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The search for *Brachyspira* outer membrane proteins that interact with the host

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Abstract

Little is known about the outer membrane structure of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* or the role of outer membrane proteins (OMPs) in host colonization and the development of disease. The isolation of outer membrane vesicles from *B. hyodysenteriae* has confirmed that cholesterol is a significant outer membrane constituent and that it may impart unique characteristics to the lipid bilayer structure, including a reduced density. Unique proteins that have been identified in the *B. hyodysenteriae* outer membrane include the variable surface proteins (Vsp) and lipoproteins such as SmpA and BmpB. While the function of these proteins remains to be determined, there is indirect evidence to suggest that they may be involved in immune evasion. These data may explain the ability of the organism to initiate chronic infection. OMPs may be responsible for the unique attachment of *B. pilosicoli* to colonic epithelial cells; however, the only *B. pilosicoli* OMPs that have been identified to date are involved in metabolism. In order to identify further *B. pilosicoli* OMPs we have isolated membrane vesicle fractions from porcine strain 95-1000 by osmotic lysis and isopycnic centrifugation. The fractions were free of contamination by cytoplasm and flagella and contained outer membrane. Inner membrane contamination was minimal but could not be completely excluded. An abundant 45-kDa, heat-modifiable protein was shown to have significant homology with *B. hyodysenteriae* Vsp, and monoclonal antibodies were produced that reacted with five *B. pilosicoli*-specific membrane protein epitopes. The first of these proteins to be characterized is a unique surface-exposed lipoprotein.

Introduction

Pathogenic spirochetes that inhabit the large intestine have an intimate association with the colonic mucosa. In the case of the agent of swine dysentery, *Brachyspira hyodysenteriae* (formerly *Serpulina hyodysenteriae*), the spirochetes localize within colonic crypts, penetrate the protective mucus barrier, and invade the epithelium and the lamina propria. In contrast, the weakly β-hemolytic intestinal spirochete *Brachyspira pilosicoli* (the agent of intestinal spirochetosis) attaches by one cell end to the colonic epithelium, but it also may localize within the colonic crypts and invade beyond the epithelium.

By analogy with other pathogenic organisms, it is probable that proteins expressed on the surface of *B. hyodysenteriae* and *B. pilosicoli* play an important role in colonization and disease expression. This interaction may be direct (for example by mediating attachment to host cells) or indirect (for example by being the targets for the host immune response). It is interesting to note that relatively few *Brachyspira* outer membrane proteins (OMPs) have been characterized, especially when compared to other species of pathogenic spirochete such as *Borrelia* and *Treponema*. Determinants of *B. hyodysenteriae* pathogenicity have thus far been restricted to motility and hemolysins, and *B. pilosicoli* has only recently been characterized and remains an open research field.

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Identification and characterization of unique *Brachyspira* OMPs will advance our understanding of swine dysentery and intestinal spirochetosis pathogenesis, yield improved methods for diagnosis and identify potential targets for novel vaccine development. Identification of OMPs is achieved by isolating the spirochete outer membrane relatively free from other cellular components. Specific proteins and their corresponding genes can then be characterized to determine surface localization, function, immune reactivity and whether they are present in all strains of the species. This paper will review the structure of the *Brachyspira* outer membrane, including the characterization of known OMPs. It will also compare methods that have been used to isolate outer membranes from the major spirochete genera. It will conclude with a summary of our recent research findings concerning the isolation of *B. pilosicoli* membrane vesicles and the characterization of unique outer membrane epitopes in this species.

The *Brachyspira* outer membrane

The spirochete outer membrane has a typical Gram-negative structure, but is much more labile and easily disrupted by detergents, osmotic shock or pH changes than those of typical Gram-negative bacteria. It appears to be only loosely attached to the underlying protoplasmic cylinder. An important difference between spirochetes and Gram-negative bacteria is the location of the peptidoglycan layer. In Gram-negative bacteria, peptidoglycan is covalently anchored by lipoproteins to the outer membrane, whereas in spirochetes it forms the outermost layer of the cell wall–cytoplasmic membrane complex (Joseph *et al.*, 1973). The outer membrane ultrastructure of intestinal spirochetes has been reviewed previously by Sellwood and Bland (1997) and appears to be similar to other spirochete membranes; however, recent findings have highlighted some important differences (Fig. 1).

Cholesterol

Stanton (1987) first showed that cholesterol is incorporated into *B. hyodysenteriae* membranes and that it constitutes approximately 9% of total membrane lipids. Plaza and co-workers (Plaza *et al.*, 1997) extended these observations to show that cholesterol is partitioned exclusively into the outer membrane. Treatment of cells with low concentrations of digitonin, a detergent that interacts with cholesterol, caused the complete disruption of the *B. hyodysenteriae* outer membrane but had little effect on *Borrelia burgdorferi*. Cholesterol is also present in the outer membrane of *B. pilosicoli* (D.J. Trott and T.B. Stanton, unpublished data) and may be a unique characteristic of the outer membrane of all *Brachyspira* species. Plaza and co-workers (Plaza *et al.*, 1997) suggested that cholesterol is responsible for the unusually low density of the *B. hyodysenteriae* outer membrane (1.10 gm/cm^3).

Lipo-oligosaccharide

The lipopolysaccharide-like material of *B. hyodysenteriae* has been well characterized. It forms the basis for the serological typing scheme for differentiating *B. hyodysenteriae* strains (Hampson *et al.*, 1990), and reacts strongly with convalescent sera, suggesting that it is the prime

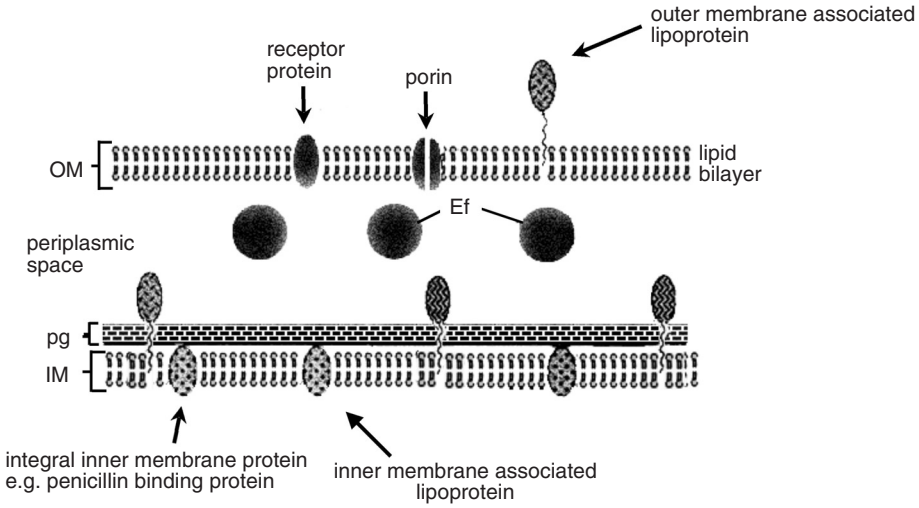

Fig. 1. Proposed intestinal spirochete ultrastructure. The outer membrane (OM) is likely to contain lipoproteins, porins and receptor proteins. Lipoproteins and integral membrane proteins may also be located on the inner membrane (IM), which is covalently anchored to the peptidoglycan (pg). Periplasmic flagella (Ef) are located between the outer and inner membranes within the periplasmic space. (Adapted from Cox *et al.*, 1992).

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target for the host immune response (Wannemuehler *et al.*, 1988). *B. hyodysenteriae* lipopolysaccharide-like material is rough or semi-rough in nature and has been termed lipo-oligosaccharide (LOS) as it does not exhibit the typical lipopolysaccharide (LPS) ladder of Gram-negative bacteria (Sellwood and Bland, 1997). It consists of a prominent lipid A core region of approximately 10–16 kDa with some strains possessing an irregularly spaced, partial ladder of short O-antigen side chains.

The LOS of *B. pilosicoli* has recently been characterized (Lee and Hampson, 1999). A prominent, non-immunogenic 16-kDa lipid A core region was observed in eight strains of *B. pilosicoli*, and some of these strains possessed a regular ladder-like O-antigen structure that was more prominent in Western blots than in silver-stained SDS-PAGE gels. This suggests that *B. pilosicoli* LOS is more smooth in nature than *B. hyodysenteriae*. The LOS profile of each strain was unique and did not cross-react antigenically, confirming that there is a high degree of LOS antigenic diversity in *B. pilosicoli*. This suggests that immunity generated against one strain may not confer immunity to another strain, and may reflect the high overall genetic diversity within the species (Trott *et al.*, 1998).

Outer membrane proteins

Freeze fracture analysis has shown that *B. hyodysenteriae* has the highest number of intramembranous particles (analogous to surface-exposed proteins) on the outer membrane surface when compared to *Treponema pallidum, B. burgdorferi, Borrelia hermsii* or *Treponema denticola* (Walker *et al.*, 1989, 1991). Preliminary freeze–fracture analysis of *B. pilosicoli* strain 95-1000 has shown fewer intramembranous particles and more regular (non-random) arrays in the concave face than *B. hyodysenteriae*. It is possible that these are the result of lipid phase transition (an artifact of fixation temperature); however, the same phenomenon was not observed on the fractured inner membrane and further analysis is required (K.W. Bourell, D.J. Trott and J.D. Radolf, unpublished results). The protein content of *B. hyodysenteriae* isolated outer membranes was shown to be 0.25 mg/mg dry weight, which is less than half the protein content of isolated inner membranes. Approximately 30–35 distinct protein bands were identified in the outer membrane of strain B204 (Plaza *et al.*, 1997) but only a fraction of these have been characterized.

The recognized species within the *Brachyspira* genus are all genetically closely related, and share cross-reactive protein epitopes, reflecting their common ecological niche within the large intestine (Chatfield *et al.*, 1988b). It is not currently known which proteins are unique to each species, and whether specific OMPs are related to pathogenicity in both *B. hyodysenteriae* and *B. pilosicoli*. Several prominent proteins identified in the *B. hyodysenteriae* outer membrane are unique amongst bacteria, and although their differential expression may play a role in immune evasion, their true physiological function can only be surmised or is not known at this stage. The end-on attachment of *B. pilosicoli* to the colonic epithelium appears to be initiated by a protein–ligand interaction. A cross-hatched structure observed on the tip surface by electron microscopy may be a possible candidate for an attachment protein, but could also possibly be associated with the inner membrane (Fig. 2). *Brachyspira* OMPs that have been identified to date are shown in Table 1.

Sellwood and co-workers (Sellwood *et al.*, 1989) found a prominent 16-kDa *B. hyodysenteriae* antigen that was solubilized in low concentrations of sodium dodecyl sulfate (SDS) and that reacted strongly with hyperimmune and convalescent pig sera. Subsequent cloning and characterization of this protein (designated SmpA) showed it to be a unique lipoprotein that was expressed on the cell surface *of B. hyodysenteriae* (Thomas *et al.*, 1992; Thomas and Sellwood, 1992, 1993). Potential existed for the development of diagnostic reagents or subunit vaccines using recombinant SmpA; however, DNA hybridization studies and PCR showed that not all virulent strains of *B. hyodysenteriae* possess this protein or that it is significantly modified (Turner *et al.*, 1995). Additionally, Sellwood *et al.* (1995) utilized a capture ELISA to monitor the expression of SmpA in swine challenged with *B. hyodysenteriae*. The results suggest the possibility that SmpA is environmentally regulated, as the protein was not expressed during the onset of clinical signs of swine dysentery. Downregulation of OMPs during the acute stages of swine dysentery may be a mechanism for immune evasion and the establishment of chronic infection. One mechanism for immune evasion by *T. pallidum* is believed to be the sequestration of lipoproteins on the inner membrane, leaving very few targets on the outer surface. Sellwood and Bland (1997) made the important observation that the low number of intramembranous particles in *T. pallidum* could reflect the fact that this organism has not been cultivated *in vitro*. In the light of the results obtained for SmpA, the environmental regulation of *Brachyspira* OMPs *in vivo* may also be an important mechanism for evading the host immune response and must be considered when identifying unique OMPs in intestinal spirochetes grown in artificial media. For example, it is possible that OMPs that act directly upon the host may only be expressed in response to environmental stimuli, such as low iron concentrations (Li *et al.*, 1995).

The identification of the *B. hyodysenteriae* Vsp family has also shed light on other possible mechanisms of immune evasion by intestinal spirochetes. Gabe *et al.* (1995) used increasing concentrations of the non-ionic detergent Tween 20 to release extracytoplasmic proteins from *B. hyodysenteriae* strain B204. While most of these

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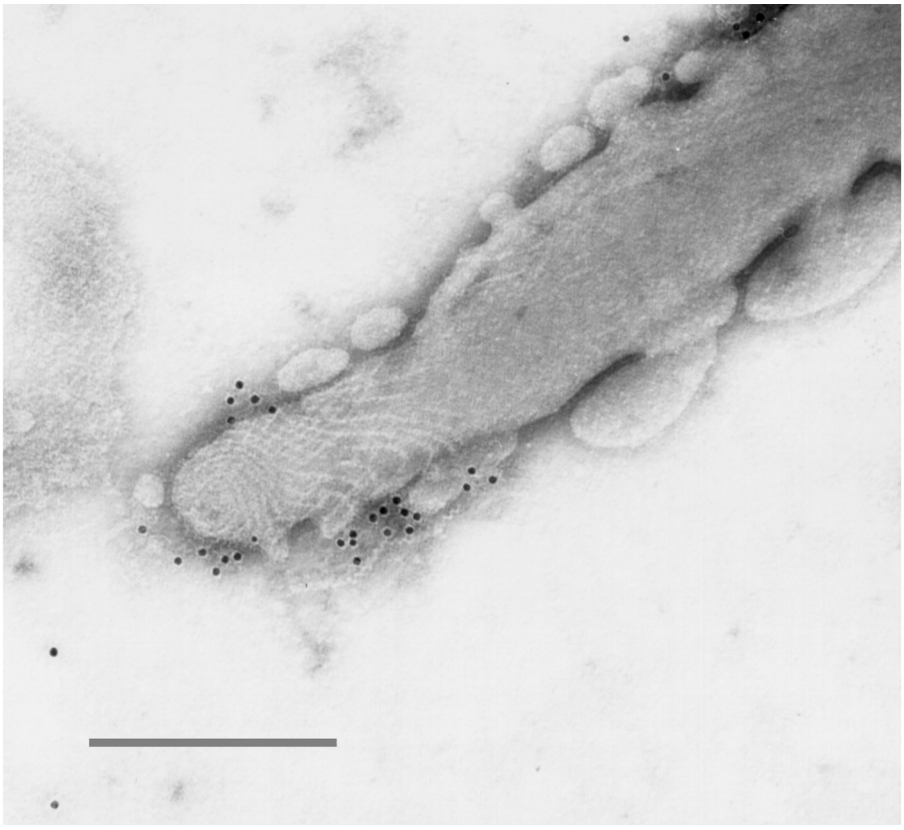

Fig. 2. Transmission electron micrograph of a *Brachyspira pilosicoli* porcine strain 95-1000 cell, showing immunogold labeling of an outer membrane lipoprotein. The outer membrane is forming discrete vesicles and a cross-hatched structure can be seen clearly at the spirochete tip. It is not clear, however, whether the cross-hatched structure is located on the inner or the outer membrane. (Marker bar = 0.20 μm.)

proteins were flagellar in nature, a sedimentable, urea-insoluble, heat-modifiable 39-kDa protein was shown to be surface exposed (Gabe *et al.*, 1998). Attempts to identify the gene sequence uncovered a linked set of at least four highly conserved genes encoding proteins that resembled, but did not match, the partial amino-acid sequence obtained for the 39-kDa protein. The gene that faithfully encodes the 39-kDa protein is still to be identified and may reside on another part of the genome (Gabe *et al.*, 1998; McCaman *et al.*, 1999). The cloned genes were identified as *vspA–D* (for variable surface proteins). A hydrophobicity plot of VspA–D showed that the proteins have highly variable hydrophilic regions (possible surface-exposed epitopes) located between conserved hydrophobic regions (possible membrane-spanning regions). This suggests a possible role in immune evasion. Vsp proteins are novel and do not share any appreciable sequence homology with recognized proteins in GenBank.

Plaza *et al.* (1997) showed that the most prominent protein in purified outer membranes of *B. hyodysenteriae* is a 39-kDa heat-modifiable protein that is likely to belong to the Vsp family. Previous studies have also shown that this protein is prominent in Sarkosyl membrane extracts and that it is reactive with convalescent sera (Joens *et al.*, 1993). We have identified a prominent, heat-modifiable, 45-kDa protein in *B. pilosicoli* strain 95-1000 that closely resembles the Vsp family of proteins by N-terminal amino-acid sequencing (D.J. Trott and T.B. Stanton, unpublished data). It is not currently known whether Vsp proteins are present in all species of intestinal spirochete. Vsp proteins may be unique to the *Brachyspira* genus and their continued study may lead to a greater understanding of how intestinal spirochetes are able to colonize the colonic mucosa for extended periods without being eliminated by the host immune response.

Several *B. hyodysenteriae* and *B. pilosicoli* proteins have been identified by producing monoclonal (MAbs) and polyclonal antibodies (PAbs) against whole-cell proteins, Triton X-114 extracts, or specific proteins eluted from SDS-PAGE gels. Most of these have been localized to the outer membrane by immunogold labeling. Additionally, screening DNA libraries with convalescent sera also has identified genes that encode for OMPs. MAbs against OMPs have been used mainly for diagnostic purposes; however, a *B. pilosicoli*-specific MAb directed against a 29-kDa protein (Lee and Hampson, 1995) has been used to screen a genomic DNA library (Rayment *et al.*, 1998). An immunopositive clone that contained an insert with an open reading frame encoding a putative pyruvate oxidoreductase was identified. However, the gene was not cloned into *Escherichia coli*

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Table 1. Characteristics of *Brachyspira* outer membrane proteins

Species	Protein	Size (kDa)	Function and location	References
B. hyodysenteriae	SmpA	16	Lipoprotein, strongly antigenic, reacts with convalescent sera. Environmentally regulated ('switched off' during disease expression). Surface associated by immunogold labeling	Thomas *et al.* (1992), Thomas and Sellwood (1992, 1993), Sellwood *et al.* (1995)
B. hyodysenteriae	Vsp family	39	Vsp A–D thus far identified. Encode closely related proteins with conserved hydrophobic and variable hydrophilic regions. Reacts with convalescent sera. Possible role in immune evasion. Surface association by ^{125}I labeling	Gabe *et al.* (1995, 1998), McCaman *et al.* (1999)
B. hyodysenteriae	BmpB	30	MAb reacts with *B. hyodysenteriae*-specific epitope. Surface associated by immunogold labeling	Lee *et al.*, 2000, Lee and Hampson (1996)
Brachyspira spp., *T. succinifaciens*	Serine protease	BmpB	Activity resides in membrane fractions and not in cell-free supernatant	Muniappa and Duhamel (1997)
B. hyodysenteriae and *B. innocens*	Unknown	26–45	Cross-reactive epitopes. Surface associated by immunogold labeling	Achacha and Mittal (1995)
B. pilosicoli	Putative pyruvate oxido-reductase	29	IgM MAb against 29-kDa OMP reactive with all strains of *B. pilosicoli*. Surface associated by immunogold labeling	Lee and Hampson (1995), Rayment *et al.* (1998)
B. pilosicoli	Unknown	72	Reacts with absorbed *B. pilosicoli* hyperimmune sera. Surface associated by immunogold labeling	Tenaya *et al.* (1998)
B. pilosicoli	MglB	35	Lipoprotein, strongly antigenic. Homology with glucose/galactose chemoreceptor/transporter	Zhang *et al.* (2000)

and then shown to react with the MAb, so it is possible that the pyruvate oxidoreductase is not the 29-kDa *B. pilosicoli* OMP protein.

Zhang *et al.* (1999) showed that Sarkosyl soluble *B. pilosicoli* proteins of 64, 54 and 47 kDa were detected by hyperimmune and convalescent pig sera. The 64- and 54-kDa proteins also were soluble in the Triton X-114 detergent phase, suggesting that they are integral membrane proteins associated with the outer membrane. Zhang *et al.* (2000) also cloned and sequenced a 36-kDa *B. pilosicoli* lipoprotein by screening a plasmid library of *B. pilosicoli* DNA with hyperimmune and convalescent swine sera. The protein has significant homology with glucose/galactose transporter/chemoreceptor MglB lipoproteins of *E. coli*, *Salmonella typhimurium* and *T. pallidum*. Homologous DNA sequences have been found in some weakly β-hemolytic *Brachyspira* species but not in porcine *B. hyodysenteriae*, human *B. aalborgi* and porcine *Treponema succinifaciens* (Zhang *et al.*, 2000).

A 72-kDa *B. pilosicoli* OMP was identified by screening Triton X-114 extracts with porcine hyperimmune sera absorbed with extracts from *B. hyodysenteriae* and *Brachyspira innocens*. MAbs raised against the protein were cross-reactive with other *Brachyspira* species or reacted with an 80-kDa *B. pilosicoli*-specific protein epitope (Tenaya *et al.*, 1998). Immunogold labeling has not been performed using the 80-kDa MAb; however, the protein does not appear to be associated with the *B. pilosicoli* outer membrane as it is enriched in soluble (non-membrane) protein fractions (D.J. Trott and T.B. Stanton, unpublished data). Achacha and Mittal (1995) produced MAbs against four *B. hyodysenteriae* OMPs ranging in size from 26 to 45 kDa; however, the epitopes were also present in other *Brachyspira* species. In contrast, Lee and Hampson (1996) produced MAbs against a 30-kDa protein epitope that was only present in *B. hyodysenteriae*. The gene encoding this protein has recently been cloned and expressed in *E. coli*. It has been shown to encode a lipoprotein that has been designated BmpB (Lee *et al.*, 2000).

A heat-stable, subtilisin-like serine protease has been identified in the outer membrane of all intestinal spirochete species (Muniappa and Duhamel, 1997). This protease may contribute indirectly to mucosal damage caused by pathogenic species and possibly to protein autolysis in isolated membrane fractions. It is therefore recommended that protease inhibitors such as phenylmethylsulfonyl fluoride be used in studies involving the fractionation of *Brachyspira* membranes.

Techniques for isolating the outer membrane of spirochetes

The selective removal of the spirochete outer membrane is hampered by its labile nature and by the periplasmic location of the flagella. Considerable structural heterogeneity exists amongst the spirochetes and a technique described for one organism may not be applicable to, or must be suitably modified when working with, an organism of a different genus or even a different species. The presence of cholesterol in the outer membrane of intestinal spirochetes may cause them to be even more fragile and less amenable to treatments involving fractionation or detergent solubilization. Additionally *B. pilosicoli* has been shown to form cyst-like structures *in vitro* (Barber *et al.*, 1995) that may disrupt the uniformity of the cell suspension prior to fractionation.

Detergent solubilization

Techniques employing the solubilization of spirochete outer membranes in detergents such as SDS, Sarkosyl or Triton X-100 tend to selectively release the periplasmic flagella or cause cell lysis and release of cytoplasmic proteins and inner membrane components (Chatfield *et al.*, 1988a; Wannemuehler *et al.*, 1988; Joens *et al.*, 1993; Fisher *et al.*, 1997). Triton X-114 has been used to release selectively and identify spirochete outer membrane proteins (e.g. Cunningham *et al.*, 1988; Zuerner *et al.*, 1991; Zhang *et al.*, 1999;) and its low cloud point permits the partitioning of hydrophobic (membrane spanning) proteins into the detergent phase and hydrophilic proteins into the aqueous phase. However, in some species of spirochete, lipoproteins anchored to the inner membrane by their lipid moieties and periplasmic proteins are also likely to be solubilized unless very low concentrations are used (Blanco *et al.*, 1994). As discussed previously, Triton X-114 treatment of *B. pilosicoli* cells enabled the identification of a 29-kDa OMP but also released an 80-kDa protein that does not appear to be anchored to a membrane.

Membrane vesicle fractionation

In recent years a number of techniques have been published for *Borrelia* and *Treponema* that involve osmotic lysis or French pressure cell disruption of the spirochete cells followed by isopycnic centrifugation (Table 2). These methods take advantage of the difference in density between the outer and inner membranes, or the resistance of the cell wall–cytoplasmic membrane complex to osmotic stress. Generally, spirochete outer membranes are lighter than the corresponding inner membrane, which is possibly due to the reduced number of proteins, but also may be due to the outer membrane lipid composition, as discussed previously for intestinal spirochetes. To determine the identity and purity of the isolated vesicles, known markers of the cytoplasm, flagella, inner and outer membrane are applied to each fraction. Compared to most Gram-negative bacteria, markers for spirochetes are poorly defined

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and often several are required to confirm the cellular identity of a particular fraction. The fractionation of membrane vesicles maintains membrane proteins in their native state within the lipid bilayer, which provides an advantage over detergent-based solubilization techniques, particularly for the preservation of epitopes that are targets for the immune system. The relative merits of each particular technique will not be discussed here; however, it must be stressed that differences in the density and protein composition of spirochete membrane fractions have been observed between techniques applied to the same organism. For instance, Bledsoe *et al.* (1994) used the French pressure cell disruption technique to show that the density of *B. burgdorferi* isolated outer membrane vesicles was 1.19 gm/cm^3. In studies using osmotic lysis, the density was much lower and the number of protein bands was reduced (Radolf *et al.*, 1995a; Skare *et al.*, 1995). A second problem is that in some studies, proteins identified in purified outer membrane fractions have subsequently been localized to the inner membrane. For instance proteins enriched in outer membrane vesicle preparations and considered rare outer membrane pore-forming proteins of *T. pallidum* (Tromps) (Blanco *et al.*, 1995, 1996), have been claimed to be anchored to the inner membrane and involved in divalent cation transport (Akins *et al.*, 1997). *T. pallidum* is clearly an unusual case due to the paucity of proteins in its outer membrane, but the results do highlight the fact that proteins enriched in outer membrane preparations may need verification of their outer membrane location by several techniques.

Isolation of membrane vesicles from Brachyspira hyodysenteriae

Plaza *et al.* (1997) used French pressure cell disruption and sucrose density ultracentrifugation to isolate inner and outer membranes of *B. hyodysenteriae* B204. The fractionation and purification of *B. hyodysenteriae* proved to be more difficult than that of previously studied bacteria due to the low density of the outer membrane and the propensity of the isolated membrane vesicles to form inner and outer membrane hybrids. Four membrane fractions (density range 1.10–1.16 g/cm^3) and two flagella fractions (density 1.22 g/cm^3) were obtained. The membrane fractions were free of cytoplasmic (β-NADH oxidase activity) and flagella contamination (PAbs to FlaA1 and FlaB). On the basis of outer (MAbs to SmpA and LOS and cholesterol) and inner (PAbs to the F_1F_0 ATPase C subunit of *E. coli*) membrane markers, the lowest density fraction was shown to be purified outer membrane, the highest to be purified inner membrane and the remaining fractions were hybrids between the two. Two-dimensional non-equilibrium pH gradient electrophoresis showed the protein profiles of outer and inner membrane fractions to be different, with approximately 7–10 polypeptides shared between the two.

Fractionation of Brachyspira pilosicoli *by osmotic lysis and identification of unique outer membrane epitopes*

For *B. pilosicoli* fractionation studies, cholesterol, the 29-kDa putative pyruvate oxidoreductase, and LOS were identified as outer membrane markers and *B. hyodysenteriae* FlaA1 and NADH oxidase as respective flagella and cytoplasmic markers. Inner membrane markers were problematic as PAbs against the *E. coli* F_1F_0 ATPase C subunit were cross-reactive with several *B. pilosicoli* proteins. We therefore used digoxigenin-labeled ampicillin to identify penicillin-binding proteins (Weigel *et al.*, 1994) and measured diamino pimelic acid (DAPA) content as an indication of peptidoglycan contamination.

Initially we attempted to isolate purified outer membrane vesicles from *B. pilosicoli* using techniques published for other spirochete species. Using French pressure cell disruption, we obtained hybrid membrane fractions that differed in density but had the same protein profile and contained equal amounts of inner and outer membrane markers. Clearly, the outer membrane of *B. pilosicoli* is different in structure to that of *B. hyodysenteriae*. For example, the tendency to form hybrid membrane structures following French pressure cell disruption may be due to the higher outer membrane density of *B. pilosicoli*. We had observed in our laboratory that mixing *B. pilosicoli* cells in distilled water caused rapid lysis of the outer membrane, while maintaining the integrity of the protoplasmic cylinder and the periplasmic flagella (Fig. 3). We therefore developed a membrane vesicle isolation technique based on osmotic lysis in distilled water followed by isopycnic centrifugation (Trott *et al.*, 1999). Isolated membrane vesicles were separated by density into four fractions: low-density membrane fractions A and B with densities of 1.12–1.14 g/cm^3 and high-density membrane fractions A and B with densities of 1.16–1.18 g/cm^3. The SDS-PAGE profiles of high- and low-density fractions differed, with the high-density fractions characterized by prominent, heat-modifiable 45-kDa and 55-kDa proteins. The 45-kDa protein was subsequently shown to have high N-terminal amino-acid homology with *B. hyodysenteriae* VspA–D. Markers showed that the membrane vesicles were free of cytoplasmic and flagella contamination. Low-density membrane fractions were enriched for LOS and the 29-kDa protein, but no fraction was completely free of inner membrane contamination.

Low-density membrane fraction B contained less than 10% of the penicillin-binding protein activity of total membranes (outer membrane plus inner membrane and flagella) and was therefore selected as antigen for the production of MAbs (Trott *et al.*, 2000). A large number

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Table 2. Techniques for isolating spirochete outer membranes

Species	Technique	Markers	Notes	Reference
Borrelia burgdorferi	French pressure cell disruption and isopycnic centrifugation to yield outer membrane, hybrid membrane and inner membrane and flagella fractions	Outer membrane: freeze–fracture analysis. Inner membrane: F_1/F_0 ATPase C subunit. Cytoplasmic: lactate dehydrogenase	Outer membrane density: 1.19 g/cm^3. Outer membrane protein content: 0.15 mg/mg dry weight. Inner membrane density: 1.12 g/cm^3. Inner membrane protein content: 0.60 mg/mg dry weight	Bledsoe *et al.*, (1994)
Borrelia burgdorferi	Osmotic lysis in low pH citrate buffer plus BSA followed by isopycnic centrifugation	Outer membrane: porin activity. Inner membrane: β-NADH oxidase	Outer membrane density: 1.13 g/cm^3	Skare *et al.*, (1995)
Borrelia burgdorferi	Osmotic lysis in 20% sucrose to separate outer membrane vesicles from protoplasmic cylinders followed by isopycnic centrifugation	Outer membrane: freeze–fracture analysis. Inner membrane: β-NADH oxidase. Flagella: flagellin. Cytoplasmic: 60-kDa heat-shock protein	Outer membrane density: 1.12 g/cm^3	Radolf *et al.*, (1995a)
Borrelia hermsii	Osmotic lysis in low pH citrate buffer plus BSA followed by isopycnic centrifugation	Outer membrane: porin activity. Inner membrane: β-NADH oxidase	Outer membrane density: 1.15 g/cm^3	Shang *et al.*, (1998)
Treponema vincentii	Osmotic lysis in low pH citrate buffer followed by isopycnic centrifugation	Outer membrane: freeze–fracture analysis lipopolysaccharide	Outer membrane density: 1.13 g/cm^3	Blanco *et al.*, (1994)
Treponema pallidum	Osmotic lysis in low pH citrate buffer followed by isopycnic centrifugation. Staining of cells with octadecyl rhodamine B chloride	Outer membrane: freeze fracture analysis. Inner membrane: penicillin-binding proteins, 19- and 47-kDa lipoprotein. Flagella: *T. pallidum* FlaA	Outer membrane density: 1.05 g/cm^3	Blanco *et al.*, (1994)
Treponema pallidum	Osmotic lysis in 20% sucrose to separate outer membrane vesicles from protoplasmic cylinders followed by isopycnic centrifugation	Outer membrane: freeze–fracture analysis. Inner membrane: penicillin-binding proteins, 47-kDa, 38-kDa and 34-kDa lipoproteins. Flagella: *T. pallidum* FlaA. Cytoplasmic: Tpn 60	Outer membrane density: 1.05 g/cm^3	Radolf *et al.*, (1995b)
Brachyspira hyodysenteriae	French pressure cell disruption and isopycnic centrifugation to yield outer membrane, hybrid membrane and inner membrane and flagella fractions	Outer membrane: SmpA, lipo-oligosaccharide, cholesterol. Inner membrane: F_1/F_0 ATPase C subunit. Flagella: FlaA1, FlaB. Cytoplasmic: NADH oxidase	Outer membrane density: 1.10 g/cm^3. Outer membrane protein content: 0.25 mg/mg dry weight. Inner membrane density: 1.16 g/cm^3. Inner membrane protein content: 0.55 mg/mg dry weight	Plaza *et al.*, (1997)

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of hybridomas produced antibodies to proteinase K resistant cellular components that resembled the O-antigen side-chains of LOS. A further group of hybridomas reacted with membrane proteins present in at least one other *Brachyspira* species. Five cloned hybridomas produced MAbs that reacted strongly with *B. pilosicoli*-specific proteinase K sensitive bands with estimated molecular weights of 79, 61, 35, 23/24 doublet and 23 kDa. Immunogold labeling with concentrated MAb 2E10 directed against the 23-kDa protein confirmed that the protein was located on the outer membrane. The protein reacting with MAb 2E10 has been cloned, sequenced and confirmed to be a unique lipoprotein with a predicted size of 19 kDa and a highly similar initial N-terminal amino-acid sequence to *B. hyodysenteriae* SmpA. This suggests the possibility of a common mechanism for anchoring of the lipid moiety into the outer membrane (Alt *et al.*, 2000). We are currently determining whether the 19-kDa protein is recognized by convalescent pig sera and if it is expressed in all *B. pilosicoli* strains. We are also determining the cellular location and identifying the genes that encode the four remaining proteins identified by *B. pilosicoli*-specific monoclonal antibodies.

Conclusion

It is clear that the outer membrane structure of the *Brachyspira* genus is unique when compared to other spirochete genera. The detection of cholesterol in the outer membrane has not been reported in other procaryotes (although cholesterol has been detected in *Mycoplasma* membranes), and a number of unique OMPs have been identified. Apart from the abundant variable surface proteins, four of the five *Brachyspira* OMPs that have been characterized to date are lipidated. The location of lipoproteins in *Brachyspira* may be analogous to those of pathogenic *Leptospira*, where lipoproteins also have been shown to be surface exposed (e.g. Haake *et al.*, 1999). In *T. pallidum*, lipoproteins are sequestered on the inner membrane and in *B. burgdorferi* only a small proportion of the major lipoproteins OspA and OspB are expressed on the

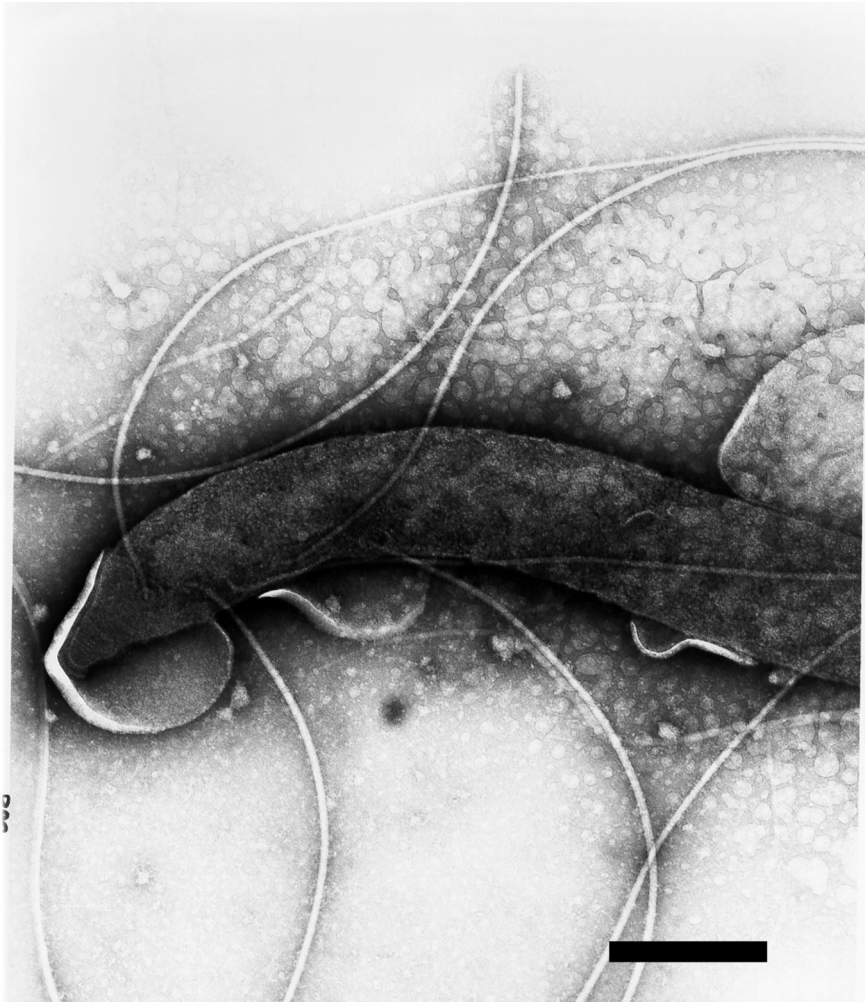

Fig. 3. Electron micrograph of osmotic lysis of *Brachyspira pilosicoli* 95-1000 outer membrane in distilled water. The flagella have unwound from the protoplasmic cylinder but are still attached to the inner membrane. (Marker bar = 0.30 μm.)

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outer surface, with the majority associated with the inner membrane. Many spirochete lipoproteins are potent immunogens and they may be environmentally regulated *in vivo*. Further investigation of the structure and function of intestinal spirochete lipoproteins is therefore recommended, particularly to determine their expression *in vivo* and their serological and mucosal immunoreactivity. Identification of unique intestinal spirochete OMPs will be important in terms of future vaccine and diagnostic test development.

As more *Brachyspira* outer membrane proteins are identified and sequenced, it may be possible to determine their physiological function and their effect on the host. Genetic systems now exist for the insertion of antibiotic resistance cassettes into *B. hyodysenteriae* by electroporation, to create knockout mutants with deletions in major virulence genes. Such techniques have been applied already to *B. hyodysenteriae* virulence determinants, including *tlyA* (ter Huurne *et al.*, 1992), *flaA1* and *flaB1* (Rosey *et al.*, 1995), and the β-NADH oxidase gene (Humphrey *et al.*, 1997), and should be applicable to *B. pilosicoli*. Additionally, the discovery of VSH-1, a defective phage that acts as a natural gene transfer agent, has significantly improved our ability to manipulate these strains genetically (Humphrey *et al.*, 1997). We are continuing to search for OMPs that mediate the unique attachment of *B. pilosicoli* to the epithelium, and their identification may be the key to unlocking the pathogenic mechanisms of this intriguing spirochete.

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