Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*

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Summary. *Helicobacter pylori* flagellar sheaths were isolated by sucrose density-gradient centrifugation and analysed by electronmicroscopy, SDS-PAGE and gas-liquid chromatography. Electronmicroscopy of thin sections of flagella showed an internal electron-dense filament and a surrounding flagellar sheath with the typical bilayer structure of a membrane. The flagellar filaments could be disintegrated by acid treatment and the resulting isolated flagellar sheaths formed vesicles, sometimes with characteristic structures. Centrifugation of flagellar preparations after acid treatment resulted in the enrichment of flagellar sheaths in the pellet. SDS-PAGE analysis of the pellet showed a reduction of the flagellin band and a number of protein bands of 150, 76, 67, 65, 53, 51, 49, 29.5, 18, 17 and 16 kDa. However, there were no major protein bands characteristic for the sheath. Differences between the protein profiles of Sarkosyl-insoluble membranes and flagellar sheaths appeared in the lower M_r range of 30–14 kDa. Major fatty acids of isolated flagellar sheaths were C 14:0, C 19:0 cyc, C 18:0, and the LPS-specific fatty acids 3-OH C 16:0 and 3-OH C 18:0. The results demonstrate that the flagellar sheaths of *H. pylori* are membranes and contain LPS and proteins.

Introduction

Helicobacter pylori is the aetiological agent of chronic type b gastritis in man. *H. pylori* gastritis is a major predisposing factor for peptic, duodenal and gastric ulcers^{1–5} and is possibly involved in the pathogenesis of gastric cancer.^{6,7} Several components of this bacterium have been suggested to be virulence factors but only urease expression⁸ and motility⁹ have been shown to be associated with bacterial virulence in animal models.

Motility is conferred on the bacteria by two to six polar flagella with a very characteristic and unusual morphology. Each flagellum consists of a central filament enveloped by a flagellar sheath. The filament consists mainly of polymers of a 53-kDa flagellin and the gene encoding this protein has recently been cloned and sequenced.¹⁰ Whereas the filaments of *H. pylori* have been characterised in detail^{11–13} there are almost no data on the structure and function of the flagellar sheath. Flagellar sheaths have been described in only a few bacterial species such as *Vibrio cholerae*,¹⁴ *Pseudomonas stizolobii*¹⁵ and *Bdellovibrio bacteriovorus*.¹⁶ However, they seem to be common to all species of the genus *Helicobacter*.¹⁷

The only flagellar sheaths that have been characterised in detail are those of *V. cholerae* and *B. bacteriovorus*. Fuerst and Perry¹⁸ have clearly demonstrated by protein A-gold immuno-electronmicroscopy that the flagellar sheath of *V. cholerae* contains lipopolysaccharides (LPS). Hranitzky *et al.*¹⁹ reported that the sheath contains a protein, composed of three polypeptides, which is also present in the outer membrane of *V. cholerae*. Thomashow and Rittenberg²⁰ found that the flagellar sheath of *B. bacteriovorus* had a characteristic composition of lipids and LPS when compared with whole bacteria.

Although there are as yet no data to support the hypothesis, it is likely that the sheath of *H. pylori* plays a role in the pathogenic process. Possible functions are protection of the filament from disintegration in the acidic environment of the stomach²¹ and promotion of adherence. We have performed ultrastructural and biochemical studies of the *H. pylori* flagellar sheath to obtain information about the composition of this structure, which will eventually contribute to a better understanding of its function.

Materials and methods

Bacteria

H. pylori strain 898-1 was used. To obtain sufficient bacteria for sheath preparation, 2-L Erlenmeyer flasks

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were filled with 1.6 L of supplemented brain heart infusion broth¹¹ and inoculated with 120 ml of a liquid culture of *H. pylori*. Flasks were incubated for 3 days in air with CO₂ 9% v/v at 37°C and agitated with a magnetic stirrer (100 rpm). By this method, a yield of 1 g (wet weight) of bacterial culture was achieved. Bacteria were harvested by centrifugation (7000 *g* for 20 min at 10°C), washed once in NaCl 0.9% w/v, centrifuged again, and resuspended in 0.01 M Tris-HCl, pH 7.2, to a concentration of *c.* 0.05 g of bacteria/ml of buffer.

Preparation of flagellar sheaths

Bacteria (6.3 g wet weight) were sheared in a Sorvall Omni-Mixer for 5 min on ice. Bacterial cells were removed by centrifugation (3500 *g* for 10 min at 10°C) and the flagella were harvested from the supernate by ultracentrifugation (270 000 *g* for 1 h at 10°C). Flagella were suspended in 0.01 M Tris-HCl, pH 7.2, and transferred to a linear sucrose density gradient (sucrose 25–65% w/w in 0.01 M Tris-HCl, pH 7.2). The gradients were centrifuged for 22 h at 100 000 *g* at 10°C. A fluffy layer that had formed at the top of the gradients was removed with a pasteur pipette and the gradient was subsequently separated into fractions of 0.7–1.2 ml with an Auto-Densi Flow device (Haake-Buchler, Saddle Brook, USA). The OD₂₈₀ was monitored continuously with a Uvicord (LKB, Bromma, Sweden). Fractions 14–19 and 22–26 (fig. 1, peaks I and II, respectively) were each pooled, diluted with water, pelleted by ultracentrifugation (270 000 *g* for 2 h at 10°C), and resuspended in water.

Disintegration of filaments

For disintegration by acid treatment, peak I material was mixed slowly with drops of 0.01 N HCl until the pH reached 2.0. The solution was centrifuged in Eppendorf tubes (15 800 *g* for 30 min at 4°C), the pellet was resuspended in distilled water, and both pellet and supernate were neutralised with 1 N NaOH. Other samples of peak I material were treated with proteinase K (5 mg/ml) for 60 min and 120 min at 37°C or at room temperature. Trypsin (200 IU/ml) was mixed with peak I material and incubated for 30, 60 and 180 min at 37°C.

Preparation of outer membranes by detergent solubilisation

Sarkosyl-insoluble membranes were prepared by a modification of the method of Achtman *et al.*²² Briefly, bacteria were harvested from broth cultures and washed twice with phosphate-buffered saline or NaCl 0.9% w/v. The bacterial pellet was resuspended in 10 mM Tris-HCl, pH 7.2, sheared in an Omni-Mixer and then sonicated for four periods of 30 s in ice (Branson sonifier). Unbroken cells were removed by centrifugation (3000 *g* for 10 min) and membranes

were pelleted by ultracentrifugation (48 000 *g* for 1 h at 10°C). The pellet was resuspended in 12.5 mM Tris-HCl, pH 7.6 containing Sarkosyl 1.88% w/v and incubated for 1 h at room temperature with agitation. Sarkosyl-insoluble outer membranes were collected by ultracentrifugation (48 000 *g* for 90 min at 20°C) and resuspended in distilled water.

Analytical methods

Protein content was assessed by the method of Markwell *et al.*²³ or Smith *et al.*²⁴ and SDS-PAGE was as described by Lugtenberg *et al.*²⁵ The concentration of acrylamide in the separating gel was 11% w/v and a 60-μg sample of protein was applied to each slot of the gel. Protein mixtures of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as standards for mol. wt determination.

Fatty acid methyl esters were prepared according to method A of Gmeiner and Martin²⁶ and analysed by gas liquid chromatography as described previously.²⁷

Electronmicroscopy

Negative staining was performed by applying samples to formvar-carbon coated grids and contrasting with phosphotungstic acid, pH 7.

For thin sectioning, flagella (peak I material) were pelleted by ultracentrifugation. After overnight fixation in Karnowsky buffer, flagellar sheaths were post-fixed in osmium tetroxide 1% w/v, dehydrated in a graded series of ethanol, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

Results

Preparation of H. pylori flagellar sheaths

Flagella were detached mechanically from the cells by shearing, and the flagella-containing material was subjected to sucrose density-gradient centrifugation (fig. 1). Two major peaks were observed and analysed by electronmicroscopy. Peak I (buoyant density 1.187 g/cm³) contained flagella and sheaths (fig. 2) and showed a marked (*c.* 5–10-fold) enrichment of sheaths as compared with peak II (buoyant density 1.213 g/cm³), which contained predominantly naked filaments (not shown). The peak I components could not be separated further by subsequent gradient centrifugations.

Ultrastructure

Ultrathin sections of peak I material confirmed the large amount of flagellar sheaths in this preparation (fig. 3). The sheath exhibited the typical picture of a

Fig. 1. Distribution of flagellar preparation after sucrose density-gradient centrifugation. Fractions were pooled (peaks I and II) as indicated below the curve.

Fig. 2. Electronmicrograph of the material contained in peak I. Sheathed flagella can be distinguished from filaments by their greater diameter. Note the high amount of sheathed flagella (→) and empty sheaths (◄).

bilayered membrane with two parallel electron-dense lines. Inside the membrane rings, the flagellar filament appeared as an electron-dense structure 12–14 nm in diameter. Contaminating membrane vesicles could be distinguished easily from flagellar sheaths because of their greater size and the absence of internal filaments. The diameter of sheath cross-sections varied considerably, consistent with the different degrees of detachment of the sheath from the filament seen in negatively-stained preparations of isolated flagella.

Since the filaments could not be removed by physical methods, attempts were made to separate the sheaths and filaments by differential solubilisation methods. Treatments with SDS 0.1–0.4% w/v resulted in the parallel dissolution of both filaments and sheaths. Proteinase K and trypsin treatments did not lead to

structural changes of the material. In contrast, incubation in the presence of 0.01 M HCl (pH 2.0) resulted in the dissolution of the filaments while the sheaths retained their membranous appearance. Fig. 4 shows the effects of HCl treatment on the partially-purified flagellar sheaths (peak I material). Isolated flagellar sheaths formed vesicles after filament disintegration. Most vesicles had a rather characteristic “cherry-shaped” appearance with a stem-like part, in which the sheath had collapsed, and a second, vesicular part.

SDS-PAGE analysis of flagellar sheaths

Isolated flagella containing flagellar sheaths and flagellar filaments were subjected to HCl treatment

Fig. 3. Thin section of a preparation of sheathed flagella (peak I material). Note the bilayer structure of flagellar sheath () and the internal electron-dense filament (→).

Fig. 4. Electronmicrograph of partially purified flagellar sheaths (peak I material after HCl treatment). There are no filaments. The sheaths form vesicles some of which carry stem structures.

and centrifugation. Protein profiles (fig. 5) obtained by SDS-PAGE showed that the flagellar filaments had been almost completely solubilised by acid treatment leading to a heavily enriched flagellin band (53 kDa) in the supernate (lane 3). The corresponding pellet (lane 4) showed a multitude of protein bands and only slightly prominent major bands with molecular masses of 76, 67 and 65 kDa (possibly the large urease subunit²⁸), 53 kDa (flagellin band), 51, 49 and 29.5 kDa (possibly the small urease subunit²⁸), 18, 17 and 16 kDa, and a characteristic band of 150 kDa. The most remarkable differences between the protein profiles of the supernate (lane 3) and the pellet (lane 4) were the reduction of the flagellin band and the enrichment of the 150, 72, 67, 51, 49 and 29.5 kDa bands (arrowheads) in the pelleted material. However, the 150-kDa band could not be seen with equal intensity in protein profiles of different sheath prepara-

tions. For comparison with outer-membrane profiles, lane 5 shows the protein profile of Sarkosyl-insoluble membranes (outer membranes). Major Sarkosyl-insoluble membrane protein bands were of M_r 150, 72, 67, 65, 53, 51, 49, 22, 21 and 18 kDa. Nearly all protein bands in the M_r range 200–30 kDa present in the Sarkosyl-insoluble material were also present in the sedimentable material after HCl treatment, but in different amounts. Major differences appeared in the lower M_r range of 30–14 kDa where the 22- and 21-kDa protein bands were remarkably enriched in the Sarkosyl-insoluble membranes.

Fatty acid composition of flagellar sheaths

The fatty acid composition of *H. pylori* flagellar sheaths was analysed to determine whether LPS-specific fatty acids were present in the sheaths and if

Fig. 5. Protein profiles of *H. pylori* flagella, flagellar sheaths after HCl treatment, and Sarkosyl-insoluble membranes, obtained by SDS-PAGE. Lane 1: standard proteins; 2: flagella isolated by shearing and centrifugation steps; 3: supernate; 4: pellet after centrifugation of HCl-treated flagella; 5: Sarkosyl-insoluble membranes.

there were fatty acids characteristic for the sheath. The most prominent fatty acid of the sheath material was myristic acid (C 14:0). Other major fatty acids were 19-carbon-cyclopropane fatty acid (C 19:0 cyc), β -hydroxypalmitic acid (3-OH C 16:0), β -hydroxystearic acid (3-OH C 18:0) and stearic acid (C 18:0). Minor amounts of palmitic acid (C 16:0), oleic acid (C 18:1) and linoleic acid (C 18:2) were also detected (table). Previously published data²⁷ about LPS and phospholipid fatty acids are also given in the table to facilitate data interpretation. The relatively high amounts of the LPS-specific fatty acids (3-OH C 16:0,

3-OH C 18:0, C 18:0) strongly suggest that LPS is a part of the sheaths. There was no sheath-specific fatty acid, although the profiles differed significantly from those obtained for cellular fatty acids or phospholipid or LPS fatty acids.

Discussion

The flagellar filaments of *H. pylori*,²⁹ *H. mustelae*,³⁰ *H. cinaedi*,^{17,31} *H. fennelliae*^{17,31} and *H. felis*³² are enveloped by sheaths and are surface structures possibly involved in the pathogenicity of these bacteria. Until now there has been no information concerning the structure and function of *H. pylori* flagellar sheaths, probably because of difficulties in the isolation of sufficient amounts for qualitative and, especially, quantitative studies. There is also very little information concerning flagellar sheaths of other bacteria and only those of *V. cholerae*^{18,19} and *B. bacteriovorus*²⁰ have been characterised in some detail.

Since it seems likely that these surface structures play a role in pathogenesis, we have performed ultrastructural and biochemical studies of the flagellar sheath of *H. pylori* to find out if it contains characteristic components. An important pre-requisite was the isolation of intact flagellar sheaths. After sucrose density-gradient centrifugation of flagella, one peak (I) was enriched in flagellar sheaths and contained only minor amounts of filaments.

Further separation of flagellar sheaths and filaments was not possible by physical methods, so HCl treatment was used to release and disintegrate flagellar filaments. Acid treatment has been used successfully with *Salmonella*,³³ *Wolinella*³⁴ and *Campylobacter* spp.³⁵ to disintegrate flagellar filaments. With *H. pylori*, disintegration of isolated flagella with HCl showed that flagellar-sheath vesicles were still visible and the sheaths sometimes even kept their linear structure, possibly because of interactions between the protein constituents. However, isolated flagella could not be separated completely into flagellar sheaths and flagellin molecules. Centrifugation of the acid-treated flagella and SDS-PAGE analysis of the resulting pellet, corresponding mainly to flagellar sheaths, showed a

Table. Fatty acid composition of *H. pylori* flagellar sheaths in comparison to cellular fatty acids and fatty acids of isolated phospholipids and LPS

Preparation	Percentage of total fatty acids* that were							
	C 14:0	C 16:0	C 18:0	C 18:1	C 18:2	C 19:0 cyc	3-OH C 16:0	3-OH C 18:0
Whole cells	45	4	8	6	tr	23	5	2
Flagellar sheaths	38	5	8	3	2	11	9	8
LPS†	4	2	21	< 0.5	< 0.5	< 0.5	25	24
Phospholipids†	41	7	5	7	2	28	< 0.5	< 0.5

tr, Traces, i.e., 0.5–1.9%.

* The number before the colon refers to the number of carbon atoms, and the number after the colon refers to the numbers of double bonds; cyc, cyclopropane fatty acid; OH, hydroxyl group.

† Data taken from Geis *et al.*²⁷

great reduction of the flagellin band, but it was still visible. This is in contrast to the fact that the filaments could not be seen by electronmicroscopy. A possible explanation could be a "trapping" of the flagellin molecules in the sheath vesicles. This is supported by the finding that a further reduction of the flagellin contamination could be achieved by sonication (data not shown).

Comparisons of sheath preparations with outer membranes of sheared bacteria showed that there were no protein bands typical of the flagellar sheath, but that some protein bands of 150, 51 and 49 kDa were enriched. However, we could not always detect the 150-kDa protein band in different sheath preparations (data not shown). The absence of typical flagellar sheath proteins may be compared with the finding of Hranitzky *et al.*¹⁹ who reported that flagellar sheaths of *V. cholerae* contain "sheath-proteins" of 61.5, 60 and 56.5 kDa, which are also present in the *V. cholerae* outer membrane.

Biochemical analyses of fatty acids were performed to clarify the existence of LPS as a constituent of *H. pylori* flagellar sheaths, because this point has been discussed controversially in relation to *V. cholerae*.^{18,19} *H. pylori* LPS contains very characteristic fatty acids²⁷ and these can serve as markers for the existence of LPS in the sheaths. Analyses revealed that fatty acids 3-OH C 16:0 and 3-OH C 18:0 were present in flagellar sheaths, leading to the conclusion that the sheaths of *H. pylori* do indeed contain LPS. For *V. cholerae*, it was originally postulated that the flagellar sheaths do not carry LPS,¹⁹ but Fuerst *et al.*¹⁸ have clearly demonstrated the presence of LPS in *V. cholerae* flagellar sheaths by immuno-gold electronmicroscopy. In *H. pylori*, comparison of the fatty acid composition of flagellar sheaths and whole cells showed higher amounts of the LPS-specific fatty acids 3-OH C 16:0

and 3-OH C 18:0 in the sheaths, but less C 18:0 than might be expected if the flagellar sheath were enriched in LPS. Thomashow and Rittenberg²⁰ showed that the LPS of flagellar sheaths of *B. bacteriovorus* has a composition different from total LPS, with significantly enriched amounts of nonadecanoic acid. Our results demonstrate that there are no fatty acids that are typical of the flagellar sheath of *H. pylori*, but that the fatty acid composition of the sheath is different from the fatty acid composition of whole cells, and has an intermediate position between that of isolated LPS and phospholipids. This composition may be a consequence of the special requirements on the sheath for rotation of the flagellar core in a flexible sheath.³⁶

It was not possible to identify components that are characteristic of the *H. pylori* flagellar sheath. The quantitative distribution of isolated substances remains unclear because, by conventional methods, it is not possible to isolate sufficient amounts of flagellar sheaths for these determinations. However, there are some hints that protein and fatty acid composition of flagellar sheaths differ from the composition of other cell compartments.

Purification and biochemical analyses of the *H. pylori* flagellar sheath allow only limited conclusions about its function and composition. For *V. cholerae*, Richardson *et al.*^{37,38} constructed *flaA*-negative mutants which had empty sheaths. The *H. pylori flaA* gene has been cloned and sequenced¹⁰ and experiments are under way concerning the mutagenesis of this gene. Construction of mutants that do not carry flagellar filaments would lead to the identification of flagellar sheath components and clarify the role of the sheath in pathogenesis, e.g., in adherence and inhibition of acid secretion.

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