, 330–336.

Weinstock, G. M., Hardham, J. M., McLeod, M. P., Sodergren, E. J. & Norris, S. J. (1998). The genome of *Treponema pallidum*: new light on the agent of syphilis. *FEMS Microbiol Rev* **22**, 323–332.

Zhang, P., Cheng, X. & Duhamel, G. E. (2000). Cloning and DNA sequence analysis of an immunogenic glucose/galactose MglB lipoprotein homologue from *Brachyspira pilosicoli* the agent of colonic spirochetosis. *Infect Immun* **68**, 4559–4565.

Zuerner, R. L., Knudtson, W., Bolin, C. A. & Trueba, G. (1991). Characterization of outer membrane and secreted proteins of *Leptospira interrogans* serovar *pomona*. *Microb Pathog* **10**, 311–322.