Structure of the Na^+-driven flagellum from the homoacetogenic bacterium *Acetobacterium woodii*

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Abstract The Na^+-dependent flagellum of *Acetobacterium woodii* was characterised. Flagellin and whole flagella were purified and analysed by SDS-PAGE and electron microscopy. The structure and dimensions of the filament and the hook-basal body, as revealed by electron microscopy, resemble those of H^+-dependent flagella from Gram-positive bacteria. Intramembrane particle rings were present at the cell pole in freeze-fractured *A. woodii* cells, which might correspond to the mot complex.

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Key words: Flagellum; Hook-basal body; Sodium ion; *Acetobacterium woodii*

1. Introduction

Acetobacterium woodii is a Gram-positive, strictly anaerobic, Na^+-dependent homoacetogenic bacterium [1]. During acetate formation via the acetyl-CoA pathway it generates a primary electrochemical sodium ion potential which is used as driving force for ATP synthesis by a F_1F_o ATP synthase [2–4]. Motility in *A. woodii* is also strictly dependent on Na^+ and is inhibited by monensin and phenamil but not by protonophores indicating $\Delta\tilde{\mu}_{Na^+}$ as driving force for flagella rotation [5].

Na^+-dependent motility was first observed in some alkaliphilic *Bacillus* species [6] and is best studied in these and in marine *Vibrio* strains [7–9]. Molecular studies and electron microscopy revealed differences as well as similarities between Na^+- and H^+-driven flagella [10–15], but a detailed analysis of the structure of Na^+-dependent flagella is still missing. Furthermore, the subunit composition of the hook-basal body of Na^+-dependent flagella has not been determined. Therefore, we have isolated and studied the flagellum and the hook-basal body from the neutrophilic, Na^+-dependent bacterium *A. woodii*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. woodii (DSM1030) was grown at 30°C on fructose (20 mM) as described [16] and was frequently inoculated on swim agar tubes to improve motility for filament and flagella preparations as described previously [5].

2.2. Preparation of filaments

A. woodii was harvested from 1-l cultures at an optical density OD_{600} of 1–2 and resuspended in 200 ml 10 mM Tris-HCl (pH 8.0). The filaments were sheared from the cell body in a blender (90 s). The cells and filaments were separated by centrifugation at $8000\times g$ and 4°C for 20 min, and the filaments were pelleted at 4°C at $70\,000\times g$ for 1 h. The pellet containing the filaments was resuspended in 1 ml 10 mM Tris-HCl (pH 8.0). A further purification was achieved by a KBr density-gradient centrifugation at $210\,000\times g$ for 24 h at 4°C (50% KBr in 10 mM Tris-HCl, pH 8.0) but this step could be omitted in most cases. Glycosylation of filaments was examined as described in [17], and by staining with the Dig Glycan Detection Kit (Boehringer, Mannheim, Germany) with maltose binding protein as negative and transferrin as positive control.

2.3. Preparation of whole flagella

The isolation of whole flagella was based on the protocol developed by Aizawa et al. [18] for *Salmonella typhimurium*. To generate protoplasts, *A. woodii* was grown in four 1-l cultures in medium supplemented with 420 mM sucrose, 8.4 mM $MgSO_4$ and 0.4% glycine. At an OD_{600} of 0.6–0.8 70 μg penicillin/l medium was added. After 16–20 h the protoplasts were harvested and resuspended in 100 ml 10 mM Tris-HCl (pH 8.0), 420 mM sucrose and 8.4 mM $MgSO_4$. The protoplasts were then solubilised with 1% Triton X-100 for 30 min on ice. Unlysed cells were removed by low-speed centrifugation ($8000\times g$, 20 min, 4°C) and the flagella were pelleted from the supernatant by high-speed centrifugation ($70\,000\times g$, 1 h, 4°C). The pellet was resuspended in 1 ml 10 mM Tris-HCl (pH 8.0), 5 mM EDTA and dissolved overnight. The solution was loaded on 10 ml 50% CsCl in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA and centrifuged for 22 h at $70\,000\times g$ and 4°C. Two bands occurred in the gradient, the upper one containing flagella and the lower one containing mainly surface layer, as judged by electron microscopy, built up by a 90-kDa polypeptide. The upper band was withdrawn and desalted by dialysis. Preparations were analysed by SDS-PAGE on 12% T, 3% C gels, and silver staining was done as described [19,20]. Proteins were blotted on PVDF membranes [21]. N-terminal sequencing of proteins was done at the Institut for Biochemistry II (Georg-August-Universität Göttingen) by Edman degradation with a 477A pulsed phase protein/peptide sequencer (Applied Biosystems, Weiterstadt, Germany).

2.4. Electron microscopy

Negative staining was performed as described in [22]. Freeze-fracture of *A. woodii* cells was done as described in [23] in 60% glycerol. Electron microscopy was performed using a Philips EM 301 electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV.

3. Results and discussion

In an ongoing project dealing with the structure-function analysis of Na^+-dependent energy converters in *A. woodii* we determined the structure of its Na^+-dependent flagellum. As controls, non-motile and filamentless mutants (Fla−) were isolated from cultures of *A. woodii* grown under non-selective conditions for years that were apparently non-motile as judged by light microscopy. They were plated on solid agar plates and incubated at 37°C in anaerobic jars under an H_2+CO_2 atmosphere. After 8 days single colonies were picked, and non-motile mutants were identified by their failure to swarm on semi-solid agar plates. Filamentless mutants were identified by electron microscopy. Loss of motility in

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these mutants was irreversible, and not accompanied by a change of the growth rate during growth on fructose, methanol or H_2+CO_2.

To determine their protein composition filaments were prepared from the motile wild type and a Fla− strain. The preparations from the wild type contained a dense suspension of filaments as judged by electron microscopy. Analysis of such a preparation by SDS-PAGE revealed a single protein with an apparent molecular mass of 50 kDa (Fig. 1). When the same isolation procedure was applied to the Fla− mutant this protein was not detected. The N-terminus of the 50-kDa protein was determined and is identical to the flagellin from *Bacillus subtilis* [24]. These experiments demonstrate that the 50-kDa polypeptide indeed represents flagellin from *A. woodii*.

To determine the physicochemical properties of the filament they were incubated under different conditions for 1 h, pelleted by high-speed centrifugation, and then analysed by SDS-PAGE and electron microscopy. The filament is degraded by incubation for 1 h at pH 2, 10 and 12, but it is equally stable at pH 4–8. It is unstable at temperatures higher than 50°C. The filaments are largely fragmented in the presence of 1% Triton X-100, but stable in the presence of 1% CHAPS or octylglucoside. This behaviour to Triton X-100 was unexpected since an effect of detergent on filament stability was only reported for glycosylated archaeal filaments before [25]. Therefore, we examined filaments for glycosylation by treatment with trifluoromethanesulphonic acid [17] or by a staining procedure as described above. With both methods we did not obtain indications for glycosylation of the filaments (data not shown).

To determine the protein composition of the flagellum, whole flagella were isolated based on the protocol of Aizawa et al. [18] for *S. typhimurium*. These preparations contained pure flagella, as judged by electron microscopy, consisting of a filament, a hook, and a basal body. The basal body was often extended by structures below the MS ring (see below). Since these structures are, in *Escherichia coli*, very sensitive to extreme ion concentrations [26] we tried to get optimal preservation by substituting the CsCl gradient in the Aizawa protocol by milder methods. Instead, a Percoll gradient [26], gel filtration [27] and differential centrifugation was used. Unfortunately, these procedures did not separate flagella from the surface layer protein (data not shown).

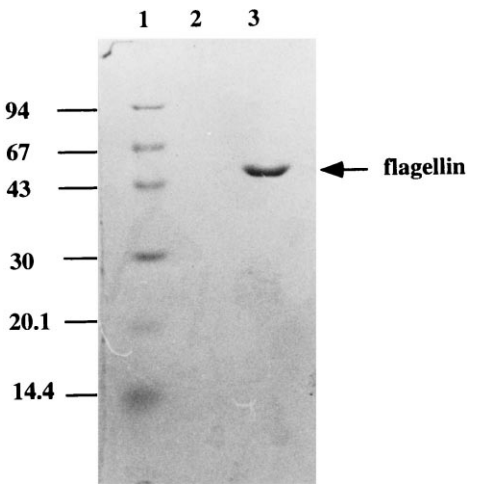


Fig. 1. SDS-PAGE of flagellin purified from *A. woodii*. The preparations were done with the non-motile mutant strain Fla− (lane 2) and the wild type (lane 3) in parallel. 20 μg protein was applied to the gel and stained with silver nitrate. The molecular mass standard is given in kDa (lane 1).

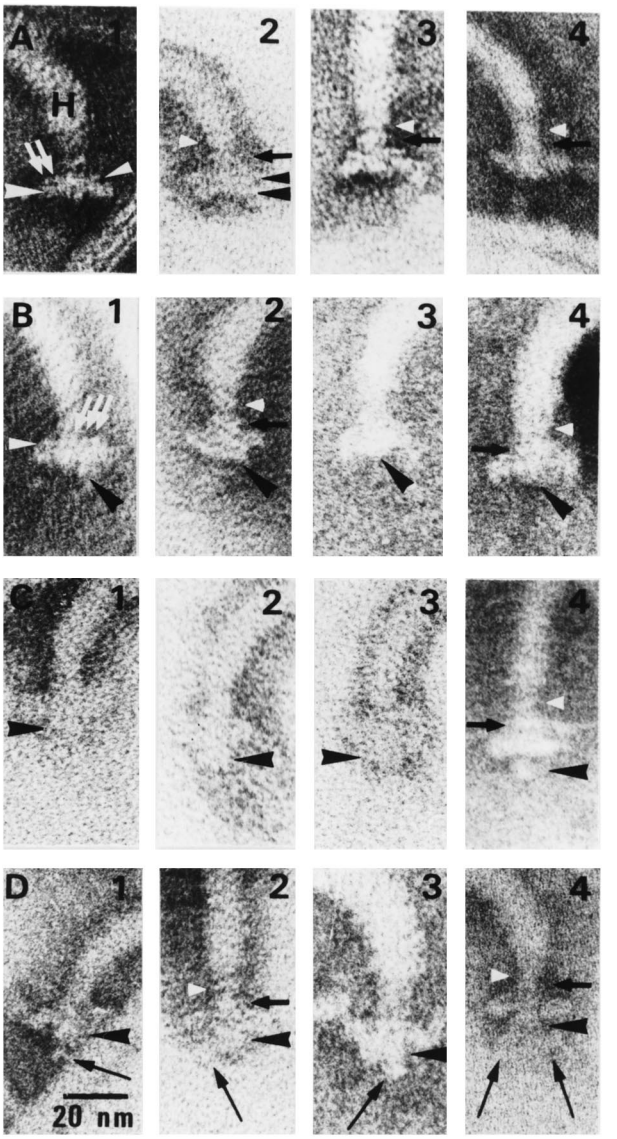


Fig. 2. Negatively stained isolated flagellar base complexes of *A. woodii* exhibiting different states of preservation. Arrows and arrowheads indicate different structural features of the flagellar basal body as described in the text. H: hook.

The flagella preparations were negative stained with uranyl acetate and examined by electron microscopy at higher magnification. The hook-basal body consists of a hook (16 nm diameter), a rod (10 nm diameter) and a MS ring (25 nm diameter). A typical gallery is presented in Fig. 2. The sample A1 exhibits the flagellar hook (H) together with the rod element to which the MS ring is attached. The MS ring is composed of an upper (small arrowheads in A1/2 and B1) and a lower (large arrowheads in A1/2) structural element, and the upper element exhibits a periodic ultrastructure (arrows in A1 and B1).Two structures are visible above the MS ring (black short arrow and small white arrowhead), that may constitute small ring-like structures since they are wider than the rod. In *E. coli*, *S. typhimurium*, and *Wolinella succinogenes* a C-ring located below the MS ring was identified [28–30]; it consists

of at least three proteins, FliM, FliN and FliG [31], and FliG was shown to interact directly with the mot complex [32–36]. In some cases we could detect such structures below the MS ring of *A. woodii*: a small extrusion of the rod into the cytoplasm at the centre of the MS ring (Fig. 2, large black arrowhead in B1–B4), a big central mass extruding 10 nm into the cytoplasm (Fig. 2, large black arrowhead in C1–D4), and additional structures at the periphery of the MS ring and on the big central mass (Fig. 2, long arrows in D1–D4). In *S. typhimurium* the C-ring protrudes 17 nm into the cytoplasm and is wider than the MS ring, and therefore, physically be able to interact with the mot complex. In contrast, the knob-like structure below the MS ring of *A. woodii* is not as wide as the MS ring but this may result from preparation artefacts due to the harsh purification procedure, as has been reported for other bacteria [26].

The analysis of hook-basal body complexes from *S. typhimurium* revealed nine [18], that of *B. subtilis* eight proteins [37]. Analysis of the preparations from *A. woodii* by SDS-PAGE revealed, apart from the flagellin, six additional proteins with molecular masses of 7, 16, 19, 23, 27, and 63 kDa (Fig. 3) which appeared consistently in our preparations. Dissociation of the filament by extreme pHs led to preparations in which no flagellin but the 7-, 16-, 19-, 23-, 27-, and 63-kDa proteins were still present, but the structure of the hook-basal body as seen in electron micrographs was also destroyed by this treatment. Unfortunately, the N-terminal sequences of the polypeptides present in the hook-basal body preparations from *A. woodii* showed no similarities to known flagellar genes or to any other proteins.

The MS ring is surrounded by the mot complex that constitutes the ion-translocating part of the flagellar motor [38]. The mot complex was seen in electron micrographs of *E. coli* as a ring-like structure consisting of 9–12 particles embedded in the cytoplasmic membrane [39]. We were able to detect such particle rings in the cytoplasmic membrane of freeze-fractured *A. woodii* only rarely (Fig. 4), which was not unexpected, because *A. woodii* has only one flagellum per cell. These particle rings resemble the *E. coli* mot complex in structure and size. They consist of 10–12 particles, each 5 nm in diameter, which surround a central mass. The diameter of the inner hole of this complex is ~25 nm, which is in good agreement with the diameter of the MS ring. The particle rings are located at the cell pole, which is in accord with the polar location of the flagella in *A. woodii*. Therefore, we suppose these particle rings to be the mot complex of the Na^+-dependent flagellum of *A. woodii*.

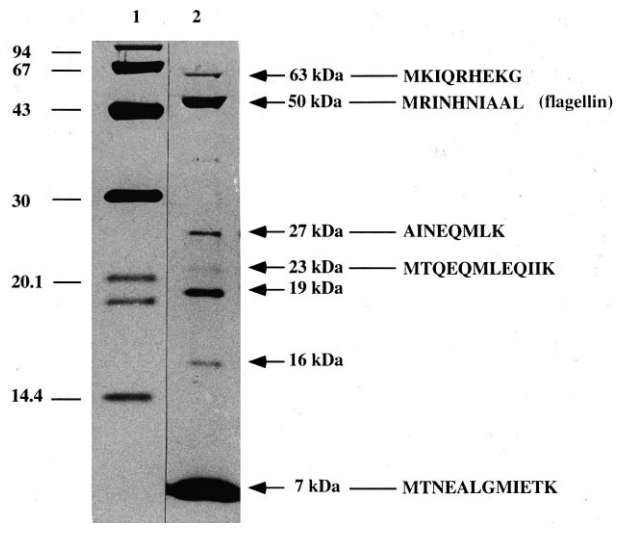


Fig. 3. SDS-PAGE of a flagella preparation from *A. woodii* (lane 2) with molecular masses and N-terminal amino acid sequences of the proteins. 50 μg protein was applied to the gel and stained with silver nitrate. The molecular mass standard is given in kDa (lane 1).

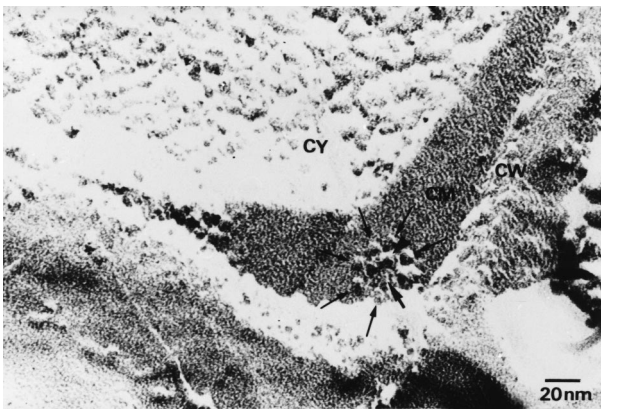


Fig. 4. Freeze-fractured cell of *A. woodii*. In the plane of the cytoplasmic membrane (CM), a ring-like structure can be seen which is composed of a number of spherical elements (small arrows) and an additional mass in its center (large arrow). CY: cytoplasm; CW: cell wall.

4. Conclusion

The structure of the Na^+-dependent flagellum from *A. woodii* is in general agreement with that of H^+-driven flagella. The dimensions of the elements are comparable to those from flagella of other organisms. The mot complex from *A. woodii* is comparable in size and structure to that of *E. coli* and *Bacillus firmus* [14,39] indicating that the difference in ion specificity of this ion channel is not reflected by its ultrastructure, as revealed in electron micrographs. The same was shown to be true for Na^+- and H^+-dependent ATP synthases [40,41].

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