The periplasmic flagella of *Serpulina* (*Treponema*) *hyodysenteriae* are composed of two sheath proteins and three core proteins.

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The major components of the periplasmic flagella of the spirochaete *Serpulina* (*Treponema*) *hyodysenteriae* strain C5 were purified and characterized. We demonstrate that the periplasmic flagella are composed of five major proteins (molecular masses 44, 37, 35, 34 and 32 kDa) and present their location, N-terminal amino acid sequence and immunological relationship. The 44 kDa and the 35 kDa protein are on the sheath of the periplasmic flagellum, whereas the 37, 34 and 32 kDa protein reside in the periplasmic flagellar core. The two sheath flagellar proteins are immunologically related but have different N-terminal amino acid sequences. The N-terminus of the 44 kDa protein shows homology with the sheath flagellins of other spirochaetes, but the 35 kDa protein does not. The three core proteins are immunologically cross-reactive and their N-terminal amino acid sequences are almost, but not completely, identical, indicating that the core proteins are encoded by three distinct genes. The core proteins show extensive N-terminal sequence similarities and an immunological relationship with periplasmic flagellar core proteins of other spirochaetes.

Introduction

The spirochaete *Serpulina* (*Treponema*) *hyodysenteriae* is the aetiological agent of swine dysentery (Harris *et al.*, 1972; Taylor & Alexander, 1971; Stanton, 1992), a severe mucohaemorrhagic diarrhoeal disease which primarily affects young pigs. The bacterium is highly motile by means of two bundles of seven to nine periplasmic flagella that are wound around the protoplasmic cylinder and are completely contained within the cell envelope in the periplasmic space. These periplasmic flagella are inserted subterminally at each pole and their ends overlap in the central portion of the bacterium (Canale-Parola, 1978; Holt, 1978).

In a number of pathogenic bacteria, flagella appear to be an important virulence factor (Attridge & Rowley, 1983; Drake & Montie, 1987; Guentzel & Berry, 1975; Holder *et al.*, 1982; Morooka *et al.*, 1985; Ochi *et al.*, 1991; Pavlovskis *et al.*, 1991; Richardson, 1991; Šadžiene *et al.*, 1991; Wassenaar *et al.*, 1991). Flagella may

also be an important virulence determinant of *S. hyodysenteriae* since they are involved in the typical corkscrew-like movement that facilitates the traverse by this bacterium of the viscous mucous lining the gut. They may therefore aid in the colonization of the gut and thus in causing disease. In addition, vaccination with a cloned periplasmic flagellar protein of *S. hyodysenteriae* was protective in a mouse model for swine dysentery (Boyden *et al.*, 1989).

Most periplasmic flagella of spirochaetes contain multiple protein species, which form an inner core surrounded by a sheath or outer layer (Brahamsha & Greenberg, 1988; Cockayne *et al.*, 1987; Holt, 1978; Joseph & Canale-Parola, 1972). However, the periplasmic flagella of *Borrelia burgdorferi* (Coleman & Benach, 1989) and *Treponema zuelzerae* (Bharier & Rittenberg, 1971) resemble other bacterial flagella: they do not have a sheath and consist of a single protein species. In spite of their role as a potential virulence factor and protective antigen little is known of the structure and composition of *S. hyodysenteriae* periplasmic flagella and conflicting data have been presented. Miller *et al.* (1988) reported the presence of six different proteins in periplasmic flagella of *S. hyodysenteriae*, ranging from 39 to 18.5 kDa. However, Kent *et al.* (1989) identified five

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major proteins, with molecular masses from 43.8 to 29.4 kDa. No further characterization of these proteins was presented. We therefore isolated periplasmic flagella of *S. hyodysenteriae* strain C5 in order to characterize their composition and structure in more detail. The major components of the periplasmic flagella were purified, their N-terminal amino acid sequence was determined and their location on the flagella and immunological relationship were studied.

Methods

Bacterial strains and growth conditions. The origin and relevant characteristics of the *S. hyodysenteriae* strains used in this investigation are listed in Table 3 (see Results). Strongly haemolytic isolates were isolated from the gut of pigs severely affected by swine dysentery. Weakly haemolytic isolates originated from stools of pigs that suffered from diarrhoea but showed no other clinical signs of swine dysentery. *S. hyodysenteriae* was cultured at 40 °C in an anaerobic hood, either in 250 ml Trypticase Soy Broth supplemented with 10% foetal calf serum, 0.05% RNA core type II C from *Torula* yeast (Sigma) and 400 µg spectinomycin ml⁻¹, or on Trypticase Soy Agar (Becton Dickinson) plates supplemented with 10% (v/v) sheep blood, 400 µg spectinomycin ml⁻¹ and 0.06% yeast extract (Oxoid).

Isolation and purification of the periplasmic flagella. A modification of the method described by Kent *et al.* (1989) was used. Bacterial cultures (250 ml) containing 10⁹ bacteria ml⁻¹, were harvested by centrifugation at 15000 g for 30 min at 4 °C and washed once with phosphate-buffered saline (PBS; 0.14 M-NaCl, 2.7 mM-KCl, 0.01 M-Na₂HPO₄, 1.76 mM-KH₂PO₄, pH 7.3). Pellets were suspended in 20 ml of PBS and outer envelopes were removed by addition of 10% (w/v) SDS to a final concentration of 0.1% and incubating the suspension for 30 min at room temperature with gentle shaking. The organisms were collected by centrifugation at 25000 g for 30 min at 4 °C and resuspended in PBS. Periplasmic flagella were removed from the cells by shearing in a blender for a total of 10 min. Pauses were included in which the suspension was cooled on ice to prevent overheating of the samples. Periplasmic flagella were separated from the bacterial bodies by collective pelleting of the latter at 30000 g for 30 min at 4 °C. Sodium lauroylsarcosinate (Sarkosyl) was added (0.2%) to the supernatant and flagella were sedimented by centrifugation at 94000 g for 60 min at 4 °C, resuspended in water and stored at -20 °C.

Electrophoresis and Western blotting. Proteins were separated by SDS-PAGE (12.5% w/v, acrylamide), essentially as described by Laemmli (1970). Proteins were visualized by staining with Coomassie Brilliant Blue R 250 and molecular masses were estimated by comparison with low molecular mass markers (Pharmacia). Proteins were transferred to nitrocellulose sheets (Schleicher and Schuell) according to the method of Towbin *et al.* (1979) using a Bio-Rad Transblot cell. After transfer, the nitrocellulose was blocked with 0.5% gelatin and 0.5% Tween 20 in PBS (PBS+) for 1 h on a rocking platform. The nitrocellulose was then incubated with an appropriate dilution of antiserum in PBS+ for 1 h. The filter was washed three times for 5 min with PBS+ and further incubated for 1 h with a 1:3000 dilution of an alkaline phosphatase conjugate of either goat anti-rabbit immunoglobulin G (Sigma) or a 1:2000 dilution of goat anti-mouse immunoglobulin G (Promega) in PBS+. After three washings with PBS+, bound antibodies were visualized by development in nitro blue tetrazolium (0.37 mM) and 5-bromo-4-chloro-3-indolyl phosphate (0.34 mM) solubilized in 100 mM-Tris/HCl, 100 mM-NaCl, 5 mM-MgCl₂, pH 9.5. Incubations and washings were performed at room temperature on a rocking platform.

Electron microscopy. *S. hyodysenteriae* cells were grown for 3 d on Trypticase Soy Agar plates and collected in 2 ml 0.03% sucrose, 0.01 M-MgCl₂, 0.01 M-CaCl₂. Samples were applied to Pioloform-coated copper grids (200 mesh, Bio-Rad) for 10 min, then washed briefly three times on drops of water and negatively stained with phosphotungstic acid (PTA). For immunogold labelling, bacteria were adsorbed onto grids and rinsed briefly three times with water. Subsequently, they were incubated for 30 min on drops of 1:1000-diluted antiserum in PBT (PBS containing 1% BSA, 0.05% Tween 20 and 0.1% gelatin). After three rinses with PBT, grids were further incubated for 30 min with protein A conjugated with 10 nm gold particles (Janssen Life Sciences). After rinsing the grids twice with water, the samples were negatively stained with PTA. All samples were examined with a Philips 201 electron microscope operating at 60 kV and an objective aperture of 30 µm.

Antisera. Rabbit antisera to the individual components of purified periplasmic flagella of *S. hyodysenteriae* C5 were obtained as follows. Approximately 600 µg of flagellar proteins was separated by SDS-PAGE, blotted onto nitrocellulose filters and visualized by staining with 0.6% Ponceau Red in 3% trichloroacetic acid. The areas of the filters containing the desired proteins were cut-out, shock-frozen in liquid nitrogen and homogenized with a Braun Mikro-dismembrator. The homogenized material was suspended in 3 ml of water and stored at -20 °C. For immunization, 1.5 ml of this material was mixed with an equal volume of Freund's complete adjuvant and inoculated intramuscularly into a New Zealand White rabbit on day 0. The rabbit was boosted with 1.5 ml of homogenized antigen/nitrocellulose in an equal volume of Freund's incomplete adjuvant on day 28. Sera were collected at days -1, 20, 34, 41 and 48.

Rabbit antiserum to *Treponema phagedenis* biotype Reiter periplasmic flagella was a gift from G. Noordhoek, RIVM Bilthoven, The Netherlands. Mouse monoclonals .1H11G7 and .2A6B4 and rabbit serum against *Spirochaeta aurantia* periplasmic flagella were obtained from E. P. Greenberg, University of Iowa, USA (Brahamsha & Greenberg, 1988). Mouse monoclonal mCC9 to the 37 kDa *Treponema pallidum* periplasmic flagellar protein was from C. W. Penn, University of Birmingham, UK (Bailey *et al.*, 1987).

N-Terminal sequence analysis. Periplasmic flagellar proteins were separated by SDS-PAGE and electroblotted on polyvinylidene difluoride membranes (Immobilon transfer membranes; Millipore) in 10 mM-3-cyclohexylamino-1,1-propanesulphonic acid, pH 11.0 and 10% methanol. The blotted proteins were stained with Coomassie Brilliant Blue R250 and bands containing the proteins of interest were cut-out. The amino acid sequence of the blotted proteins was determined with an Applied Biosystems model 470A protein sequencer, on-line equipped with a model 120A PTH Analyzer.

Results

Purification and SDS-PAGE analysis of periplasmic flagellar proteins

To identify the proteins that constitute the periplasmic flagella of *S. hyodysenteriae*, the periplasmic flagellar proteins of *S. hyodysenteriae* strain C5 were separated by SDS-PAGE. Five major proteins with apparent molecular masses of 44, 37, 35, 34 and 32 kDa were observed (Fig. 1, lane B). Bands of 44 and 37 kDa could be discerned clearly in a whole cell lysate of *S. hyodysenteriae* C5 (Fig. 1, lane A) suggesting that they are abundant proteins in the bacterium. In some periplasmic flagellar

Fig. 1. SDS-PAGE analysis of whole cell lysate (lane A) and isolated flagella (lane B) of *S. hyodysenteriae* C5 stained with Coomassie Brilliant Blue R250.

preparations the 34 kDa band appeared as a doublet (as in Fig. 1, lane B) or two additional bands of 30 and 28 kDa were visible. Since these bands were not always present and were of low intensity they may be minor periplasmic flagellar proteins, breakdown products or contaminants.

Immunoblot analysis of periplasmic flagellar protein

Further evidence for the flagellar nature of the five major proteins present in the periplasmic flagellar preparations was obtained by their reactivity on immunoblots with sera and monoclonal antibodies that had been raised against periplasmic flagellar proteins of other spirochaetes. With the exception of the 30 and 28 kDa proteins, all proteins present in the periplasmic flagellar preparation reacted with one or more of these sera (Table 1). The data obtained also show that the 37, 34 and 32 kDa proteins are immunologically related.

Rabbit antisera raised to the individual periplasmic flagellar proteins of *S. hyodysenteriae* strain C5 were tested in immunoblots for reactivity with *S. hyodysenteriae* C5 lysate and purified periplasmic flagella. An example of these immunoblots is shown in Fig. 2. Table 2 summarizes the results of the experiments. When Western blots with purified flagella were immunostained with serum raised against the 44 kDa flagellar protein, a strong 44 kDa band and a weak 35 kDa band appeared (Fig. 2, lane 1B). Other bands did not stain, even after prolonged development of the immunoblots. This confirms that the 44 and 35 kDa proteins are immunologically related. When Western blots with *S. hyodysenteriae* C5 lysate were incubated with anti-44 kDa serum, a 44 kDa band readily appeared, but a

Table 1. Western blot reactions of *S. hyodysenteriae* C5 periplasmic flagella-associated proteins and cell lysates probed with antisera against flagella of other spirochaetes

Reactivity is scored as – (no signal above background), + (weak reactivity) or +++ (strong reactivity).

Protein*	Reactivity with:†				
	RαTPRPF	RαSAPF	.2A6B4	.1H11G7	mCC9
44	+	+	–	–	–
37	+++	+++	+++	–	–
35	–	–	–	–	+
34	+++	+++	+++	+	–
32	+++	+++	–	+	–
30	–	–	–	–	–
28	–	–	–	–	–

* Molecular mass of flagella-associated proteins in kDa.

† RαTPRPF, rabbit serum against periplasmic flagella of *T. phagedenis* biotype Reiter 1; RαSAPF, rabbit serum against periplasmic flagella of *S. aurantia*; mCC9, monoclonal antibody against the 37 kDa periplasmic flagellar sheath protein of *T. pallidum* (Bailey *et al.*, 1987); .2A6B4, monoclonal antibody primarily against the 31.5 kDa periplasmic flagellar protein of *S. aurantia* (Brahamsha & Greenberg, 1988); .1H11G7, monoclonal antibody against the 31.5 and 34 kDa periplasmic flagellar proteins of *S. aurantia* (Brahamsha & Greenberg, 1988).

Fig. 2. Immunoblot analysis of *S. hyodysenteriae* C5 flagellar proteins. Rabbit antisera against the individual flagellar proteins were reacted with cell lysate of *S. hyodysenteriae* C5 (lanes A) and purified flagella (lanes B). Blots were incubated with sera to the 44 (lanes 1), 37 (lanes 2), 35 (lanes 3), 34 (lanes 4) and 32 (lanes 5) kDa flagellar proteins.

35 kDa band did not become visible (Fig. 1, lane A). This is probably because preparations of whole bacteria contain relatively low amounts of the 35 kDa protein as compared to preparations of purified flagella. Sera raised against the 37 or 34 kDa proteins reacted with the 37, 34 and 32 kDa proteins (Fig. 2, lanes 2 and 4). Sera raised against the 32 kDa protein reacted not only with the 32 kDa protein, but recognized the 37 kDa protein as well, although weakly (Fig. 2, lane 5). These findings show that the 37, 34 and 32 kDa proteins share epitopes. The anti-35 kDa serum reacted weakly with both the 35 kDa protein and the 44 kDa protein (Fig. 2, lane 3), confirming the immunological relationship observed between these two proteins in lane 1B. The serum also reacted weakly with the other proteins present in the preparation. However, this cross-reaction is probably due to the way in which the 35 kDa protein was isolated: it is plausible that the 37 kDa protein contaminated the preparation when cut-out from Western blots. Thus, immunization of rabbits with this preparation not only resulted in the production of antibodies that recognized

Table 2. Western blot reactions of *S. hyodysenteriae* C5 periplasmic flagella-associated proteins and cell lysates probed with antisera against individual flagellar components

Reactivity is scored as – (no signal above background), + (weak reactivity), ++ (moderate reactivity) or +++ (strong reactivity).

Protein*	Reactivity with:†				
	α44	α37	α35	α34	α32
44	+++	–	+	–	–
37	–	+++	+	+++	++
35	+	–	+	–	–
34	–	+++	+	+++	–
32	–	+++	+	+++	+
30	–	–	–	–	–
28	–	–	–	–	–

* Molecular mass of flagella-associated proteins in kDa.

† α44, α37, α35, α34 and α32 are rabbit immune sera raised against purified 44, 37, 35, 34 and 32 kDa periplasmic flagellar proteins, respectively, of *S. hyodysenteriae* C5.

the 44 and 35 kDa protein, but also produced antibodies that recognized both the 37, 34 and 32 kDa proteins. The fact that sera raised against either one of these three related proteins did not recognize the 35 kDa protein or the 44 kDa protein showed already that the 44 kDa and 35 kDa protein are not immunologically related to the other three proteins. The 35 kDa protein and 32 kDa protein seemed to be less immunogenic than the other proteins present in the preparation: antisera raised against these proteins reacted only weakly on immunoblots.

Immunogold labelling of *S. hyodysenteriae* periplasmic flagella

To investigate whether the proteins present in periplasmic flagellar preparations of *S. hyodysenteriae* C5 are exclusively localized in the periplasmic flagella we used the rabbit antisera raised against the individual components of the periplasmic flagella in immunogold electron microscopy experiments. In each of the 20 samples (prepared in four separate experiments) antibodies that react with the 44 kDa and the 35 kDa protein specifically decorated the periplasmic flagella along their entire length (Fig. 3a). Occasionally, periplasmic flagella were observed that were labelled except for their distal parts. When antibodies that recognize the 37, 34 and 32 kDa protein were used for immunogold labelling, reactivity with the ends of periplasmic flagella only was observed. Occasionally, the antisera labelled the flagella in scattered areas (Fig. 3b). The majority of the periplasmic flagella that were observed on the 20 grids examined (prepared in four independent experiments) were

Fig. 3. Immunogold electron-microscopy after incubation of *S. hyodysenteriae* C5 with antiserum to the 44 kDa (a) and 37 kDa (b) flagellar protein. Bars, 400 nm.

(a) <i>S. hyodysenteriae</i> 44 kDa	LSNSTLIDFA	LTGNADNLQ	
	<i>S. hyodysenteriae</i> 37 kDa	MVINNNISAI	NAQRTLKFRQ VDLKK
	<i>S. hyodysenteriae</i> 35 kDa	-ETVPYMFEN	
	<i>S. hyodysenteriae</i> 34 kDa	MIINNNSAI	NAQRTLKFRN VDL-R
	<i>S. hyodysenteriae</i> 32 kDa	MIINNNSAL	NANRQLNLTG NSMTK
(b) <i>S. hyodysenteriae</i> 44 kDa	L SNSTLIDFAL	TGNADNLQ	
	<i>T. pallidum</i> 37 kDa	DESVLIDFAK	LNADIMADKS GGMTH
	<i>T. phagedenis</i> 39 kDa	EQATLIDFGK	LNADIVDDKN GGMTQ
	<i>S. aurantia</i> 37 kDa	EQATLIDFSK	LVGEGNTGLH APTTI
(c) <i>S. hyodysenteriae</i> 37 kDa	MVINNNISAI	NAQRTLKFRQ	VDLKK
	<i>S. hyodysenteriae</i> 34 kDa	MIINNNSAI	NAQRTLKFRN VDL-R
	<i>S. hyodysenteriae</i> 32 kDa	MIINNNSAL	NANRQLNLTG NSMTK
	<i>T. pallidum</i> fla B1	MIINHNSAM	FAQRTLGHNT VQVGK
	<i>T. pallidum</i> fla B2	MIINHNSAM	FSQRTLGHNT LSVQK
	<i>T. pallidum</i> fla B3	MIINHNSAM	FAQRQGGING LAIAK
	<i>T. phagedenis</i> 34 kDa	MIINHNSAM	FAQRTLGVNT NAIGK
	<i>T. phagedenis</i> 33 kDa	MIINHNSAM	FAQRTLGNNTN LSVQK
	<i>S. aurantia</i> major 34 kDa	MIINHNSAI	NAQRVQGBVT GVTKN
	<i>S. aurantia</i> minor 33 kDa	MIINHNSAI	NAQXVQGBVT
	<i>S. aurantia</i> minor 32 kDa	MIINHNSAI	NANRVLGBT
	<i>S. aurantia</i> major 31.5 kDa	MIINHNSAI	NANRVLGBTN ADITK
	<i>B. burgdorferi</i> 41 kDa	MIINHNTSAI	NASRNNGINA NLSKT
	<i>L. borgpetersenii</i> 32 kDa	MIINHNSAV	NSHRSCLKFNE LAVDK

Fig. 4. N-terminal amino acid sequences of the *S. hyodysenteriae* C5 flagellar proteins (a) and comparison with flagellar sheath (b) and core (c) proteins of *T. pallidum* (Champion *et al.*, 1990; Isaacs *et al.*, 1989; Norris *et al.*, 1988; Pallesen & Hindersson, 1989), *T. phagedenis* (Norris *et al.*, 1988) and *S. aurantia* (Brahamsha & Greenberg, 1989; Parales & Greenberg, 1991). The single flagellin of *B. burgdorferi* (Wallich *et al.*, 1990) and a periplasmic flagellar protein of *Leptospira borgpetersenii* (Mitchison *et al.*, 1991) are included in group (c). The identity of underlined residues could not be determined with certainty.

Table 3. Western blot reactions of cell lysates of several isolates and strains of *S. hyodysenteriae* with antiserum raised against the 37 kDa and the 44 kDa periplasmic flagellar protein of *S. hyodysenteriae* C5

<i>S. hyodysenteriae</i> isolate/strain	Source*	Haemolysis†	Proteins reactive with:‡	
			anti-37	anti-44§
27164	ATCC 27164	+++	37, 34, 32	44
31212	ATCC 31212	+++	37, 34, 32	44
31287	ATCC 31287	+++	37, 34, 32	44
B8	FI	+++	37, 34, 32	44
C5	FI	+++	37, 34, 32	44
D2	FI	+/-	37, 34, 32	47
D7	FI	+/-	37, 34, 32	45
PWS/A	Hudson <i>et al.</i> (1976)	+/-	37, 34, 32	44
Z7	FI	+++	37, 34, 32	44
Z10	FI	+++	37, 34, 32	44
Z12	FI	+/-	37, 34, 32	46

* FI, Dutch field isolate.

† +/-, Weakly haemolytic; + + +, strongly haemolytic.

‡ The molecular masses given are those of proteins reactive with rabbit serum to the 37 kDa and 44 kDa flagellar proteins of *S. hyodysenteriae* C5.

§ Weak reactivity with a 35 kDa protein was noted in each of the *S. hyodysenteriae* tested.

unlabelled. These findings indicate that epitopes that are reactive with the antisera used in this experiment are not accessible on intact flagella, and only become exposed when an outer layer is damaged or removed. The finding that labelling occurs mostly at the tips of the flagella is probably due to a high susceptibility of the ends of flagella to damage caused during preparation of the samples for electron microscopy. Gold particles were not observed to be attached to the outer envelope or other structures of the bacterium with any of the rabbit

antisera raised against the individual components of the periplasmic flagella.

N-Terminal amino acid sequencing

All major proteins present in the periplasmic flagella preparation were further characterized by N-terminal amino acid sequencing. The N-terminal amino acid sequences obtained are listed in Fig. 4(a). The N-

terminal amino acid sequences of the 37, 34 and 32 kDa proteins are highly similar. This supports the conclusion, based on antigenic relatedness, that the three polypeptides are similar. The N-terminal sequences of the 44 and 35 kDa protein are distinct from each other and from those of the other proteins in the periplasmic flagellar preparation.

Similarities of the N-terminal sequences of S. hyodysenteriae flagellins to other flagellins

A comparison was made between the N-terminal sequences of the *S. hyodysenteriae* periplasmic flagellar proteins and amino acid sequences present in the NBRF (release 28.0) and Swiss-prot (release 18.0) databases, the N-terminal amino acid sequences of periplasmic flagellar proteins of *S. aurantia* (Parales & Greenberg, 1991) and a *Leptospira borgpetersenii* periplasmic flagellar subunit protein (Mitchison *et al.*, 1991). This search revealed no significant similarities to the N-terminal sequence of the 35 kDa protein. The 44 kDa protein shared some N-terminal amino acid residues with the periplasmic flagellar sheath proteins of *T. pallidum*, *T. phagedenis* and *S. aurantia* (Fig. 4*b*), whereas the N-termini of the 37, 34 and 32 kDa proteins are very similar to those of the core proteins of other spirochaetes (Fig. 4*c*).

Antigenic conservation of periplasmic flagellar proteins within various isolates of S. hyodysenteriae

We used our monospecific sera to the individual flagellins to examine the conservation of periplasmic flagellar antigens of *S. hyodysenteriae*. Cell lysates of three ATCC reference strains and seven Dutch field strains of *S. hyodysenteriae*, isolated from pigs severely affected by swine dysentery, were reacted in immunoblots with serum raised against the individual flagellins of *S. hyodysenteriae* C5. Only the results of immunoblotting experiments with the anti-37 kDa and the anti-44 kDa sera are shown (Table 3), since corresponding results were obtained with the anti-37, -34, -32 and the anti-44 and -35 kDa sera respectively.

All isolates tested possessed proteins of 37, 35, 34 and 32 kDa, which reacted with serum raised against proteins of the same molecular mass in *S. hyodysenteriae* C5. In contrast, the molecular mass of the largest periplasmic flagellar protein varied between isolates: in strongly haemolytic isolates it was 44 kDa, whereas in weakly haemolytic isolates it varied between 44 and 47 kDa.

Discussion

The objective of the present study was to characterize the structure and composition of periplasmic flagella of *S. hyodysenteriae* strain C5. Periplasmic flagella of *S. hyodysenteriae* are the organelles of motility and serve as protective antigens (Boyden *et al.*, 1989; Chatfield *et al.*, 1988; Kent *et al.*, 1989). It has been suggested that motility is the major mechanism by which *S. hyodysenteriae* colonizes the colonic mucosa of pigs (Kennedy *et al.*, 1988) and thus it may augment virulence of the bacterium. Flagellar preparations of *S. hyodysenteriae* C5 contained five major proteins with molecular masses of 44, 37, 35, 34 and 32 kDa. Electron microscopy with immunogold labelling demonstrated that these proteins form a flagellar core surrounded by a sheath. Antisera that recognize both the 44 and 35 kDa proteins decorated the periplasmic flagella along their entire length, indicating that at least one of these two proteins is located on the sheath. The 37, 34, and 32 kDa proteins reside in the core, since specific antisera to these proteins labelled the periplasmic flagella only at the tips and occasionally in scattered areas, presumably where no sheath is present or where it has been damaged or removed due to breakage of the periplasmic flagella when preparing the samples for electron microscopy. Often flagella did not label with sera recognizing the 37, 34, and 32 kDa proteins. This is in agreement with the proposal that in intact flagella these proteins are completely surrounded by an outer layer of sheath protein and thus not accessible to antibodies. With antisera recognizing both the 44 kDa and 35 kDa protein we occasionally observed flagella that were labelled entirely, except for the distal parts. This illustrates that this antiserum does not react with proteins that are exposed at the tips (i.e. those proteins which reside in the flagellar core), strongly suggesting that both the 44 kDa protein and the 35 kDa protein are located exclusively on the sheath.

In agreement with our results, Kent *et al.* (1989) identified five major proteins in periplasmic flagella of *S. hyodysenteriae*, with molecular masses of 43.8, 38, 34.8, 32.8 and 29.4 kDa. However, Miller *et al.* (1988) reported that *S. hyodysenteriae* periplasmic flagella consist of six proteins, with approximate molecular masses of 39, 29, 27, 22, 21, 18.5 kDa. The difference in number and molecular masses of periplasmic flagellar proteins, when compared to our results and to those of Kent *et al.* (1989), may be related to the different method used to isolate the periplasmic flagella and for subsequent electrophoretic analysis.

The 44 kDa flagellar sheath protein shows N-terminal amino acid similarity with the sheath flagellins of *T. pallidum* (Isaacs & Radolf, 1990) and *S. aurantia*

(Parales & Greenberg, 1991). Its N-terminal amino acid sequence does not start with a methionine residue, indicating that the protein is post-translationally modified. Most likely, a signal sequence is cleaved-off before assembly onto flagella in the periplasmic space, as reported for the sheath flagellin of *T. pallidum* (Isaacs & Radolf, 1990) and *S. aurantia* (Parales & Greenberg, 1991). Post-translational modification or degradation of the 44 kDa protein could yield the 35 kDa sheath flagellar protein. The immunological cross-reactivity of the two proteins supports such a relationship. Alternatively, the 35 kDa protein is the product of a separate gene. An indication for this is that monoclonal antibody mCC9, which specifically recognizes the sheath protein in *T. pallidum* (Bailey *et al.*, 1987) reacts exclusively with the 35 kDa protein on Western blots but not with the 44 kDa protein.

The 37, 34 and 32 kDa proteins show an extensive N-terminal sequence identity with each other and with other spirochaete periplasmic flagellar core proteins (Champion *et al.*, 1990; Coleman & Benach, 1989; Mitchison *et al.*, 1991; Norris *et al.*, 1988; Pallesen & Hindersson, 1989; Parales & Greenberg, 1991). The N-terminal region of the core proteins also shows some similarity to flagellar proteins of remotely related bacteria (DeLange *et al.*, 1976; Kuwajima *et al.*, 1986; Martin & Savage, 1988; Wei & Joys, 1985), indicating that these proteins have an important role, e.g. in the function or assembly of the flagellum. We assume that the three core flagellar proteins are encoded by different genes, since the N-terminal amino acid sequences of these proteins are similar, but not identical. This would resemble the situation in *T. pallidum* where each of the three core proteins is the product of a distinct gene (Champion *et al.*, 1990; Pallesen & Hindersson, 1989).

SDS-PAGE profiles of flagellar preparations clearly show that the various flagellar proteins are present in different quantities. This phenomenon has been observed by Kent *et al.* (1989) and also by other workers studying different spirochaetes (Blanco *et al.*, 1988; Brahmsha & Greenberg, 1989; Limberger & Charon, 1986). We cannot explain why these differences exist in *S. hyodysenteriae*. Although we cannot exclude the possibility that these differences are the result of our purification method, it may be that the flagellar proteins are present on the same periplasmic flagellum in different amounts. It is also possible that there exist different periplasmic flagella, each composed of a single type of core or sheath protein. The precise distribution of the flagellar proteins on the periplasmic flagella can be determined with antibodies that discriminate between the flagellins but at the moment these antibodies are not available. We attempted to localize the 35 kDa protein on the periplasmic flagella using monoclonal antibody

mCC9. However, in these immunogold labelling experiments reactivity was never observed. Apparently, the epitope that is recognized by the monoclonal antibody on Western blots is not available for binding on non-denatured periplasmic flagella. Cockayne *et al.* (1987) reported that a proportion of the sheathed filaments of *T. pallidum* failed to react with monoclonal antibody mCC9, and suggested that antigenic heterogeneity was present within the population of sheathed filaments.

The 44 kDa flagellin seems to be the periplasmic flagellar protein that is least constrained by functional demands. Thus, in spirochaetes isolated from swine, variation in the molecular mass of this protein was observed whereas the molecular masses of the other flagellins were conserved. It is noteworthy that variation in molecular mass was observed exclusively with weakly haemolytic isolates, originating from stools of pigs that suffered from diarrhoea but showed no other clinical signs of swine dysentery. All strongly haemolytic isolates, originating from the gut of pigs severely affected by swine dysentery, had a 44 kDa sheath protein. This suggests the intriguing possibility that there is a relationship between virulence and the structure of the sheath of *T. pallidum* subsp. *pallidum* and *pertenue* pathogen-specific determinant is located on the flagellar sheath of *T. pallidum* subsp. *P. pallidum* and *pertenue* (Lukehart *et al.*, 1985) and on invasive oral spirochaetes from dental plaque (Reviere *et al.*, 1991), but there is no evidence that this pathogen-specific determinant is directly involved in virulence. Currently, the hypothesis that the structure of the flagellar sheath protein may influence virulence remains mere speculation. Our characterization of the periplasmic flagellar proteins of *S. hyodysenteriae* will enable the isolation of the flagellar genes and allow their potential function in virulence of *S. hyodysenteriae* to be addressed.

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