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Ultrastructure and Chemical Analysis of *Campylobacter pylori* Flagella

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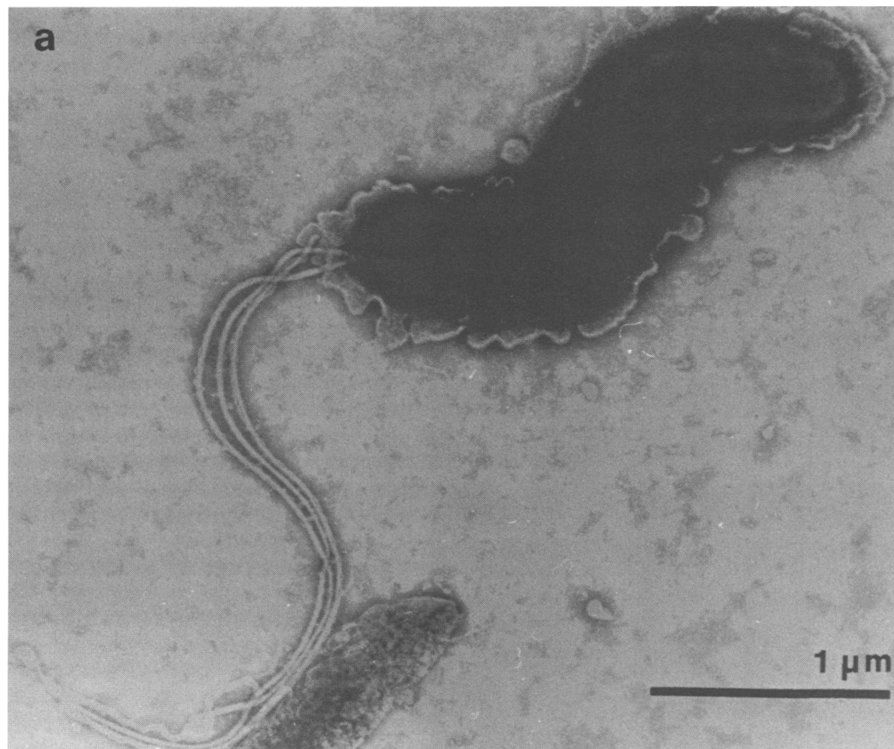
Flagella of *Campylobacter pylori* were analyzed by electron microscopy and purified, and the molecular weight of the flagellin was determined. Isolation of flagella was performed by mechanical shearing from the cell surface, sucrose density gradient centrifugation, and Sepharose CL-4B gel chromatography. The flagella of *C. pylori* differ from those of other *Campylobacter* species and of most other bacteria by the presence of a flagellar sheath. The sheath narrows at the end and is linked to a club-shaped terminal structure. The molecular weight of *C. pylori* flagellin was 51,000.

Campylobacter pylori is a curved gram-negative rod whose presence in gastric mucosa is closely associated with histologic gastritis and gastroduodenal ulcer disease (4, 13, 18, 19). The mechanisms of *C. pylori* pathogenicity and the reasons for its predilection for the colonization of gastric mucosa are still under investigation.

Bacterial flagella have been shown to be important virulence factors in some bacteria causing gastrointestinal disease (2, 6); both the motility the flagella give to the bacterium and specific adhesins seem to play a role in flagellum-associated virulence. Bacterial flagella consist of three parts: (i) the filament, which is a polymer of a single protein subunit, the flagellin, (ii) the hook, and (iii) the so-called basal structure (16).

The flagella of some bacteria, including *Vibrio cholerae* and other vibrios (7, 11), differ from their usual composition in that they are enclosed by a protein-containing flagellar sheath. *C. pylori* was recently reported to form a flagellar sheath, thus differing from all other known *Campylobacter* species (9).

Until now there have been no investigations concerning properties of purified surface structures of *C. pylori*. The



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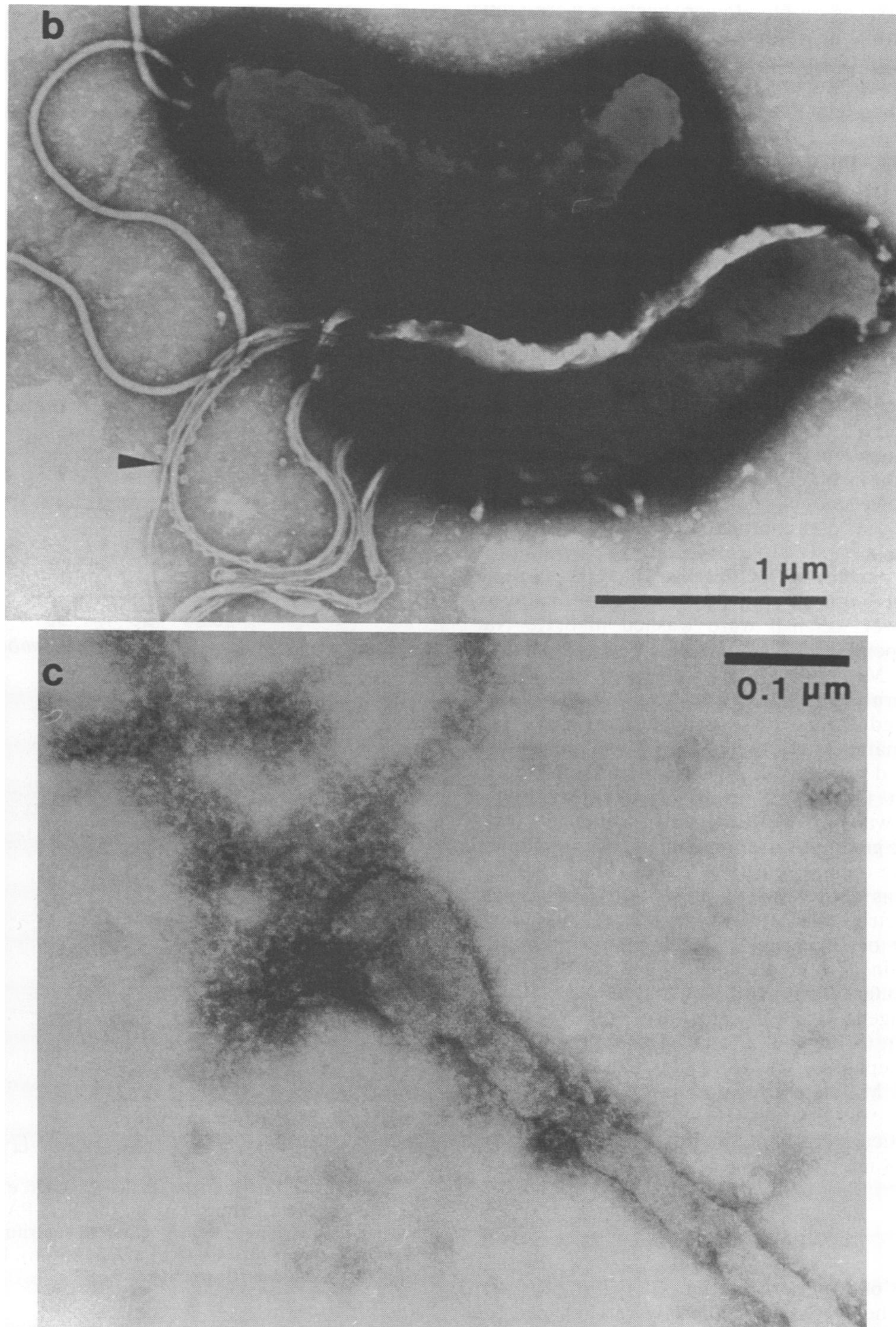

FIG. 1. Electron micrographs: (a) lophotrichously flagellated *C. pylori*, (b) broken sheath with extending filament (arrow), (c) distal end of a sheath with shaft, narrowing, and terminal club.

flagella of *C. pylori*, which are of special interest because of the sheath, have not been purified, nor has the molecular weight of *C. pylori* flagellin been determined.

The aims of this study were therefore to give a precise description of flagellar morphology by electron microscopy (EM) and to purify the flagella to gather further information about their biochemical properties.

MATERIALS AND METHODS

Bacteria. The *C. pylori* strain used for the investigations was isolated from a biopsy of corpus mucosa of a patient with a duodenal ulcer. Identification of the bacterium was performed on the basis of typical colony morphology, Gram stain, and positive oxidase, catalase, and strong urease tests.

After several passages on blood agar, bacteria were grown for 5 days in brain heart infusion broth (Oxoid, Wesel, Federal Republic of Germany) with 8% horse serum (Gibco, Eggenstein, Federal Republic of Germany), 0.25% yeast extract (Oxoid), and 0.0005% hemine (37°C, microaerophilic conditions).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Lugtenberg et al. (15). The concentration of acrylamide in the separating gel was 11%. A 50-μg sample of protein was applied to each slot of the gel. Staining was performed with Coomassie brilliant blue R-250. The molecular weights of proteins were estimated from a calibration curve prepared with a low-molecular-weight calibration kit (Pharmacia, Uppsala, Sweden) containing a protein mixture of phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400) as standards.

Protein determination. Protein content was determined by the method of Markwell et al. (17).

EM. For negative staining, samples were applied to Formvar-coal-coated grids and contrasted with phosphortungstic acid (2%, pH 7.0).

Isolation and purification of flagella. Bacteria harvested from 1.1 liter of culture broth described above (approximately 2.4 g [wet weight]) were washed in 0.9% NaCl solution and suspended in 48 ml of 0.01 M Tris hydrochloride buffer (pH 7.2). Mechanical separation of surface appendages was performed at 4°C with a Sorvall Omnimixer. Bacteria were sedimented by centrifugation (6,700 × *g*, 6 min). The supernatant was lyophilized, suspended in 0.35 ml of double-distilled water, and brought onto a linear sucrose density gradient (35 ml, 25 to 65% [wt/wt] sucrose in double-distilled water). After ultracentrifugation (90,000 × *g*, 22 h, 10°C) the gradient was fractionated under continuous monitoring of the optical density at 280 nm. The volume of each fraction was approximately 1 ml. All fractions were analyzed by electron microscopy. Flagella were found at a buoyant density of 1.25 g/cm^3. Flagellum-containing fractions were combined, diluted with 10 volumes of water, and centrifuged (290,000 × *g*, 1 h, 4°C). The pellet, which contained the flagella, was suspended in 0.5 ml of water and centrifuged again (6,700 × *g*, 2 min). The supernatant was brought onto a Sepharose CL-4B column (1.5 by 25 cm) and eluted with 0.01 M Tris hydrochloride buffer (pH 7.2) at 6 ml/h. The eluate was fractionated under continuous monitoring of the optical density at 280 nm; the volume of each fraction was 0.68 ml.

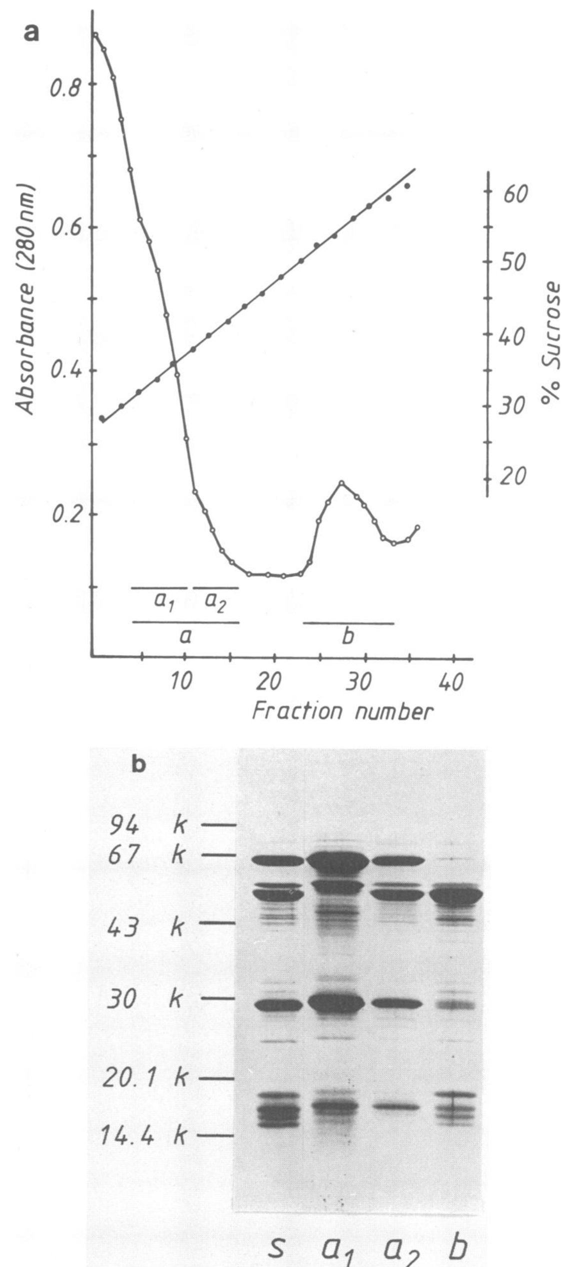

FIG. 2. (a) Distribution of the flagellar extract after sucrose density gradient centrifugation. (b) SDS-PAGE of the flagellar extract before sucrose density gradient centrifugation (lane s) and of fractions as indicated in panel a (lanes a_1, a_2, b). Peak b contained the bulk of flagellar fragments.

RESULTS

Ultrastructure of *C. pylori* flagella. *C. pylori* cells were morphologically heterogeneous. There were U-shaped cells and curved rods with two or three curves. In older cultures coccoid cells predominated. In all growth phases lophotrichously flagellated bacteria were found. The number of flagella varied between two and six (Fig. 1a). Each flagellum had its own flagellar sheath (Fig. 1b), which at its end carried a club-shaped thickening (Fig. 1c). Flagella were 3 to 5 μm long. The diameter of the whole structure (flagella plus sheath) was 30 to 35 nm; that of the flagellum alone was 9 to 12 nm. The maximum diameter of the terminal club was 100 nm. At the distal end the sheath narrowed for a short distance, where the diameter was only 22 nm (Fig. 1c). This narrowing seemed to be more fragile than the rest of the sheath, since already after centrifugation EM examination revealed many isolated terminal clubs. Also in the more proximal region the sheath could break up and separate from the flagellum, which then could be seen extending from the rest of the sheath (Fig. 1b). The sheath retained its shape when the flagellum had been torn out.

Purification and SDS-PAGE of flagella. The surface appendages were mechanically separated from the bacteria, and the bacteria were removed by centrifugation. The resulting supernatant contained flagella when analyzed by EM but demonstrated a multitude of protein bands in the SDS-PAGE (Fig. 2b, lane s). This material was further separated by

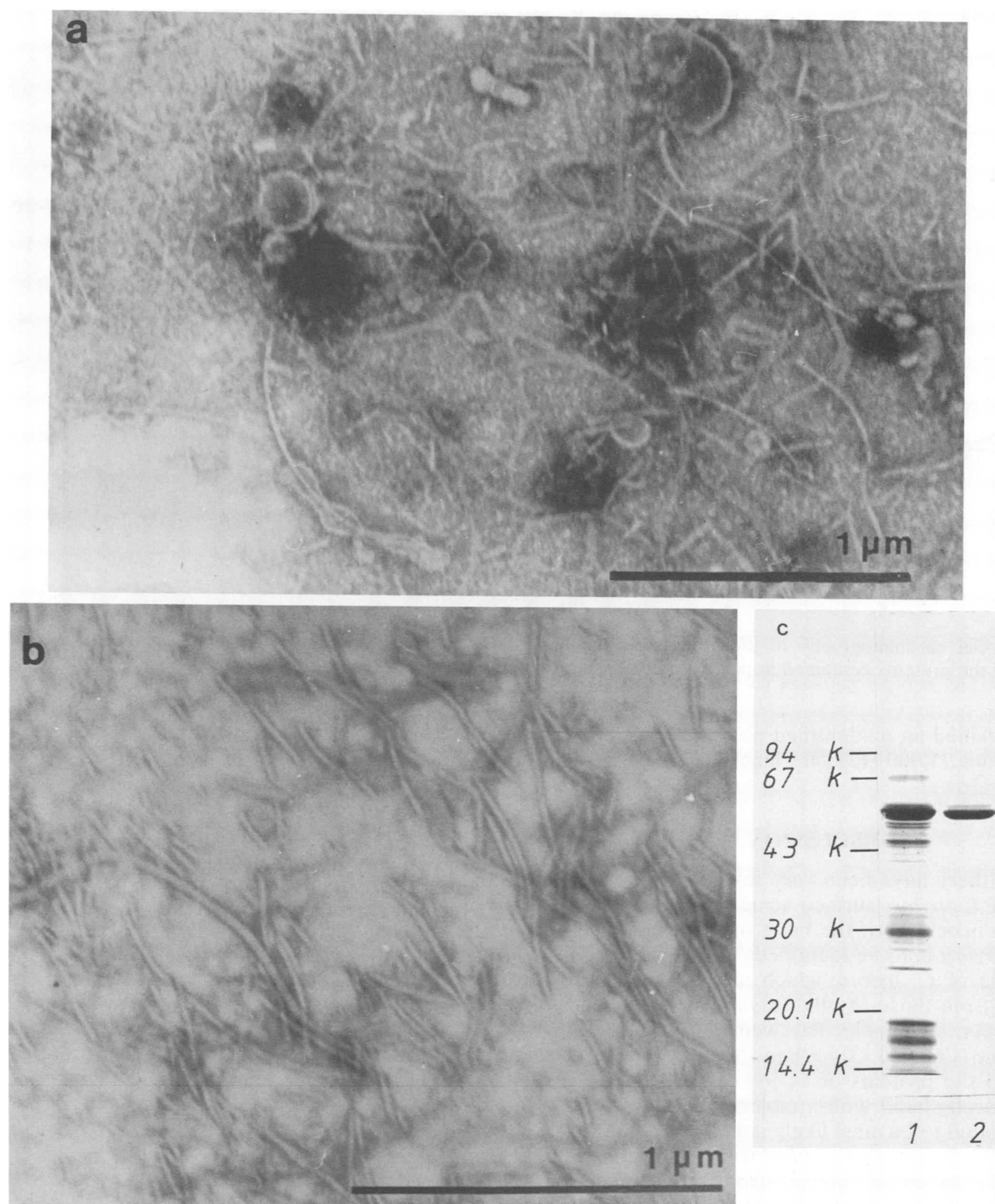

FIG. 3. Sediment and supernatant of flagella prepurified by sucrose density centrifugation, ultracentrifugation, and further centrifugation (6,700 × *g*, 2 min). (a) Electron micrograph of the sediment containing filaments as well as sheaths and membrane vesicles. (b) Electron micrograph of the supernatant predominantly containing filaments and few sheaths. (c) SDS-PAGE of the sediment (lane 1) and the supernatant (lane 2).

sucrose density ultracentrifugation (Fig. 2a). Two main fractions (a and b) were further analyzed by SDS-PAGE and EM. The SDS-PAGE protein patterns of fractions a (subdivided into a_1 and a_2) and b are shown in Fig. 2b (lanes a_1, a_2, and b). In fraction b a protein with a molecular weight of about 51,000 was concentrated (Fig. 2b, lane b). EM showed that this fraction contained the bulk of flagellar fragments.

For further purification the material of fraction b was centrifuged at 6,700 × *g* for 2 min. The sediment (Fig. 3a) contained flagellar sheaths as well as membrane fragments and flagella of different lengths. The supernatant appeared to be more homogeneous, predominantly containing flagella with few flagellar sheaths (Fig. 3b). These data were supported by the SDS-PAGE patterns. The supernatant (Fig. 3c, lane 2) contained almost only the 51,000-dalton protein, the sediment contained additional proteins (Fig. 3c, lane 1). Finally the supernatant material was further analyzed by gel chromatography (Fig. 4a). Of the three resulting peaks only peak I contained flagella; Fig. 4b shows the EM picture of this preparation. Morphologically the preparation appeared very homogeneous, containing filament fragments of rather homogeneous size. The molecular weight of *C. pylori* flagellin, the monomer of the *C. pylori* flagellum, was determined by SDS-PAGE (Fig. 4c, lane I); it was about 51,000.

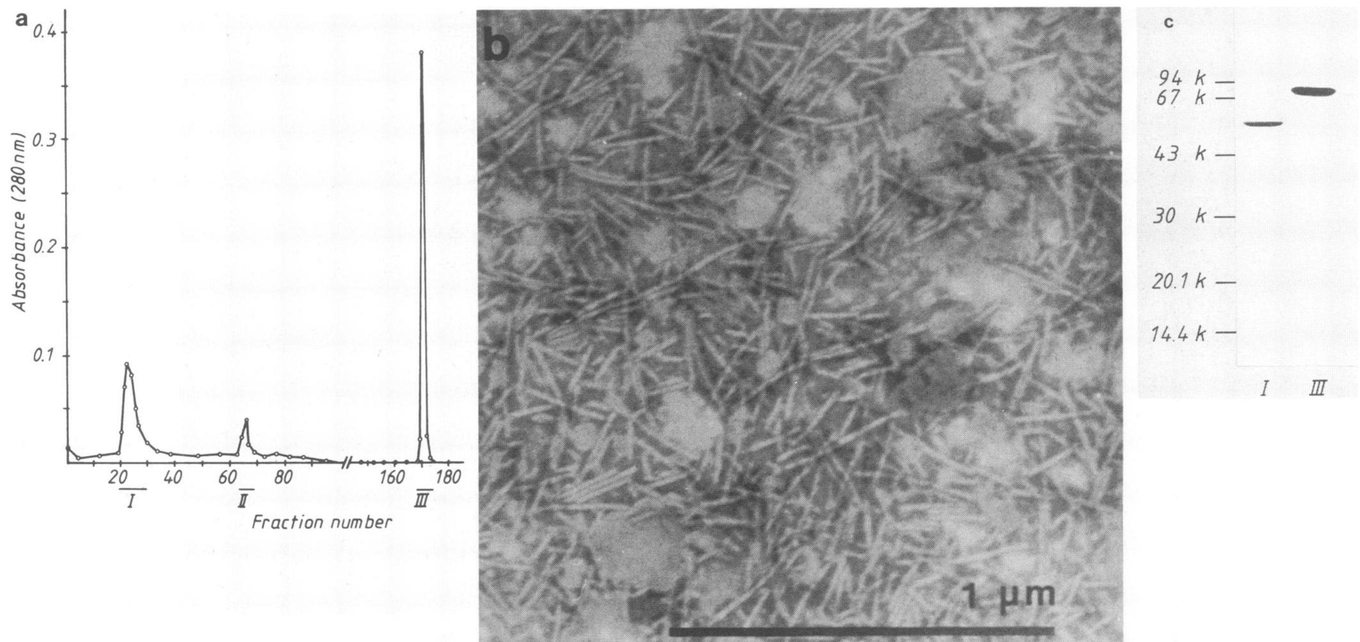

FIG. 4. (a) Gel chromatography of the prepurified flagella. (b) Electron micrograph of filament fragments contained in peak I. (c) SDS-PAGE of the proteins contained in peaks I and III.

Peak III contained an unidentified protein with a molecular weight of about 71,000 (Fig. 4c, lane III). Peak II was not further analyzed.

DISCUSSION

Until now there have been very few studies dealing with properties of *C. pylori* surface structures. However, such studies seem necessary if the basis of the specific pathogenicity of *C. pylori* is to be identified.

The flagella of *C. pylori*, which are the subject of this study, differ from those of other *Campylobacter* species in several aspects. The molecular weight of *Campylobacter jejuni* flagellin is 63,000 (3). When Perez-Perez and Blaser (22) analyzed the proteins of *C. pylori* in SDS-PAGE gels they saw a strong band with a molecular weight of 62,000, which they found to be most likely a flagellar protein. Lee et al. (14) found a protein of *C. pylori* with a molecular weight of 58,000 to 59,000 which showed strong cross-reaction with antiserum against *C. jejuni* flagellin. Newell (21) noticed two proteins with molecular weights of 56,000 and 54,000 cross-reacting with rabbit-anti *C. jejuni* flagellin antiserum. According to our data, the molecular weight of *C. pylori* flagellin is 51,000. It thus resembles in size the flagellins of *Pseudomonas aeruginosa* (molecular weight, 45,000 to 53,000 [1, 20]), *Salmonella* species (molecular weight, 48,000 to 58,000 [12]), and *Escherichia coli* (molecular weight, 51,000 to 57,000 [16]). It may be possible that some *C. pylori* strains are capable of producing flagellins of two different molecular weights, as has been described by Harris et al. (10) for some *Campylobacter coli* and *C. jejuni* strains.

The most remarkable difference between the flagella of *C. pylori* and those of other *Campylobacter* species is the flagellar sheath that encloses each flagellum of *C. pylori*. The presence of flagellar sheaths has been reported for *V. cholerae* and other *Vibrio* species (7, 11), *Bdellovibrio bacteriovorus* (23), *Pseudomonas stizolobii* (8), *Bacillus brevis* (5), and *C. pylori* (9).

The only flagellar sheath that has been characterized biochemically is that of *V. cholerae*. Hranitzky et al. (11) found it to be composed of a single protein with three polypeptide chains with molecular weights of 61,500, 60,000, and 56,500. Since antibodies against lipopolysaccharide did not react with the flagellar sheath, they concluded that it is unlikely that the sheath contains lipopolysaccharide in significant amounts. They did not give data about the phospholipid or carbohydrate content of the sheath.

The function of the flagellar sheath is unknown. It was demonstrated by Attridge and Rowley (2) that *V. cholerae* flagella carry adhesins. Most likely the adhesive molecules are part of the sheath; however, adhesive properties have also been described for the unsheathed flagella of *E. coli* (6) and therefore are not a unique feature of sheathed flagella.

The flagellar sheaths of *C. pylori* have not been purified to date. Remarkable is the pronounced club-shaped thickening at the end of each sheath. The filament does not seem to extend into the club. When the terminal club has been removed by mild shearing the filament cannot be seen sticking out of the sheath, which, however, is the case when the sheath breaks more proximally and the club is removed together with a piece of the sheath.

Further studies about the biochemistry and function of the flagellar sheath of *C. pylori* and its terminal club are certainly necessary to determine the pathological relevance of these structures.

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