1 **Structure of the archaellar motor and associated cytoplasmic cone in**

2 ***Thermococcus kodakaraensis***

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21 Keywords: electron cryotomography, cryo-EM, archaea, archaella, flagella, T4P, motility,

22 *Thermococcus kodakaraensis*, *Thermococcus kodakarensis*

23

24 **ABSTRACT**

25 Archaeal swimming motility is driven by rotary motors called archaella. The structure of these

26 motors, and particularly how they are anchored in the absence of a peptidoglycan cell wall, is

27 unknown. Here, we use electron cryotomography to visualize the archaellar motor *in vivo* in

28 *Thermococcus kodakaraensis*. Compared to the homologous bacterial type IV pilus (T4P), we

29 observe structural similarities as well as several unique features. While the position of the

30 cytoplasmic ATPase appears conserved, it is not braced by linkages that extend upward through

31 the cell envelope as in the T4P, but rather by cytoplasmic components that attach it to a large

32 conical frustum up to 500 nm in diameter at its base. In addition to anchoring the lophotrichous

33 bundle of archaella, the conical frustum associates with chemosensory arrays and ribosome-

34 excluding material and may function as a polar organizing center for the coccoid cells.

35

36 **INTRODUCTION**

37 Motility is a fundamental property of single-celled organisms. In archaea, swimming motility is

38 driven by a rotary motor called the archaellum. Archaella are functionally analogous to bacterial

39 flagella, but evolutionarily homologous to the type IV pilus (T4P) and type II secretion system

40 (T2SS) machineries of bacteria [1]. Recently, an atomic structure of the archaellum fiber

41 purified from the euryarchaeon *Methanospirillum hungatei* revealed differences compared to the

42 bacterial T4P fiber, including lack of a central pore and more extensive inter-subunit interactions

43 [2]. The structure of the archaellar basal body, and its similarity to the T4P basal body remains

44 unknown.

45

46 Unlike T4P fibers that only assemble and disassemble, archaella assemble and can then rotate in

47 both directions to either push or pull the cell [3, 4]. Light microscopy of *Halobacterium*

48 *salinarum* revealed discrete steps during rotation, likely corresponding to ATP hydrolysis events

49 by the basal body ATPase, FlaI [5]. While the bacterial T4P contains two distinct ATPases for

50 assembly and disassembly of the pilus fiber, the single ATPase FlaI drives both assembly and

51 rotation of the archaellum [6]. The N-terminal domain of the archaellum/T2SS/T4P superfamily

52 ATPases is the most variable, and the first 29 residues of FlaI, located on the outer edge of the

53 hexamer, were found to be essential for motility but not assembly, although the basis of this

54 functional separation remains unclear [6].

55

56 FlaI is predicted to interact with the integral membrane protein FlaJ [7]. Structural studies of the

57 bacterial T4P suggest that ATPase-driven rotation of the FlaJ homolog, PilC, incorporates pilin

58 subunits from the membrane into the growing fiber [8]. This is possible because the ATPase

59   itself is clamped in an integrated structure that spans the inner and outer membranes and

60   periplasm and anchors on the cell-encompassing peptidoglycan cell wall [8]. A similar cell-wall-

61   attached structure anchors the rotation of the bacterial flagellar motor [9]. Without knowing the

62   structure of the archaellar basal body, it is unclear how similar anchoring could occur in the

63   envelope of archaea, which consists of a single membrane and thin proteinaceous surface (S-

64   )layer. It was recently proposed that FlaF might anchor the archaellum through interactions with

65   the S-layer [10]. Others have suggested that a cytoplasmic structure mechanically stabilizes the

66   motor [3]. Supporting this idea, cytoplasmic structures underlying the archaella have been

67   observed by traditional electron microscopy (EM) of Halobacteria [11, 12].

68

69   Electron cryotomography (ECT) can image intact cells in a frozen, fully-hydrated state,

70   providing macromolecular-resolution (~4-6 nm) details about native cellular structures [13].

71   Here, we used ECT to visualize the structure of the archaellar basal body *in vivo* in

72   *Thermococcus kodakaraensis* cells. *T. kodakaraensis* (originally designated *Pyrococcus* sp.

73   strain KOD1 and later identified as belonging to the *Thermococcus* genus [14]; also known as *T.*

74   *kodakarensis*) is one of the best-studied archaeal species. It was isolated from a Japanese

75   solfatara in 1994 [15], and has proven readily amenable to genetics (well-developed gene

76   manipulation techniques exploit its natural competence [16]) and the isolation of thermostable

77   enzymes (e.g. high-fidelity DNA polymerase for PCR [17]). In addition to revealing the overall

78   structure of the archaellar basal body *in vivo*, we discovered a novel cytoplasmic conical

79   structure in *T. kodakaraensis* associated with archaellar motility and potentially other polar

80   organizing activities.

81

82   **RESULTS**

83   We imaged *T. kodakaraensis* cells by ECT in a native, frozen-hydrated state. Many cells

84   appeared to be lysed prior to plunge-freezing for ECT, but out of 18 apparently intact cells, we

85   observed a lophotrichous bundle of archaella in 13. Each bundle contained between four and 14

86   archaella. Due to the relatively large size of *T. kodakaraensis* cells (cells are irregular cocci ~1.5

87   μm in diameter), only a portion of the cell was visible in the limited field of view of our high-

88   magnification cryotomograms. We therefore think it likely that in the remaining five cells, the

89   archaellar bundle was present but not located in the portion of the cell imaged. In addition, we

90   observed well-preserved archaellar bundles in eight apparently lysed cells.

91

92   We consistently observed a prominent conical structure associated with the archaellar bundle in

93   the cytoplasm (Figure 1). We never observed archaella unassociated with a cone, or vice versa.

94   The conical structure showed a consistent morphology and localization inside the cell: closely

95   associated with, but not touching, the cytoplasmic membrane at its narrow end and expanding a

96   variable length to a wide base, which varied from 220 to 525 nm in diameter. The central axis

97   was perpendicular to the membrane, as seen in cross-sectional side views (Figure 1A-C,

98   additional examples in Figure EV1). The edges, seen in cross-section, frequently exhibited

99   periodic densities suggestive of individual protein subunits (Figure 1C), with a thickness of 3-4

100   nm. We observed that while cones in different cells had different heights, the opposite edges

101   within each cone were always symmetric (of similar lengths). Tomographic slices capturing the

102   central axis of the conical structure in side view showed an angle of $109 \pm 6^{\circ}$ (mean $\pm$ s.d., n=5)

103   between opposite edges. The structures were not complete cones but rather conical frusta: they

104   did not taper fully to a point, but exhibited a blunt tip. In top-views, we observed a ring situated

105   in the throat of the frustum, just below the tip (Figure 1D,E). These rings comprised 19 subunits

106   (Figure 1D inset), each again 3-4 nm thick, with an overall ring diameter of 31 ± 2 nm (mean $\pm$

107   s.d., n=10). The position of the ring in the conical frustum was clearest in tomograms of lysed

108   cells, which were thinner and contained less cytoplasmic material (Figure 1F-H). Even in such

109   tomograms, however, we could not visualize a well-defined connection between the two portions

110   of the structure, so it is unclear if and how the components are connected.

111

112   Conical structures were surrounded by an ~30-45 nm wide ribosome-excluding zone (REZ)

113   (Figure 1E, Figure 2). In nearly all cells, both intact and lysed, we observed filament bundles

114   near or associated with this REZ (Figure 2B-E). The bundles were more extensive in lysed cells.

115   Each filament was ~12 nm wide and made up of a series of disk-like densities spaced ~7 nm

116   apart. Chemosensory arrays were also consistently observed near the conical structures (Figure

117   2A,B). In one cell, we observed two attached conical structures, each associated with archaella

118   and each approximately 250 nm in diameter at its base (Figure EV2).

119

120   To characterize the interaction between the archaellar bundle and the conical structure, we

121   measured the distance from the base of each archaellum in the membrane to the cone. The

122   structure of the cone means that the distance between it and the membrane varies – shortest at the

123   tip of the cone and longest at the base. Since archaella were located at various radial positions

124   along the cone, we expected their distance to vary similarly. Interestingly, however, we

125   measured a much more consistent distance of 44 ± 5 nm (mean $\pm$ s.d., n=29) from the cone to the

126   base of each archaellum in the membrane (Figure 3). Consistent with this, we observed a variety

127   of orientations of archaella in the cell envelope, frequently not perpendicular to the S-layer,

128   allowing the conserved distance to the cone (Figure 3, Figure EV3). In a few cases, we observed

129   continuous densities connecting the archaella and the cone (Figure EV3E).

130

131   To determine the structure of the archaellar basal body, we calculated a subtomogram average

132   (Figure 4). 30 particles were used, and an axial two-fold symmetry was applied. The resulting

133   average revealed several layers of density extending into the cytoplasm. Immediately adjacent to

134   the membrane-embedded density was a ring-like structure (L1 in Figure 4A). Below the ring

135   was a disk of similar diameter (L2), followed by a larger diameter component (L3) and finally, at

136   a greater distance, a less well-defined density. This density was 44 nm away from the

137   membrane, corresponding to the cone. Consistent with our observation that archaella exhibited

138   various orientations with respect to the S-layer, we did not observe a strong density

139   corresponding to the S-layer in the average. As seen in individual particles, the component in L3

140   does not appear to be a ring, but rather comprises distinct legs, seen on one or both sides, that

141   appear symmetric in the average (Figure EV4). Similarly, the density of the cone is more

142   prominent in individual particles; different angles of the structure in different particles wash out

143   in the average (Figure EV4).

144

145   **DISCUSSION**

146   ***Structure of the basal body of the* T. kodakaraensis *archaellum***

147   Here we describe the structure of the archaellar motor in *T. kodakaraensis* (Figure 4). We think

148   it is almost certain that density L1 in the *T. kodakaraensis* basal body corresponds to the ATPase,

149   FlaI, since its size and shape match those of the homologous ATPases in the T4P and all three

150   interact directly with integral membrane proteins. More specifically, FlaI shares domain

151 homology with the assembly/disassembly ATPases, PilB and PilT, of the bacterial T4P. The size

152 of L1 is comparable to that of the PilB/PilT ring in the bacterial T4P (Figure 4B), consistent with

153 their conserved hexameric oligomerization [7] and similar sizes of the protein monomers (540

154 amino acids for FlaI and 566 for PilB). FlaI is predicted to interact directly with the polytopic

155 integral membrane protein FlaJ [6, 7]. FlaJ shares sequence homology with the ATPase-

156 interacting inner membrane protein PilC of the T4P [18]. The relative locations of these

157 components are therefore predicted to be the same in the basal bodies of the archaellum and the

158 bacterial T4P (Figure 4) [8, 19], and the size and shape and position of density L1 seen here

159 support that expectation, and the corollary that these two systems likely share a similar assembly

160 mechanism.

161

162 The identities of the proteins making up L2, L3, and the cone remain unclear. In the T4P, no

163 structures were observed in the cytoplasm below the ATPase [8, 19, 20]. In Crenarchaeota, only

164 one accessory component is not membrane-bound (FlaH). In Euryarchaeota like *T.*

165 *kodakaraensis*, however, additional soluble proteins, FlaC/D/E, are thought to be components of

166 the archaellum that receive switching signals from the chemotaxis machinery [21]. All of these

167 proteins, and potentially others, are candidates for the densities we observed. It will therefore be

168 of great interest to obtain a structure of the crenarchaeal basal body, which lacks FlaC/D/E, for

169 comparison, and/or to dissect the *T. kodakaraensis* basal body structure through analysis of

170 deletion mutants.

171

172 ***Conical structures anchor* T. kodakaraensis *archaella***

173   We observed that *T. kodakaraensis* archaella associate with a large conical structure in the

174   cytoplasm. In a few cases, we observed direct connections between archaella and cone. The fact

175   that we did not see such a connection for every archaellum may simply reflect variations in

176   image clarity and orientation of the structures between cells in different cryotomograms. The

177   conserved distance from the cone to the archaellar basal body in the membrane suggests a rigid

178   interaction. It is an interesting question how archaella are attached to the cone. We did not

179   observe strong densities connecting L3 and the cone in the averaged basal body structure, but in

180   individual particles we observed heterogeneity. Also, the resolution of the average may be too

181   low to detect such connections. If, for example, the links are thin (such as coiled-coils), they

182   would not be resolved; similar coiled-coil linkages in the bacterial flagellar motor between FlaH

183   and the C-ring were not resolved even in higher-resolution subtomogram averages [22].

184

185   We propose that the *T. kodakaraensis* cone anchors the archaellar basal body in part to provide

186   leverage for rotation. In the bacterial T4P, the ATPase is clamped by extensive interactions up

187   through the cell envelope that anchor it to the peptidoglycan cell wall [8] (Figure 5). Signals

188   governing disassembly are thought to be processed by sensory elements in the periplasm [8]. In

189   the absence of a peptidoglycan cell wall and outer membrane, the *T. kodakaraensis* archaellum

190   appears to turn the system upside down, with components stacking nearly 50 nm into the

191   cytoplasm to anchor onto a large cone (Figure 5). Signals governing rotation and direction are

192   likely integrated by sensory components in the cytoplasm.

193

194   Similar leveraging structures may exist in other Archaea. More than twenty years ago, it was

195   observed that when archaella are dissociated from lophotrichous *H. salinarum* cells by detergent,

196   the bundles remain intact, connected to a large (~500 nm diameter) structure [11]. A similar

197   structure was also observed below the cell membrane in cell ghosts [12]. More recently, a

198   spherical structure was observed anchoring Iho670 fibers, T4P-like filaments in *Ignicoccus*

199   *hospitalis*. This structure is thought to be located in the cytoplasm of the cell and contains a

200   central ring of similar dimensions to the one observed here [23]. It is possible that either or both

201   of these structures are related to the *T. kodakaraensis* cone. Large cytoplasmic structures have

202   not been described in other motile archaeal species to date, however, so it will be interesting to

203   determine how archaella may be anchored in those systems.

204

205   It will also be of great interest to identify the proteins that form the *T. kodakaraensis* cone and

206   associated ring. These subunits must be capable of interacting both circumferentially around the

207   cone as well as radially with subunits making up the next (larger or smaller diameter) ring.

208   While it is possible that the conical structure is an assembly of stacked rings, we think it more

209   likely that the subunits assemble into a filament spiral, similar to what has been proposed for

210   ESCRT-III polymers [24, 25]. Interestingly, an architecturally similar spiral has been observed

211   in the basal body of the bacterial flagellar motor: in *Wolinella succinogenes*, an Archimedian

212   spiral forms a bushing for the motor in the periplasm, allowing the flagellum to rotate in the cell

213   wall. This spiral is formed by protein subunits interacting both circumferentially and laterally

214   through nonspecific interactions [26]. While that spiral takes the form of a disk, similar protein

215   interactions may give rise to a cone in *T. kodakaraensis*.

216

217   **T. kodakaraensis *cones are potential polar organizing structures***

10

218   In addition to a potential role in rigidly anchoring the basal body of the archaellum, the *T.*

219   *kodakaraensis* cone may function to gather the archaellar bundle to maximize efficiency, either

220   by concentrating molecules for assembly or signaling, or by concentrating force at one point on

221   the coccoid cell for directional swimming. The cone's structure may also help distribute the

222   force from archaellar rotation to the larger bulk of the cell's contents. This might be more

223   efficient in a pushing than a pulling mode; swimming speed in another euryarchaeon, *H.*

224   *salinarum*, was found to be approximately twice as fast when the archaella push as when they

225   pull the cell body [5]. The structurally-similar spiral basal disk in the bacterial flagellar motor of

226   *W. succinogenes* was suggested to play a role in dispersing lateral forces created by flagellar

227   rotation [26].

228

229   Our results suggest a further role for the cone in breaking the symmetry of the coccoid cell. In

230   many rod-shaped bacterial cells, proteins and other macromolecules are specifically localized to

231   the cell pole for various purposes ranging from cell motility and adhesion to differentiation and

232   division [27]. One well-studied example of this polar organization occurs in *Caulobacter*

233   *crescentus*, where the oligomeric protein PopZ defines an asymmetric pole, localizing many

234   cytoplasmic proteins and tethering the chromosomal centromere to facilitate division [28-30]. In

235   *Vibrio cholerae*, the HubP protein organizes the polar localization of the chromosomal origin,

236   chemotaxis machinery, and flagella [31]. Perhaps the *T. kodakaraensis* cone similarly defines a

237   pole in the spherical cells, anchoring the chemotaxis and motility machinery. An intriguing

238   feature observed in our cryotomograms is the cone-associated REZ. In bacterial cells, such

239   REZs are commonly interpreted to be the nucleoid [32, 33]. Supporting this assignment, we

240   observed bundles of filaments (most extensive in lysed cells) associated with the REZ (Figure 2).

11

241 Such filaments are reminiscent of nucleoprotein filaments formed by various bacterial DNA-

242 binding proteins in stress conditions [34-36].

243

244 A spatial organizer analogous to PopZ may be especially important for a polyploid species like

245 *T. kodakaraensis* (chromosome copy number varies depending on growth phase, from 7 to 19

246 copies [37]). Fluorescence imaging suggests that the nucleoid is relatively compact in log phase

247 growth, and nucleoids appear to separate before the cells are deeply constricted [38]. Perhaps

248 the cones segregate attached structures, including the archaella and possibly chromosomes. This

249 function is consistent with the duplicated cone structure we observed in one cell (Figure EV2),

250 which could represent an intermediate after replication and prior to segregation, or may simply

251 represent an aberrant structure. Further studies imaging cells throughout the cell cycle could

252 shed light on whether, and how, cones function to coordinate archaellar and chromosomal

253 segregation.

254

255 Understanding the prevalence of this structure among Euryarchaeota and across different

256 archaeal kingdoms may illuminate its function. If it is restricted to lophotrichous species, it may

257 simply be an anchoring mechanism for the archaella in the absence of a peptidoglycan cell wall.

258 In that case, monotrichous or peritrichous species may exhibit a less extensive plate underneath

259 the basal bodies of individual archaella. If it is more widely found in coccoid, and/or highly

260 polyploid, cells, it may serve an added role in polar specification.

261

262 **METHODS**

263 ***Growth***

12

264   *Thermococcus kodakaraensis* strain KOD1 [JCM 12380] was grown anaerobically in MA-YT

265   medium supplemented with elemental sulfur as previously described [14, 39].

266

267   ***Electron cryotomography and image analysis***

268   Samples of cell cultures in growth media were mixed with bovine serum albumin-treated

269   colloidal gold fiducial markers (Sigma) and applied to Quantifoil R2/2 200 copper EM girds

270   (Quantifoil Micro Tools). After blotting excess liquid, grids were plunge-frozen in a mixture of

271   liquid ethane and propane [40], and subsequently kept at liquid nitrogen temperature. Images

272   were acquired using either an FEI Polara G2 or Titan Krios 300 keV transmission electron

273   microscope (FEI Company) equipped with a field emission gun, image corrector for lens

274   aberration, energy filter (Gatan), and K2 Summit direct electron detector (Gatan). Cumulative

275   electron dose was 160 e$^-$/Å^2 or less for each tilt-series. Tilt-series were acquired using UCSF

276   Tomography software [41]. Images were contrast transfer function corrected, aligned, and

277   reconstructed by weighted back projection with the IMOD software package [42]. SIRT

278   reconstructions were calculated with TOMO3D [43], subtomogram averages generated using

279   PEET [44], and segmentations generated with Amira software (FEI Company).

280

281   **ACCESSION CODES**

282   The subtomogram average of the *T. kodakaraensis* archaellar basal body was deposited into the

283   Electron Microscopy Data Bank (entry number EMD-8603).

284

285   **ACKNOWLEDGMENTS**

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388

389

390 **FIGURE LEGENDS**

391 **Figure 1.   Cytoplasmic conical structures in *Thermococcus kodakaraensis*. (A)** A

392 tomographic slice shows a side view of a conical structure (c) in the cytoplasm, rotated and

393 enlarged in **(B)**.   **(C)** A tomographic slice shows a side view of the cone in another cell,

394 highlighting the subunit texture along the edge of the cone (arrowheads). **(D, E)** Top views of a

395 cone at different heights show the inner ring (r; enlarged in inset to highlight 19-subunit

396 structure) and outer cone. **(F-H)** Sequential slices through a side view of a cone in a lysed cell

397 show the relative location of the ring in the cone. **(I, J)** Different views of a 3D segmentation of

398 the cone shown in **(A)**, embedded in a tomographic slice. s, S-layer; m, membrane; a, archaella;

399 rib, ribosomes; rez, ribosome-excluding zone. Scale bars 100 nm; segmentation not to scale.

400

401 **Figure EV1. Additional examples of conical structures in *T. kodakaraensis* cells. (A)** shows

402 sequential tomographic slices (1-3) at different heights through a side view of the cone.

16

403   Additional examples of cones in intact **(B-C)** and lysed **(D-E)** cells are shown below. s, S-layer;

404   m, membrane; c, conical structure; a, archaella; r, ring. Scale bars 100 nm.

405

406   **Figure 2. Cones are associated with chemosensory arrays, ribosome-excluding zones and**

407   **filament bundles.** Tomographic slices show side **(A)** and top **(B, C)** views of cones (c) in three

408   cells, highlighting associated chemosensory arrays (ca), ribosome-excluding zones (rez) and

409   filament bundles (f). For a slice-by-slice view through the tomogram shown in (A), see Movie

410   S1. **(D)** and **(E)** show different views of a 3D segmentation of the structures shown in **(C)**, with

411   the conical structure in blue and the filament bundle in red. s, S-layer; m, membrane; a,

412   archaellum; o, other filaments. Scale bar 100 nm; segmentation not to scale.

413

414   **Figure EV2. Double cone structure observed in *T. kodakaraensis*.** A tomographic slice

415   through a side view shows two associated conical structures (c1 and c2), both associated with

416   archaella (a). Scale bar 100 nm.

417

418   **Figure 3. Archaellum orientation with respect to the cell envelope. (A-D)** show tomographic

419   slices through side views of cones. White lines show the angle of the archaellum with respect to

420   the surface layer, and red dashed lines show the conserved distance from the archaellum at the

421   membrane to the cone. Schematic in **(E)** depicts the 44 nm distance from the cone to the basal

422   body in the membrane for archaella at different radial positions along the cone. Since different

423   radial positions on the cone are located at different distances from the membrane (shorter at the

424   tip and longer at the base), this results in a range of archaellar orientations in the cell envelope.

425   Scale bar 100 nm.

426

Figure EV3. Association of cones with the archaellar bundle. **(A)** and **(B)** show tomographic slices through two cells, highlighting the association between the cone and the archaella. **(C)** and **(D)** show 3D segmentations of the cells in **(A)** and **(B)**, respectively, with cones in blue and archaella in red, embedded in tomographic slices. **(E)** Tomographic slices of individual archaella show the varying orientations of archaella with respect to the cell envelope, as well as apparent connections to the cone. Scale bars 100 nm in (A) and (B), 50 nm in (E); segmentations not to scale.

434

Figure 4. Structure of the *T. kodakaraensis* archaellum. **(A)** A sub-tomogram average of the archaellum reveals structural features, including four layers of density in the cytoplasm (L1-L3, cone). CM, cytoplasmic membrane. The speculated identity of densities in the archaellum is proposed: archaellum fiber = FlaA/B flagellins; integral membrane density = FlaJ; L1 = FlaI; L2/L3/cone = FlaH/FlaC/D/E. **(B)** For comparison, a subtomogram average of the type IVa pilus machine from *Myxococcus xanthus* is shown (adapted with permission from [8]). Arrows indicate components with recognized homology. OM, outer membrane; IM, inner membrane. Scale bar 10 nm.

443

Figure EV4. Individual particles from the subtomogram average show heterogeneity in the L3 density and angle of cone density. The L3 density appears as either two dots of similar (first two panels) or different intensity (third panel), a single dot (fourth panel), or a dot and an extended line (fifth panel). Scale bar 10 nm.

448

449 **Figure 5. Schematic comparing organization of the related archaellum and type IVa pilus**

450 **basal bodies.** In the bacterial T4P **(right)**, an integrated system of components spanning the

451 outer and inner membranes (OM, IM) uses the peptidoglycan cell wall (PG) to brace the ATPase,

452 allowing rotation of PilC (orange) in the membrane to assemble the pilus fiber. In the *T.*

453 *kodakaraensis* archaellum **(left)**, our results suggest that an integrated system of components

454 extends from the single membrane (CM) inward to a large conical structure in the cytoplasm to

455 similarly brace the ATPase. Sensory components (purple) are proposed to be located in the

456 periplasm for the T4P and the cytoplasm for the archaellum.

Figure 1
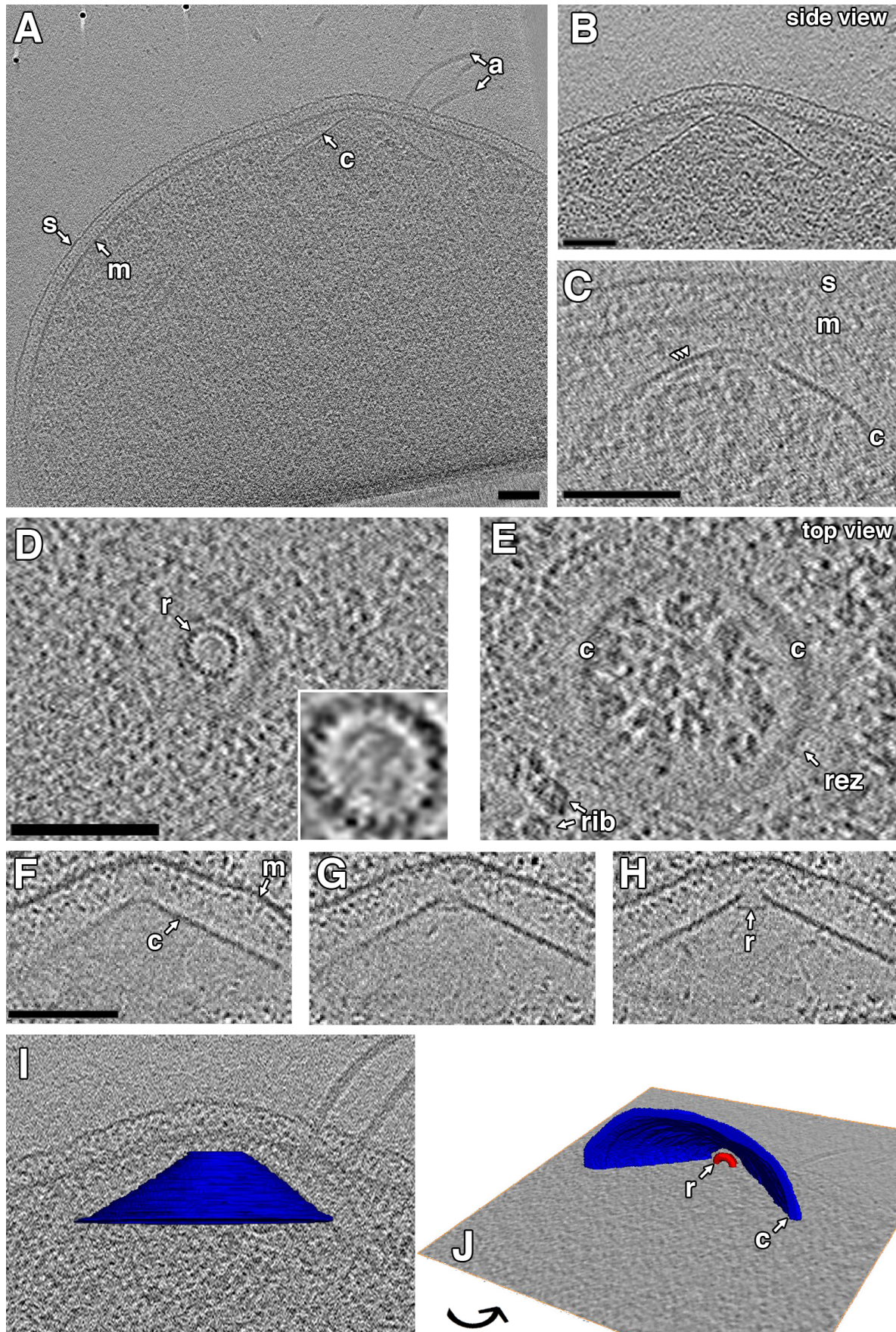
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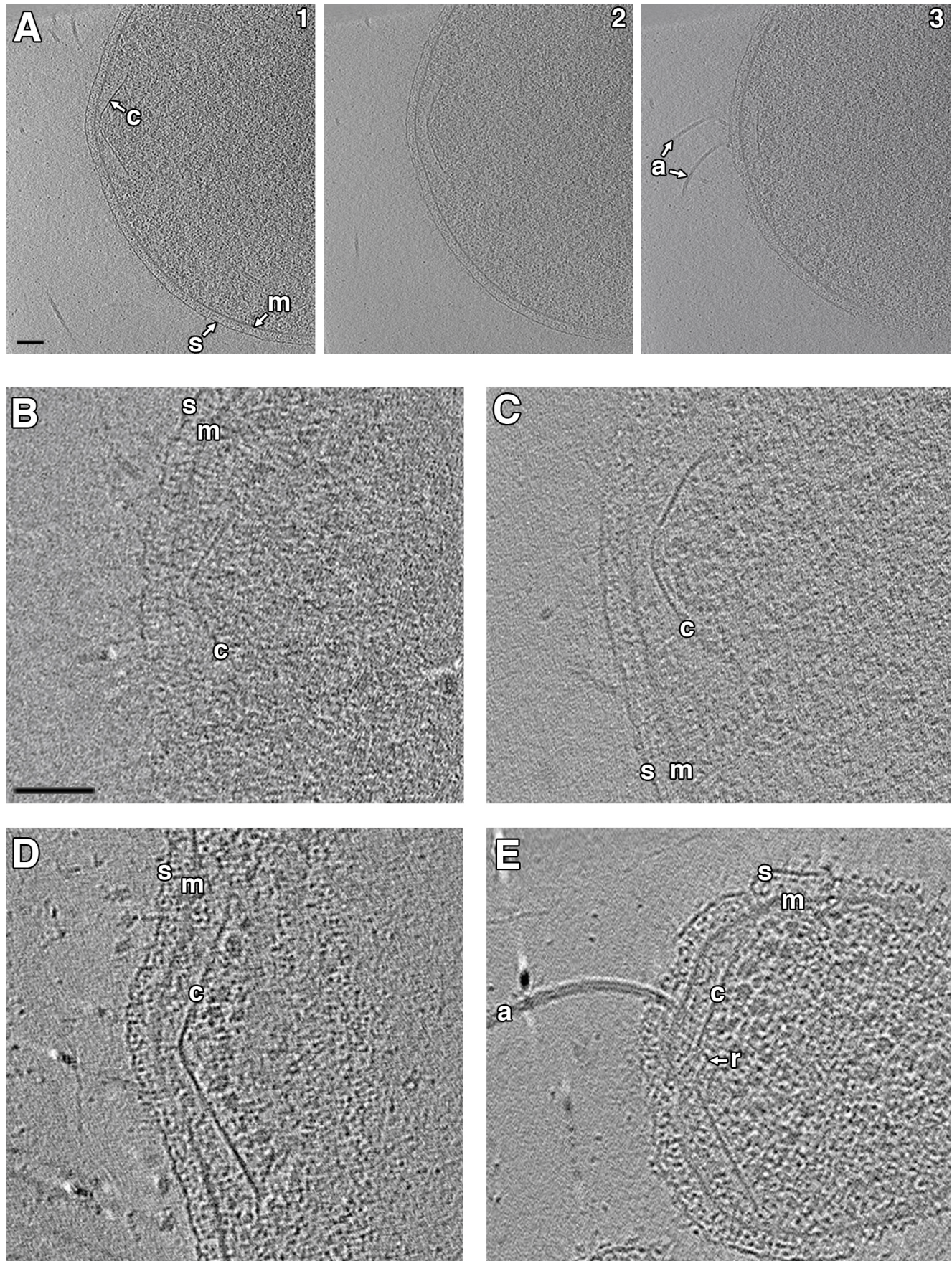
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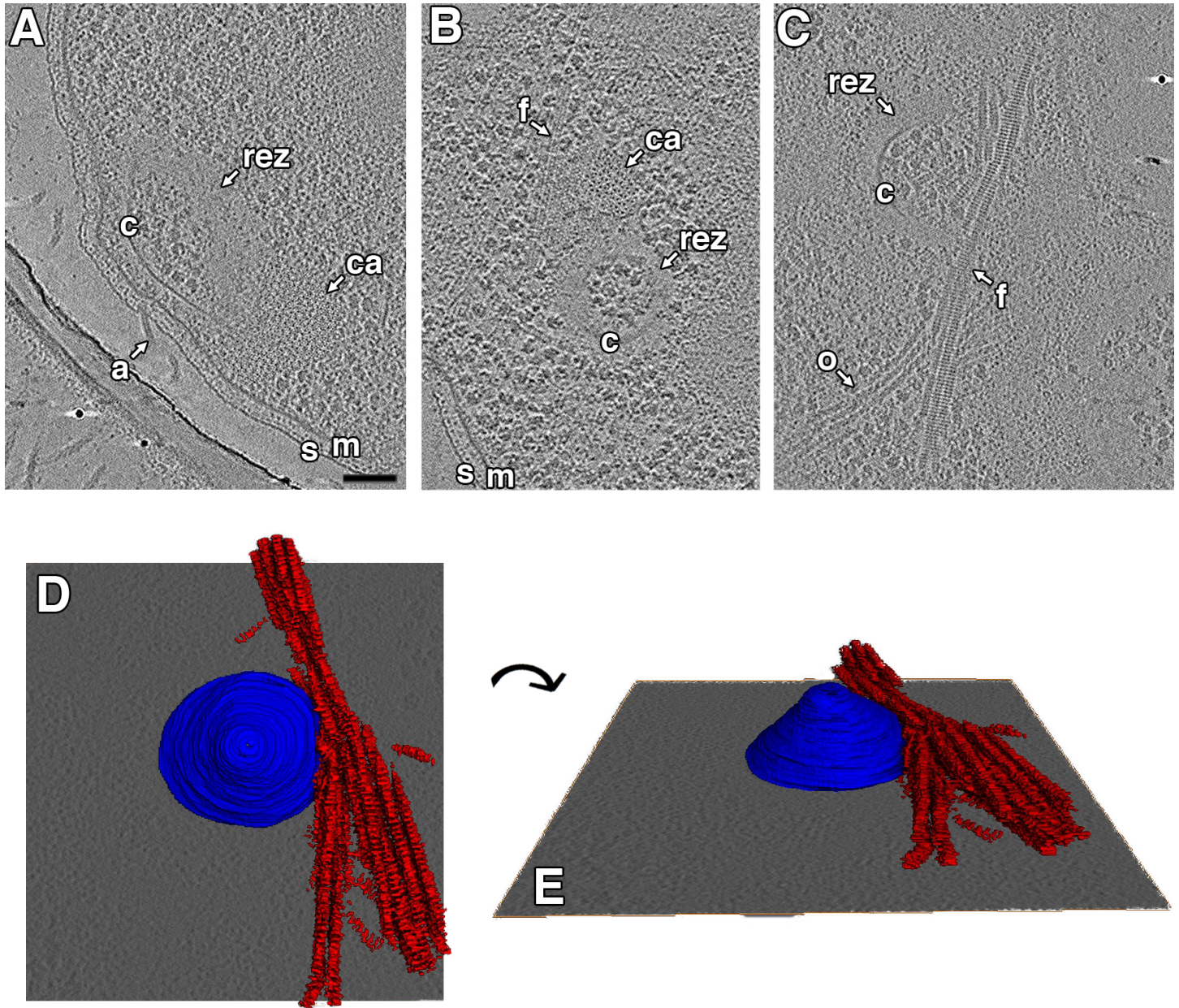
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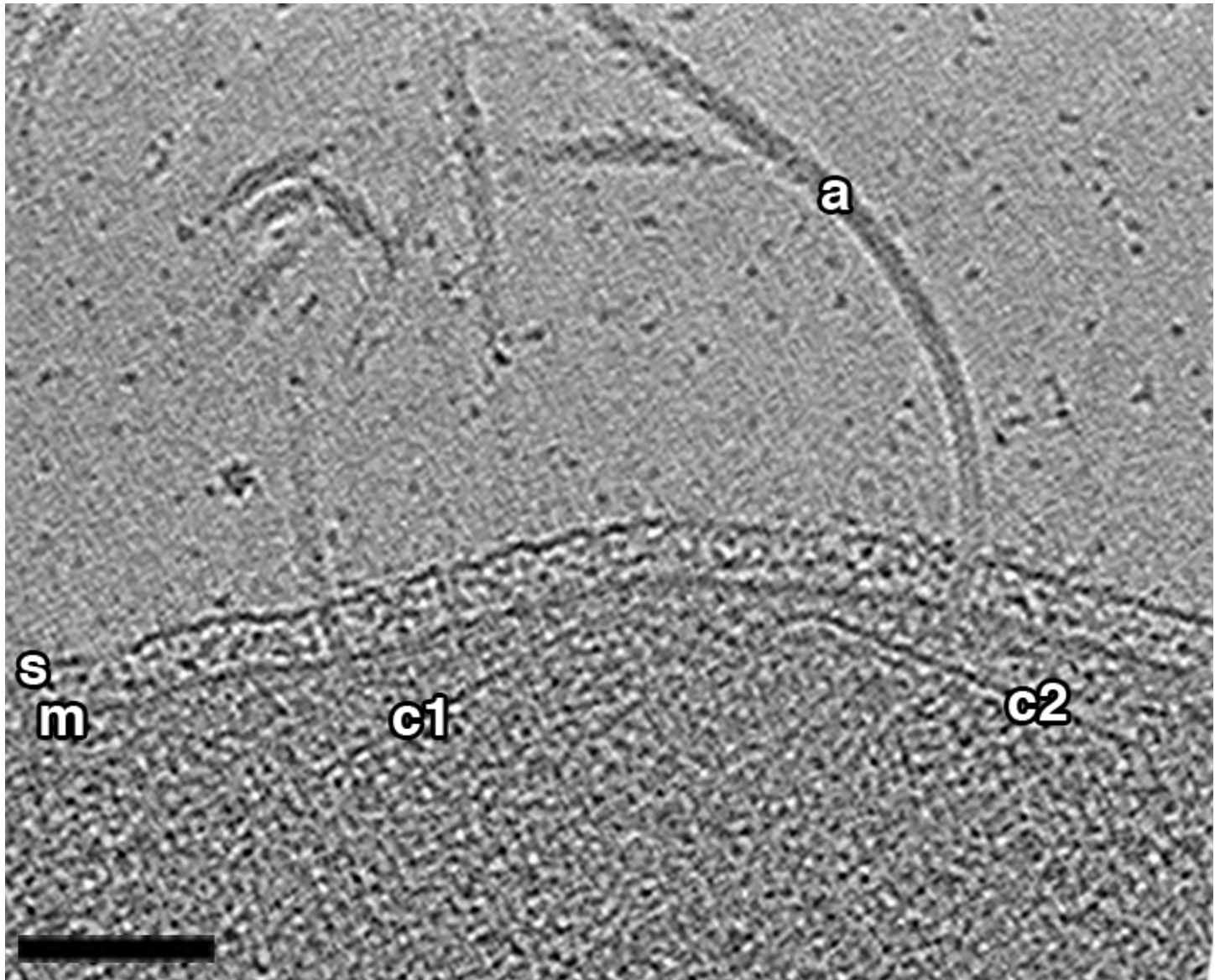
Figure 3

Figure EV3

Figure 4

Archaellum

Type IVa Pilus

Figure EV4

Figure 5