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EXPERIMENTAL ARTICLES

Structure of the Intracellular Part of the Motility Apparatus of Halobacteria

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Abstract—he electron microscopic study of the structure of the motility apparatus of the archaea Halobacterium salinarium 4W12 and Natronobacterium magadii confirmed our earlier observation that the motility apparatus of halobacteria contains an intracellular disk-shaped lamellar structure (DLS). Polar cap structures (PCSs) isolated from the halobacterium were preliminarily identified as the DLSs. The PCSs in complexes with flagella were also isolated from the haloalkaliphilic bacterium N. magadii. The specific structure of the archaeal motility apparatus is discussed.

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Bacterial flagella, which are responsible for the motility of prokaryotes, have been intensively investigated since the mid-1960s. At present, most of the structural and functional proteins of prokaryotic flagella, their localization in the flagellum, and the bioenergetics of bacterial motility are well understood [1, 2]. Macnab et al. provided evidence that some components of the flagellar export apparatus are similar to type III secretory system of virulence factors [1, 3]. Although morphologically identical to bacterial flagella, archaebacterial (or archaeal) flagella differ considerably from the latter in other respects. The conception of archaebacteria (presently called archaea), which was formulated in 1978 [4], gave an impetus to the study of the archaeal motility apparatus. Like the rotation of bacterial flagella, the rotation of archaeal flagella is provided by a proton gradient. The archaeal flagella are twice as thin as the bacterial flagella and contain several flagellins, most of which are glycosylated. The bacterial and archaeal flagella also differ in the mechanism of their assembly [5]. At the same time, the archaeal flagella and component flagellins are found to be similar to bacterial pili and component pilins [5, 6]. In fact, fundamental differences between the bacterial and archaeal flagella follow from the analysis of the full genome sequences of motile archaea, which are found to contain neither genes coding for bacterial flagella nor proteins involved in the flagellum assembly [7].

The intracellular part of bacterial flagella contains the so-called basal body (it consists of a series of rings built in the cell wall and membrane), Mot proteins (they represent a stator of the flagellar motor), the switch complex (it represents a rotor of this motor), and a special apparatus for the export of flagellar proteins [1, 8, 9]. In spite of intense studies, researchers have not thus far revealed such protein systems in the archaeal flagella, except for a hook similar to that of the bacterial flagella [6, 10]. In particular, there is no evidence for the existence of a ring system similar to the L, P, and MS disks of the bacterial flagella. Nor do we know how archaeal flagella are anchored in the cells [10].

In 1992, we presented experimental evidence that *Halobacterium salinarium* cells contain a so-called disk-shaped lamellar structure (DLS), which accommodates flagella [11]. Later, Kupper et al. succeeded in isolating polar cap structures (PCSs), or cap-shaped bodies, from this archaeon [12]. The bodies were isolated together with attached flagella.

This work is an attempt to confirm the existence of the DLSs in the motility apparatus of *H. salinarium* and to study the organization of this apparatus in another representative of the *Archaea* domain, *Natronobacterium magadii* (*Natrialba magadii*).

MATERIALS AND METHODS

Experiments were carried out with the bacteria *Halobacterium salinarum* 4W12 (a generous gift from M. Alam, University of Hawaii) and *Natronobacterium magadii* (*Natrialba magadii*) M21,3, which was kindly provided by M.G. Pyatibratov from the Institute of Protein Research, Pushchino, Moscow region, Russia. The names of the bacteria are given according to [13]. PCSs with attached flagella were isolated from *H. salinarium* 4W12 cells by a procedure described earlier [12]. To isolate analogous structures from the haloalkaliphile *N. magadii*, this procedure was considerably modified.

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N. magadii was grown at 37°C in a liquid medium on a shaker (180 rpm) for 60 h. Cells for inoculation were grown in a semiliquid medium. The liquid medium contained the following ingredients (in g/l): NaCl, 205; CH_3COONa, 13.6; KCl, 3.6; $MgSO_4 \cdot 7H_2O$, 1.0; Na_2CO_3, 19.0; Na_2HPO_4, 0.07; yeast extract, 10.0; and casamino acids, 7.5 (pH 9.5).

Cells were harvested from 100 ml of the culture by centrifugation at 12000 *g* for 30 min and suspended in 18 ml of basal saline solution (4.3 M NaCl, 81 mM $MgSO_4$, and 27 mM KCl). The suspension was lysed by adding an aliquot of DNase I, 0.3 ml of 1 M $MgSO_4$, and 0.17 ml of 0.5% taurodeoxycholate. The mixture was centrifuged at 50000 *g* for 25 min (10°C). The pellet was suspended in 6 ml of ice- cold basal saline solution. In order to isolate the PCSs with attached flagella, this suspension was mixed with 2 ml of ice-cold dissolving buffer (0.1 M Tris–HCl with pH 7.2, containing 0.4% Triton X-100) and incubated for 20 min with continuous stirring. Cell debris was removed by centrifugation at 6000 *g* for 10 min (10°C). The supernatant was centrifuged at 100000 *g* for 2 h (4°C). The pellet was resuspended in an elution buffer (50 mM Tris–HCl with pH 7.2, containing 2.75 M NaCl, 50 mM $MgCl_2$, and 0.1 wt % sodium cholate). The suspension was mounted on a carbon- coated grid, prefixed with 0.4% glutaraldehyde for 2 min (glutaraldehyde and elution buffer were mixed immediately before use), washed several times with distilled water, and stained with 2% phosphotungstic acid.

In order to obtain thin sections of halobacterial cells, they were fixed with 5% glutaraldehyde in the fresh cultivation medium. The cells were postfixed with 1% osmium tetroxide in the same medium and dehydrated in a series of alcohol solutions of increasing concentration (20, 70, 80, and 97%), 96% ethanol–acetone mixture (1 : 1), and then three times in pure acetone. After treating in 70% ethanol, the mixture was kept overnight in a refrigerator in a 2% solution of uranyl acetate in ethanol. The fixed cells were embedded in a mixture of 45% epon 812, 30% DDSA, 20% MNA, and 5% DMP-30. After curing the preparation, it was cut on an LKB III ultratome (Sweden) into thin sections, which were mounted on a formvar-coated grid, contrasted with lead citrate [14], and examined with a Hitachi-11B electron microscope (Japan).

RESULTS AND DISCUSSION

Archaeal motility has only been investigated in extreme halophiles, since other motile archaea are strict anaerobes difficult to study under vital conditions. The study of the halobacterial motility apparatus by high-resolution dark-field microscopy allowed Alam and Oesterhelt [15] to reveal the unique properties of this apparatus and to suggest the existence of an intracellular plate which attaches flagella via their proximal ends. In 1992, we confirmed this suggestion with the aid of electron microscopy, by demonstrating the occurrence of a DLS in the cell cytoplasm at a depth of approximately 20 nm from the cytoplasmic membrane [11].

In this study, we revealed a similar structure in particular and serial thin sections of cells of another halophilic strain, *H. salinarum* 4W12 (Fig. 1). This structure was not studied in detail because there was a high degree of probability that it is identical to the DLS of *H. salinarium* VKM B-1231 [11] (Fig. 1a).

Our attempts to reveal similar structures in the archaeal haloalkaliphiles *Natronobacterium pharaonis (Natronomonas pharaonis)* and *N. magadii* showed that these two archaea contain a DLS-like structure. However, technical difficulties (the precipitation of glutaraldehyde at pH values physiological to natronobacteria (approximately 9.0) and the impossibility of retaining the native ultrastructure of these archaea without glutaraldehyde prefixation) did not allow us to obtain specimens of the necessary quality. For this reason, we tried to approach the problem by isolating the PCS–flagella complex from *N. magadii* cells.

In 1994, Kupper et al. isolated unusual objects from *H. salinarium* cells. These objects looked like disks uniting the proximal ends of flagellar filaments [12]. The size of these structures corresponded to that of the DLSs observed earlier in the same cells [11]. It was reasonable to suggest that the presence of analogous structures in the cells of other halophiles would provide evidence for a common organization of the motility apparatus of extreme halophiles.

In this work, we attempted to isolate the PCS–flagella complex from the halophilic strain *H. salinarium* 4W12 (Fig. 2) and a different haloalkaliphilic bacterium, *N. magadii* M21,3 (Fig. 3). It should be noted that the results obtained for *H. salinarium* 4W12 were very close to those reported by Kupper et al. [12].

It should be noted that the isolation of the PCS–flagella complex is a delicate procedure, since the PCSs of unknown origin are very labile and may rapidly dissolve under the action of the reagents used for the isolation. As a result, the diameter of the caps gradually decreases during isolation, although their shape is preserved (Fig. 2a). The PCS–flagella complex of *N. magadii* M21,3 is particularly sensitive to Triton X-100. For this reason, the concentration of this detergent was considerably reduced in the medium used for the isolation of this complex from *H. salinarium* 4W12 cells. Eventually, we could devise an optimal and reproducible procedure for the isolation of the PCS–flagella complex.

It is known that (1) bacterial flagellar filaments are made up of flagellin molecules with conservative *N*-and *C*-termini, (2) the flagellin molecules are assembled into a filament on the distal end, and (3) the bacterial flagellar filaments have lumens [16, 17]. By contrast, the flagellin molecule of archaea contains only one conservative terminus (the *N* terminus), the diameter of archaeal flagellum filaments is twice as small as that of bacterial filaments (10–12 nm) [18, 19], and archaeal

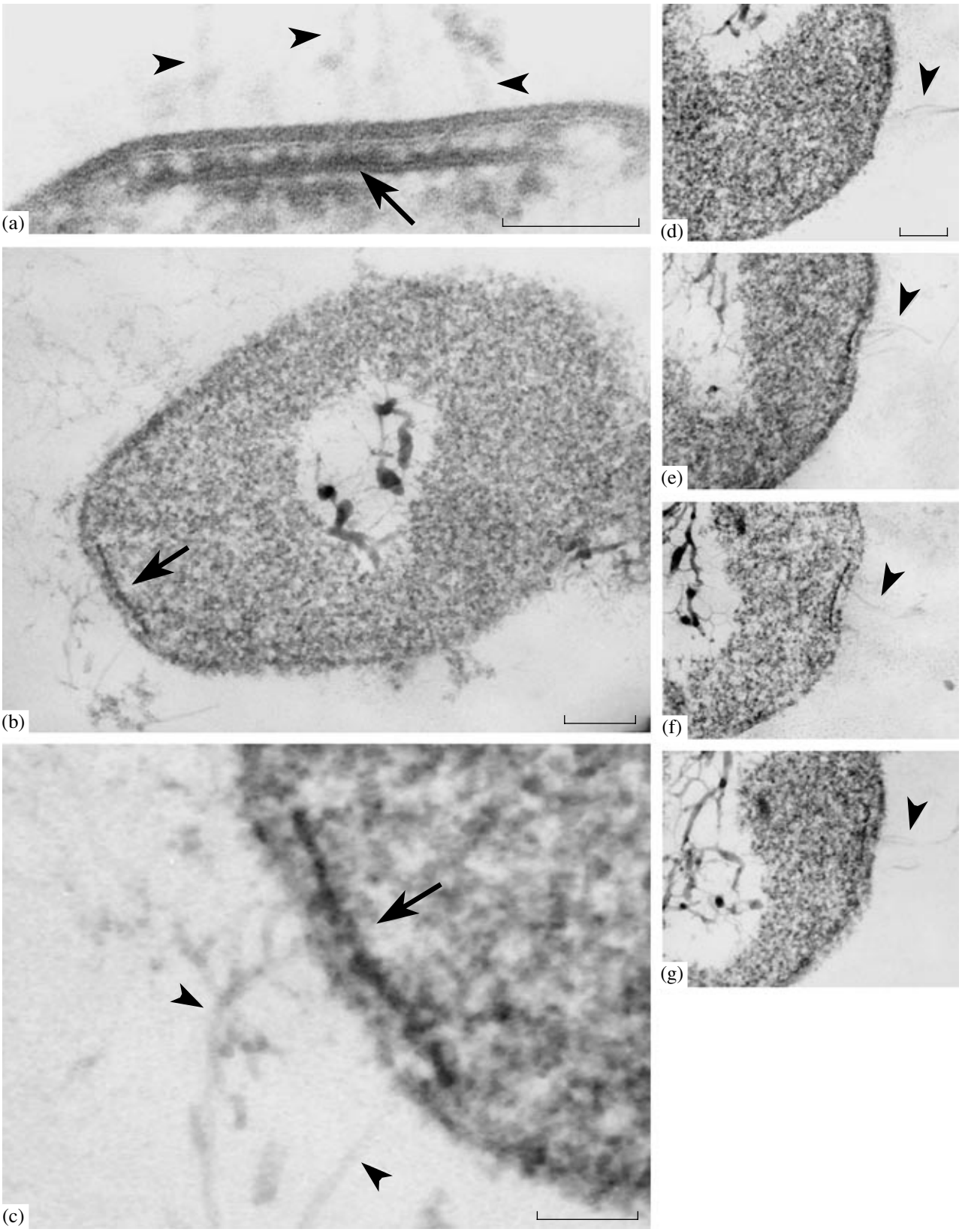

Fig. 1. Electron micrographs of *H. salinarium* VKM B-1231 and *H. salinarium* 4W12 cells: (a) thin section of a pole of an *H. salinarium* VKM B-1231 cell [11]; (b) general view of an *H. salinarium* 4W12 cell; (c) pole of an *H. salinarium* 4W12 cell at a high magnification; (d–g) pole of an *H. salinarium* 4W12 cell on serial sections. The arrows point to DLSs. The arrowheads show flagellar filaments. The scale bars represent 100 nm (a, c) and 200 nm (b, d–g).

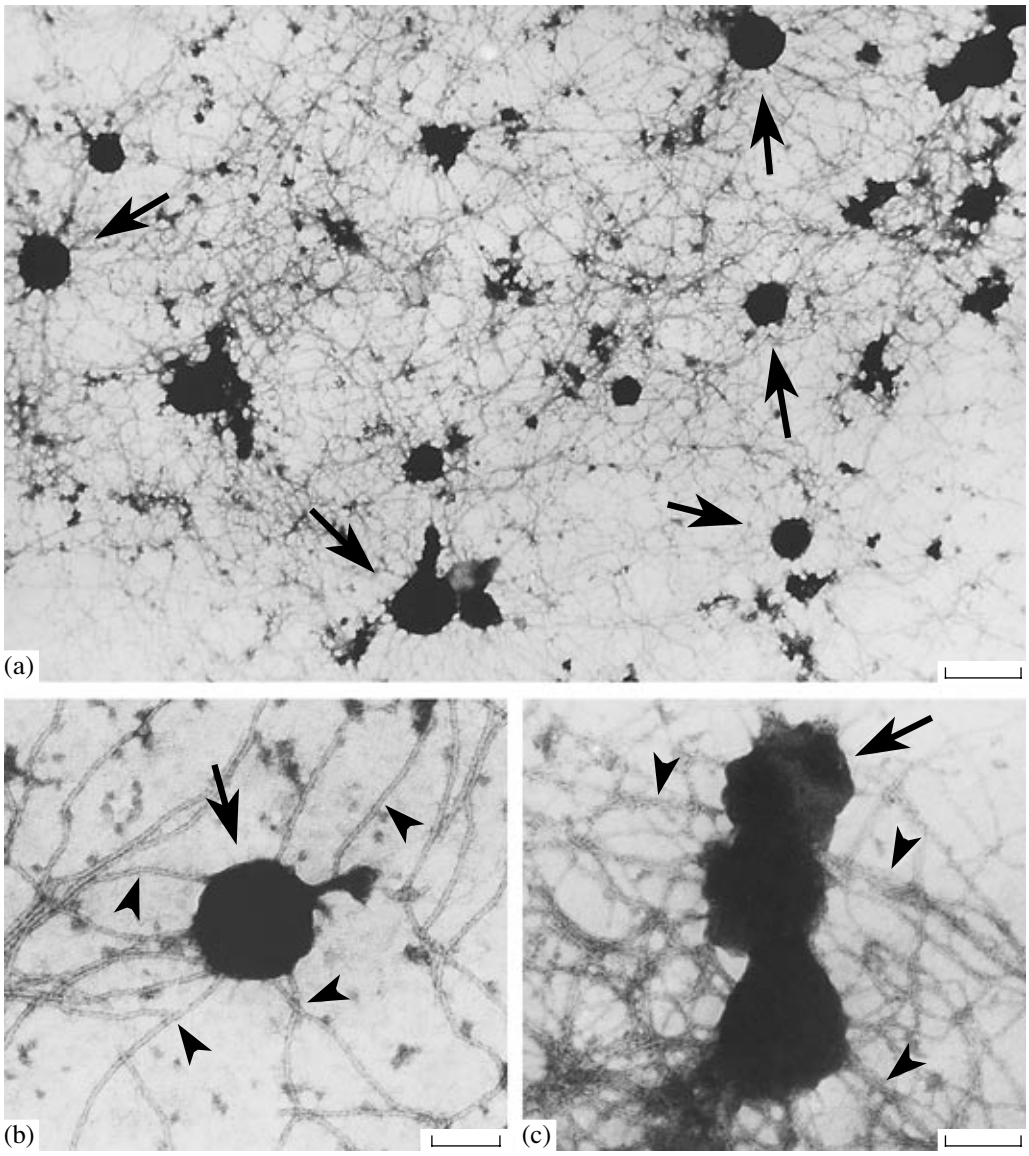

Fig. 2. Electron micrographs of the PCS–flagella complex from *H. salinarium* 4W12 cells: (a) general view; (b, c) particular PCSs with flagella at a high magnification. The arrows point to PCSs. The arrowheads show flagellar filaments. The scale bars represent 500 nm (a) and 250 nm (b, c).

filaments have no lumens [20]. However, the most important difference between bacterial and archaeal flagella lies in the structural organization of their filaments and the specific package of their flagellins. Indeed, at pH 2–3, bacterial flagellar filaments dissociate into individual flagellin molecules, which are able to reassociate into filaments at neutral pH values either spontaneously [21, 22], or in the presence of a template [23]. By contrast, the flagellar filaments of extreme halophiles dissociate into so-called protofilaments (filaments of a lower diameter), which are not able to reassociate [24].

In this context, let us compare the PCS–flagella complexes of *H. salinarium* 4W12 (Fig. 2) and *N. magadii* M21,3 (Fig. 3). The filaments of *H. salinarium* 4W12 are more distinct than the flagellar filaments of *N. magadii* M21,3 (Fig. 3a). Unlike the filaments of *H. salinarium* 4W12, all of which are as thick as 10–12 nm, the *N. magadii* M21,3 filaments may occasionally be thinner, suggesting that they represent protofilaments produced under the action of the reagents used for the isolation of the PCS–flagella complex. It should be noted that this was the first time that we observed changes in the shape and thickness of flagellar filaments during the isolation of the PCS–flagella complex from *H. salinarium* cells.

The data presented in this publication show that the motility apparatus of both halophiles under study is characterized by the presence of a disk-shaped plate which unites the proximal ends of their flagella. In spite

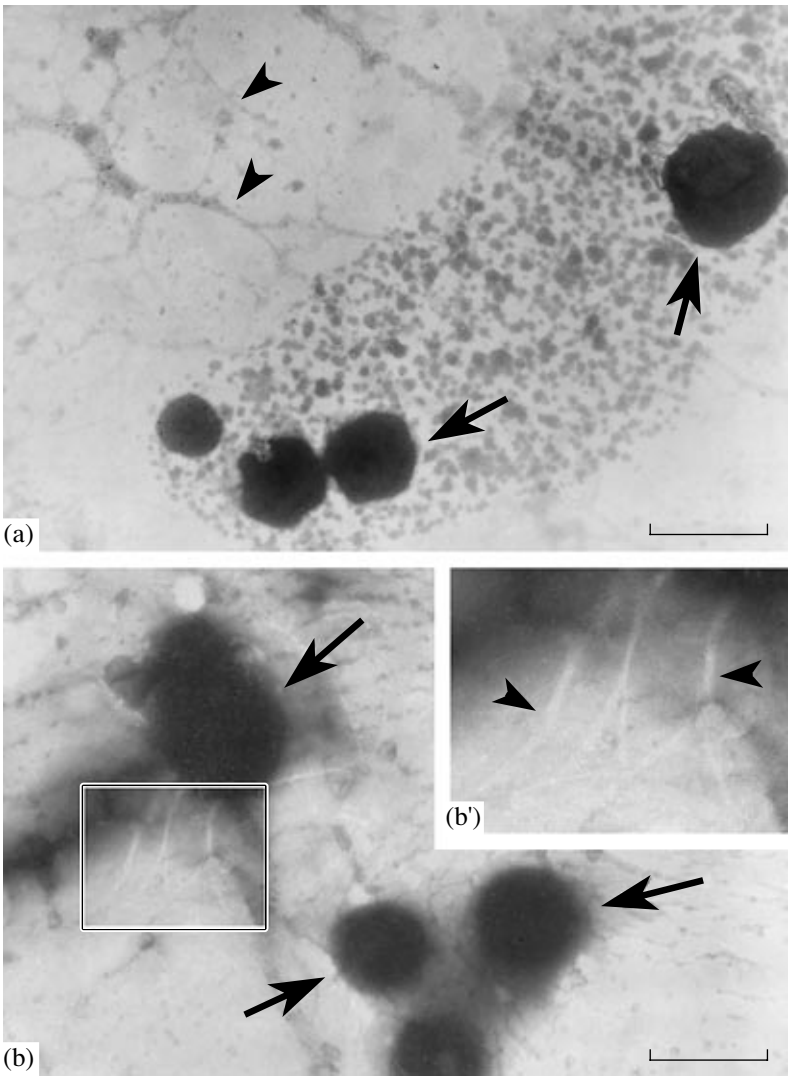

Fig. 3. Electron micrographs of the PCS–flagella complex from *N. magadii* M21,3 cells: (a, b) general view. Insertions in panel b show flagella alongside PCS at a higher magnification. The scale bars represent 400 nm.

of certain experimental difficulties, electron microscopy is a promising approach to studying the archaeal motility apparatus.

The motility of a living organism is an important feature whose study may considerably enlarge our knowledge of biological evolution. Bacteria and archaea are ideal objects for comparative studies along this line [25].

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