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2 **CesA protein is included in the terminal complex of**
3 ***Acetobacter***

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16

17 **Running title: CesA in bacterial terminal complex**

18

19 **Abstract**

20 Cellulose is a major biopolymer on the earth that is derived from cellulose synthase in the cell
21 membrane of living organisms. Cellulose synthase is a hetero-subunit complex composed of
22 several different protein subunits, and is visualized as a supermolecular complex called a “terminal
23 complex” by electron microscopy. Such supermolecular organization of an enzyme complex is
24 believed to be important for the fiber formation or crystallization of cellulose microfibrils in
25 cellulose biosynthesis. In the case of the cellulose-producing bacterium *Acetobacter*, it is
26 hypothesized that the enzyme complex includes at least six subunits given its genetic constitution.
27 However, to date, only three of these molecules have been experimentally confirmed as the
28 subunits included in the cellulose synthase complex: CesB, CesD, and ccp2. In this study, we used
29 fluorescence immuno-microscopy to show that CesA protein, the catalytic subunit, is included in
30 the terminal complex of *Acetobacter*. Furthermore we discuss the obtained microscopic data for
31 improving our understanding of the molecular organization of the bacterial cellulose synthase
32 complex.

33

34 *Keywords: CesA; cellulose synthase complex; terminal complex; immunolabeling*

35

36 Abbreviations: PFA, paraformaldehyde; EDTA, ethylenediamine tetra-acetic acid; SDS, sodium

37 dodecyl sulfate; PAGE, poly-acrylamide gel electrophoresis; PVDF, poly-vinylidene difluoride;

38 RT, room temperature; BSA, bovine serum albumin

39

40

41 INTRODUCTION

42 Cellulose is one of the major biopolymers on Earth. Despite its mass
43 production on Earth by plants and other living organisms, destructive
44 accumulation of cellulose on Earth has never been identified, although this has
45 been observed for some synthetic plastics. This indicates that the cycle of
46 synthesis and degradation shows a good balance for maintaining the cellulose
47 content on Earth, which is actually a striking feature of cellulose, suggesting it as
48 a promising material for sustainable human life.

49 All of the cellulose on Earth is produced by living organisms, and
50 originates from the cellulose synthase complex (CSC) in living cells. CSC is a
51 hetero-subunit complex in the cell membrane (Somerville 2006). Electron
52 microscopy with the freeze-fracture technique has been used to visualize CSC as a
53 terminal complex (TC), which is a characteristic array of particles found on the
54 cell membrane at the terminal of cellulose microfibrils (Kimura et al. 1999,
55 Kimura et al. 2001). Since the 1970s, TCs have been found in many of the
56 cellulose-producing organisms (a tunicate, algae, plants, and a bacterium),
57 although the arrangement of the TC particles showed a variety of patterns (Itoh et
58 al. 2007): linear type (for a bacterium, tunicate, and algae producing a giant
59 microfibril) and rosette-type (for higher plants and an alga of the order
60 *Zygnematale*). Regardless of the specific pattern, such a regular array of a
61 cellulose-synthesizing enzyme is considered to be important for cellulose
62 microfibril formation by assembling many cellulose chains into a cellulose
63 microfibril.

64 To date, several studies have identified the molecules included in the CSC
65 based on biochemical and molecular/cell biological analyses. For *Acetobacter*

66 (recently renamed for some strains as *Gluconacetobacter*, *Komagataeibacter*, and
67 so on), a popular model for studying cellulose biosynthesis, six subunits are
68 proposed to be included in the CSC given the constitution of the genes related to
69 cellulose synthesis (McNamara et al. 2015): GH-8 (also known as carboxymethyl
70 cellulase (CMC)) (Standal et al. 1994), cellulose complementing factor (ccp)
71 (Standal et al. 1994), CesA, CesB, CesC, and CesD (Saxena et al. 1994, Wong et
72 al. 1990). Among these, CesA is the catalytic subunit harboring the
73 glycosyltransferase domain of the GT-2 family in the cytosolic part (Morgan et al.
74 2013), and CesA and CesB are the minimally required subunits for cellulose-
75 synthesizing activity (Omadjela et al. 2013, Saxena et al. 1994, Wong et al. 1990).
76 CesD is considered to control the crystallization process of cellulose microfibrils
77 (Hu et al. 2010, Saxena et al. 1994), and four chains are found inside the ring
78 structure formed by the octamer of CesD protein (Hu et al. 2010). The functions
79 of the other subunits have not yet been clarified despite their clear relevance to
80 cellulose-synthesizing activity, as experimentally reported for GH-8 (Kawano et
81 al. 2002, Kawano et al. 2008, Nakai et al. 2013), ccp (Sunagawa et al. 2013),
82 CesC (Saxena et al. 1994), and CesD (Hu et al. 2010, Saxena et al. 1994,
83 Sunagawa et al. 2013).

84 The SDS-freeze replica labeling (SDS-FRL) method (Fujimoto 1995), an
85 immuno-labeling technique combined with the freeze-replica technique, is a direct
86 method to localize a specific protein in the TCs, and its application has shown that
87 CesB protein is found in the linear TC of *Acetobacter* (Kimura et al. 2001). In
88 addition, fluorescence microscopy could also be used to successfully visualize the
89 linear localization of GFP-fused CesD and ccp protein in the cells of *Acetobacter*
90 (Sunagawa et al. 2013), which confirmed that these two proteins are also included

91 in the linear TC of *Acetobacter*. These microscopic studies showed that CesB,
92 CesD, and ccp proteins form a part of the TC or CSC of *Acetobacter*. However,
93 no report has provided concrete evidence that CesA protein is included in the
94 linear TC of *Acetobacter*, although this is the widely accepted hypothesis given
95 that CesA is the core subunit of the CSC. In the present study, CesA protein was
96 successfully visualized as a linear array in the cell, and experimental evidence was
97 obtained to show that CesA protein is included in the TC of *Acetobacter*.

98

99 MATERIALS AND METHODS

100 *Chemicals*

101 Peptone and yeast extract for the culture medium were purchased from
102 Becton, Dickinson and Company Inc. (USA). Paraformaldehyde (PFA) for cell
103 fixation was paraformaldehyde EM from TAAB Inc. (UK). Poly-L-lysine
104 solution, lysozyme and BSA were purchased from Sigma-Aldrich Inc. The other
105 chemicals used in this study were purchased from Wako Pure Chemicals Inc.
106 (Japan) unless described.

107 *Cell culture*

108 Three different strains of *Acetobacter* were used in this study:
109 ATCC53264, ATCC53524, and JCM9730. For convenience, the former name
110 *Acetobacter* is used for these strains herein, although these are actually considered
111 to be different species based on the current taxonomy (*Gluconacetobacter xylinus*
112 for ATCC53264, *Komagataeibacter xylinus* for ATCC53524, and
113 *Komagataeibacter sucrofermentans* for JCM9730). The two ATCC strains were
114 provided by the American Type Culture Collection and the last strain was
115 obtained from the Japan Collection Microorganisms at BRC-RIKEN, Japan. Each

116 strain was grown in Schramm-Hestrin medium (Schramm and Hestrin 1954) at
117 30°C in a static condition for 3 to 5 days, until a sufficient amount of cellulose
118 was produced. The cells were detached from the cellulose pellicle by shaking the
119 culture medium and pressing the pellicle with a spatula, and then filtered by 37- or
120 50-µm pore-sized nylon mesh. The filtrated cells were collected by centrifugation
121 (2000×g for 10 min at RT).

122 *Antibody evaluation by western blot analysis*

123 Western blot analysis was performed to evaluate whether the primary
124 antibody has cross-reactivity with the proteins in the strains ATCC53264 and
125 JCM9730, as well as strain ATCC53524 for which cross-reactivity has already
126 been shown (Hashimoto et al. 2011). The primary antibodies used in this study
127 were the same as those used in our previous studies (Hashimoto et al. 2011, Imai
128 et al. 2014, Sun et al. 2016). In brief, each antibody is a polyclonal antibody
129 against the synthetic peptide corresponding to a part of CesA (carboxyl terminal),
130 CesB (a loop in the CBD2 domain), CesC (the part between the last six-TPR
131 repeat and the carboxyl terminal region), and the CesD subunit (the loop between
132 the β3 and β4 strands). The antigen peptide sequence for each of the proteins was
133 designed from the sequence of the strain BPR2001 (Nakai et al. 1998) or
134 JCM9730 (GenBank: AB010645) as reported in our previous study (Hashimoto et
135 al. 2011). As shown in Table 1, high sequence similarity was found for each of the
136 proteins between this strain and ATCC53264 or 1306-03 (GenBank: AAA21884 –
137 21887), and probably ATCC53524 or 1306-21, which is a derivative strain of
138 ATCC53264 (Wong et al. 1990).

139

140 **Table 1.** Amino acid sequences of the peptide antigens for the antibodies used in this study
 141 (JCM9730), together with the sequence of the corresponding part for ATCC53264. The non-
 142 identical residues are indicated with shadowing.

Protein and strain		Amino acid sequence															
CesA	JCM9730 (antigen)	S	G	Q	T	Q	E	G	K	I	S	R	A	A	S		
	ATCC53264	S	G	Q	T	Q	E	G	K	I	S	R	A	A	S		
CesB	JCM9730 (antigen)	S	P	D	L	Y	T	W	R	D	R	P	N	K			
	ATCC53264	S	P	D	L	Y	T	W	R	D	R	P	Y	K			
CesC	JCM9730 (antigen)	P	S	I	D	G	G	L	G	F	R	S	R	S	G	E	H
	ATCC53264	P	S	I	D	G	G	L	G	F	R	S	R	S	G	E	H
CesD	JCM9730 (antigen)	T	R	D	I	D	A	E	D	L	N	S					
	ATCC53264	T	R	D	I	D	A	E	D	L	N	S					

143

144 The centrifuged cells described above were resuspended in a buffer of 10

145 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.02% NaN₃, 50 µg/mL chloramphenicol.

146 Then, the cell suspension was mixed with the SDS-PAGE sample buffer. After

147 incubating at 4°C for overnight, the sample was analyzed with a precast gel with a

148 gradient of 5-20% acrylamide (SuperSep Ace, Wako Pure Chemicals Industries

149 Ltd., Japan). The band pattern was transferred from the gel to a PVDF membrane

150 (Immobilon-P, Millipore Inc.), and then the membrane was incubated with each of

151 the primary antibodies against CesA, CesB, CesC, and CesD protein. Finally, the

152 protein band was visualized on the PVDF membrane by a chemical luminescence

153 method with ECL select (GE Healthcare Inc.) and recorded by a CCD camera
154 (EZ-capture, ATTO Inc., Japan).

155 *Preparation of the cells for immunolabeling*

156 The centrifuged cells were resuspended in CBS (citrate buffered saline:
157 50 mM sodium citrate buffer (pH 5.0), 136 mM NaCl, 2.7 mM KCl) and then
158 incubated in 2% PFA in CBS at 4°C overnight to chemically fix the cells. Then,
159 the gently centrifuged cells (1000×g for 10 min at RT) were resuspended in PBS
160 (phosphate buffered saline: 10 mM phosphate buffer (pH7.4), 136 mM NaCl, 2.7
161 mM KCl) with 0.1 M glycine for quenching the PFA. The cell suspension was
162 dropped on the glass coverslip, which was made to be hydrophilic in advance by
163 dipping in 1 mg/mL poly-L-lysine solution at RT for 30 min. The coverslip
164 carrying the cells was processed with the following procedures of lysozyme
165 treatment and permeabilizing treatment, prior to the antibody treatment.

166 The cells on the coverslip were treated with 1 mg/mL lysozyme in TE
167 buffer (100 mM Tris-HCl (pH 6.7), 5 mM EDTA) at 37°C for 1 h. After four
168 repeated washes with PBS, the cells were permeabilized with 1% IGEPAL CA-
169 630 (MP Biomedicals LLC; equivalent to a detergent Nonidet P-40) in PBS at
170 30°C for 30 min. The cells were then washed four times with PBS for 5 min each
171 time. Some of these treatments were skipped to explore the subunit localization in
172 the cell.

173 *Immunolabeling of the cells*

174 Prior to the antibody treatment, the cells on the coverslip were incubated in
175 1% BSA and 1% Blocking Reagent (Roche Inc.) in PBS at RT for 1 h for
176 blocking. Then, the cells were treated with the primary antibody solution, which
177 was diluted 500-fold in the blocking buffer, at 4°C for overnight with gentle

178 shaking. After four washes with PBS every 5 min, the cells were treated with 5
179 $\mu\text{g/mL}$ of the fluorophore-conjugated anti-rabbit IgG (Alexa fluor 488, Thermo
180 Fisher Scientific Inc.) in the blocking buffer at RT for 2 h in the dark. The cells on
181 the coverslip were washed with PBS four times every 5 min. The coverslip was
182 taken out to wash the side without the cells in pure water, and then placed on the
183 slide glass to seal the cells in the anti-fading reagent (SlowFade, Invitrogen Inc.).
184 Control experiments were done for the fixed *Acetobacter* cells by using either no
185 primary antibody or fluorophore-conjugated anti-mouse IgG (Alexa fluor 488,
186 Thermo Fisher Scientific Inc.) for the secondary antibody.

187 The cells on the slide glass were observed on an IX71 microscope
188 (Olympus Inc., Japan) with an oil immersion lens. Epi-fluorescence mode with a
189 mercury lamp and the filter set FITC-2024B (Semrock Inc., USA) was used for
190 recording the fluorescence image by a CCD camera (DP73, Olympus Inc., Japan).
191 The same region of interest was also recorded with the phase-contrast mode for
192 subsequent merging with the fluorescence image.

193 *SDS-freeze replica labeling*

194 The cells collected from the pellicle as described above were quickly
195 frozen on the gold sample career by dipping into liquid ethane at -175°C with a
196 Leichert KF-80 system (Leica Inc.). The freeze-fracture replica of these cells
197 without chemical fixation was prepared using a BAF-400D system (Balzers Inc.).
198 The fracture was performed at -113°C , and then platinum/carbon was evaporated
199 on the fractured surface at an angle of 45° followed by rotary carbon coating to
200 support the platinum replica. The prepared replica was treated in the lysozyme
201 solution (1 mg/mL lysozyme in 25 mM Tris-HCl (pH 8.0), 10 mM EDTA) for 4 h
202 at RT, and subsequently in the lysis solution (2.5% SDS, 10 mM Tris-HCl (pH

203 8.0)) for 2 h at RT. After washing three times with PBS, the replica was then
204 incubated in the blocking solution (1% BSA in PBS) for 30 min at RT, and then
205 treated with the primary antibody diluted in the blocking buffer overnight at 4°C.
206 The replica was then washed in PBS with 0.05% Tween-20 (PBST) and treated
207 with the secondary antibody (anti-rabbit IgG conjugated with 15-nm colloidal
208 gold, British BioCell International, UK) for 1.5 h at RT. Finally, the replica was
209 treated with 0.5% glutaraldehyde in PBS for 15 min at RT, and then transferred on
210 the carbon-coated copper grid after washing with water.

211 The replica on the grid was observed by a JEM-2000EXII (Jeol Inc.,
212 Japan) electron microscope and the images were recorded with photo-emulsion
213 (FG film, FujiFilm Inc., Japan), which was developed by Korectol (FujiFilm Inc.,
214 Japan) for 4 min at 20°C.

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219 RESULTS

220 *CesA is present in the linear array in the bacterial cells*

221 The results of the western blot analysis with the antibodies used in this
222 study are shown in Figure 1. These antibodies basically showed cross-reactivity to
223 the proteins extracted from *Acetobacter* in the three different strains used in this
224 study (ATCC 53524, ATCC53264, and JCM9730). We then used these antibodies
225 for immunolabeling fluorescence microscopy, as shown in Figure 2.

226

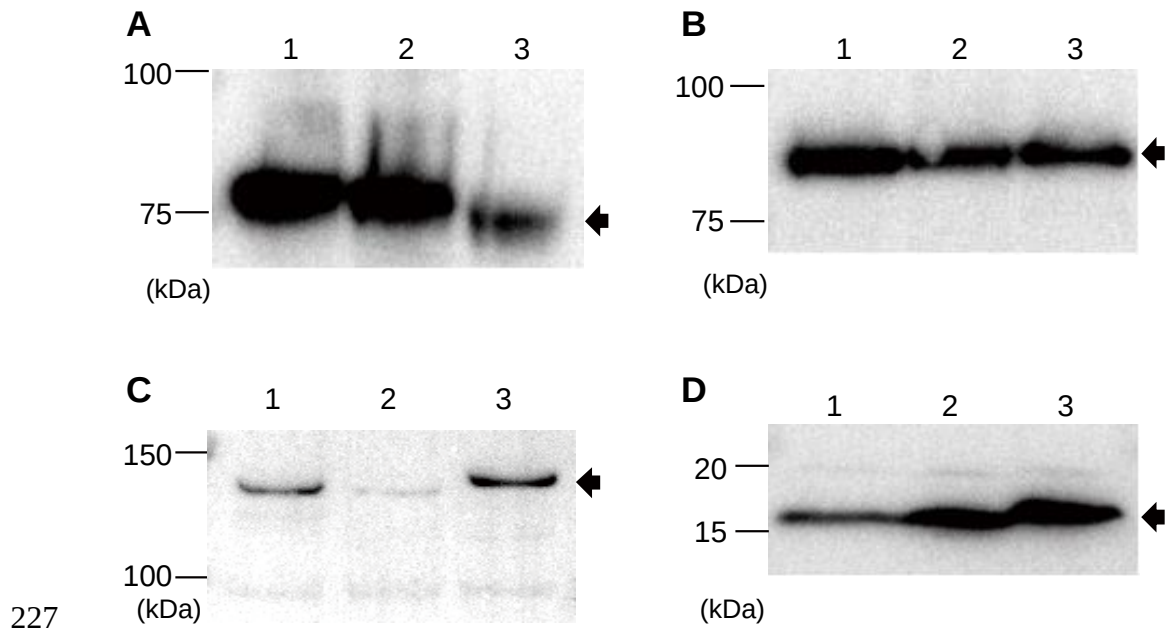
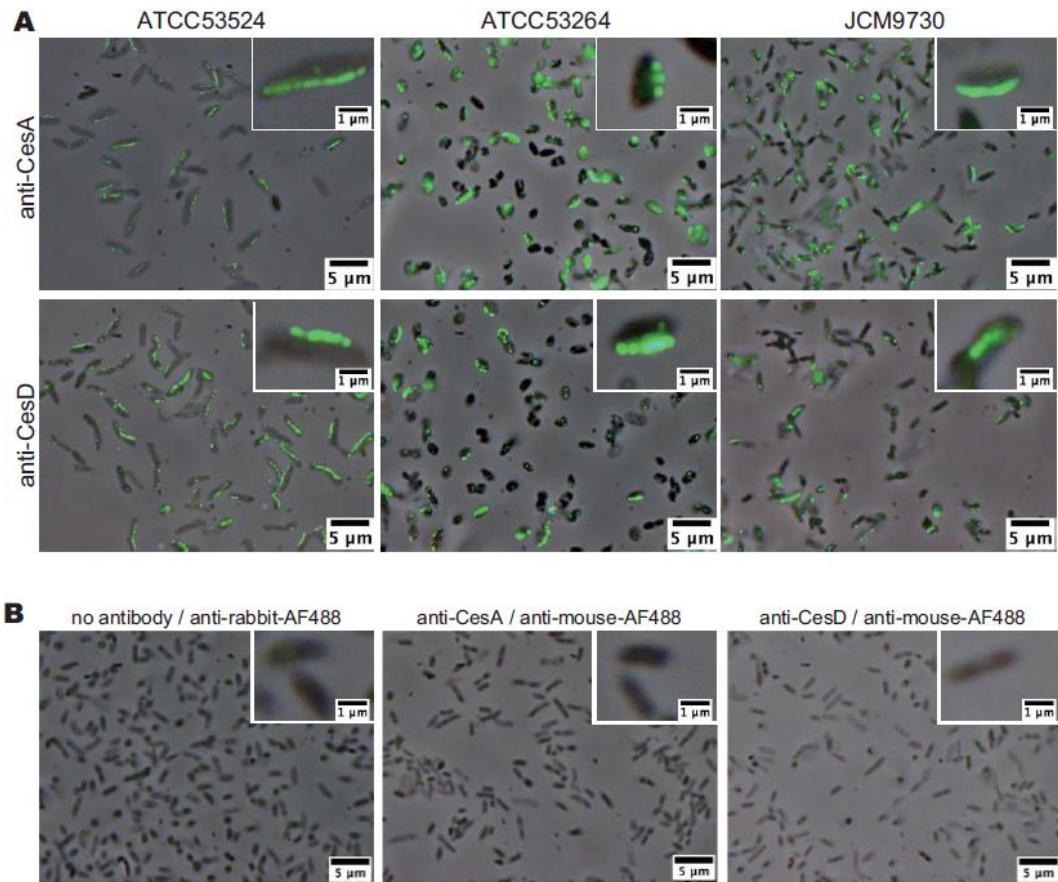


Figure 1. Western blot analysis with SDS-PAGE for the whole cell sample of *Acetobacter* cells. A, B, C, and D show the results with the antibody against CesA, CesB, CesC, and CesD protein, respectively. The sample of ATCC53524, ATCC53264, and JCM9730 was loaded into the lane 1, 2, and 3, respectively. Roughly the same number of cells, measured by the optical density at 600 nm, were loaded. The arrow indicates the band of interest.

As a result of optimizing the pretreatment of the cells (fixation, lysozyme treatment, and detergent treatment) as well as the antibody treatment, we could successfully label CesA and CesD proteins as a linear array in the cell (Figure 2A) whereas no labeling was found in negative controls (Figure 2B). Such a linear labeling pattern was not found for the immunolabeling of CesB and CesC. The linear labeling pattern of CesA and CesD was observed for all of the strains used in this study. This clearly indicates that CesA and CesD proteins are the subunits included in the linear TC of *Acetobacter*. Furthermore the linear signal was sometimes found at the lateral edge of the cell on the micrograph, indicating that the labeled protein is not on the inside but rather at the boundary of the *Acetobacter* cell. Therefore, the linear immunolabeling pattern shown in Figure

246 2A provides the experimental evidence that CesA and CesD are included in the
 247 linear TC on the cell membrane, the bacterial CSC.

248



249

250 Figure 2.

251 Fluorescence micrographs of *Acetobacter* cells with immunolabeling by the antibodies against
 252 CesA and CesD proteins. The phase-contrast images and the epi-fluorescence image are merged.
 253 (A) Three different strains (ATCC53524, ATCC53264, and JCM9730) were labeled using the
 254 identical protocol with a correct choice of the antibodies. The inset shows the image at a higher
 255 magnification. (B) Control experiments with a strain ATCC53524. Combination of the
 256 primary/secondary antibodies was used as indicated. Almost no labeling was found in neither
 257 conditions.

258

259 *Change of the immunolabeling efficiency for CesA and CesD protein.*

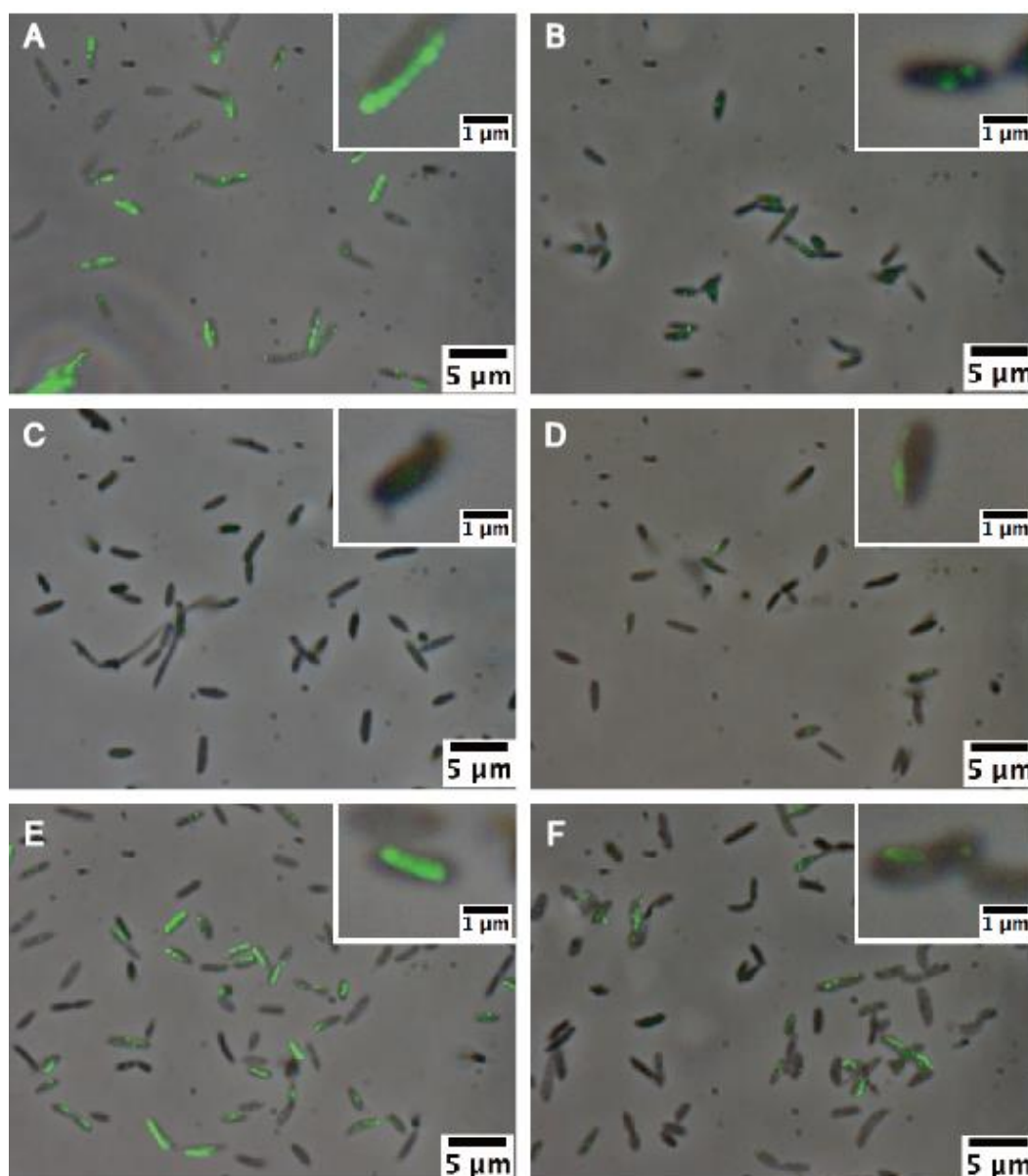
260 For efficient immunolabeling, the cells are usually treated with an

261 adequate procedure prior to labeling. In the case of the bacterial cell, lysozyme

262 treatment is commonly used for disintegrating the peptidoglycan layer beneath the
263 outer membrane, and detergent treatment is used for permeabilizing the outer and
264 inner membrane. Therefore, in principle, the protein exposed to the outside of the
265 cell will be labeled without any pretreatment. We then surveyed the change in the
266 immunolabeling efficiency depending on the pretreatment applied to explore the
267 location of CesA and CesD proteins, which were successfully immunolabeled in
268 this study. The strain ATCC53524 was used for this purpose given the fact that
269 this strain showed the highest immunolabeling efficiency.

270 We tested five different pretreatments, in addition to the optimized
271 condition shown above (Figures 3A and 4A): (i) no treatments, (ii) EDTA
272 treatment, (iii) detergent treatment, (iv) EDTA treatment followed by detergent
273 treatment, and (v) lysozyme treatment (Figures 3 and 4, and summarized in Table
274 2). First, the cells with no pretreatment showed almost no immunolabeling for
275 neither CesA nor CesD (Figures 3B and 4B), indicating that CesA and CesD are
276 not exposed to the outside of the cell. Notably, EDTA treatment alone allowed for
277 the immunolabeling of CesD but not CesA (Figures 3C and 4C). Given the
278 relatively mild disturbance of the outer membrane only by depletion of divalent
279 cations with EDTA, CesD is probably located in the periplasmic space and was
280 immunolabeled due to access of the antibody. By contrast, CesA protein is a
281 transmembrane protein, with its carboxyl terminal (the epitope of the antibody
282 used in this study) facing to the cytoplasm. Therefore, it is reasonable that CesA
283 was not immunolabeled for cells whose outer membrane is mildly disturbed by
284 EDTA alone, which is not harsh enough to allow for cell lysis.

285



286

287 Figure 3.

288 Fluorescence micrographs with immunolabeling of the strain ATCC53524 by the antibody against
 289 Cesa protein, merged on the phase-contrast image. Pretreatment of the cell prior to the primary
 290 antibody treatment was as follows: (A) lysozyme treatment followed by detergent treatment (the
 291 optimized condition in this study), (B) no pretreatment, (C) EDTA treatment, (D) detergent
 292 treatment, (E) EDTA treatment followed by detergent treatment, (F) lysozyme treatment. The inset
 293 shows the image at a higher magnification.

294

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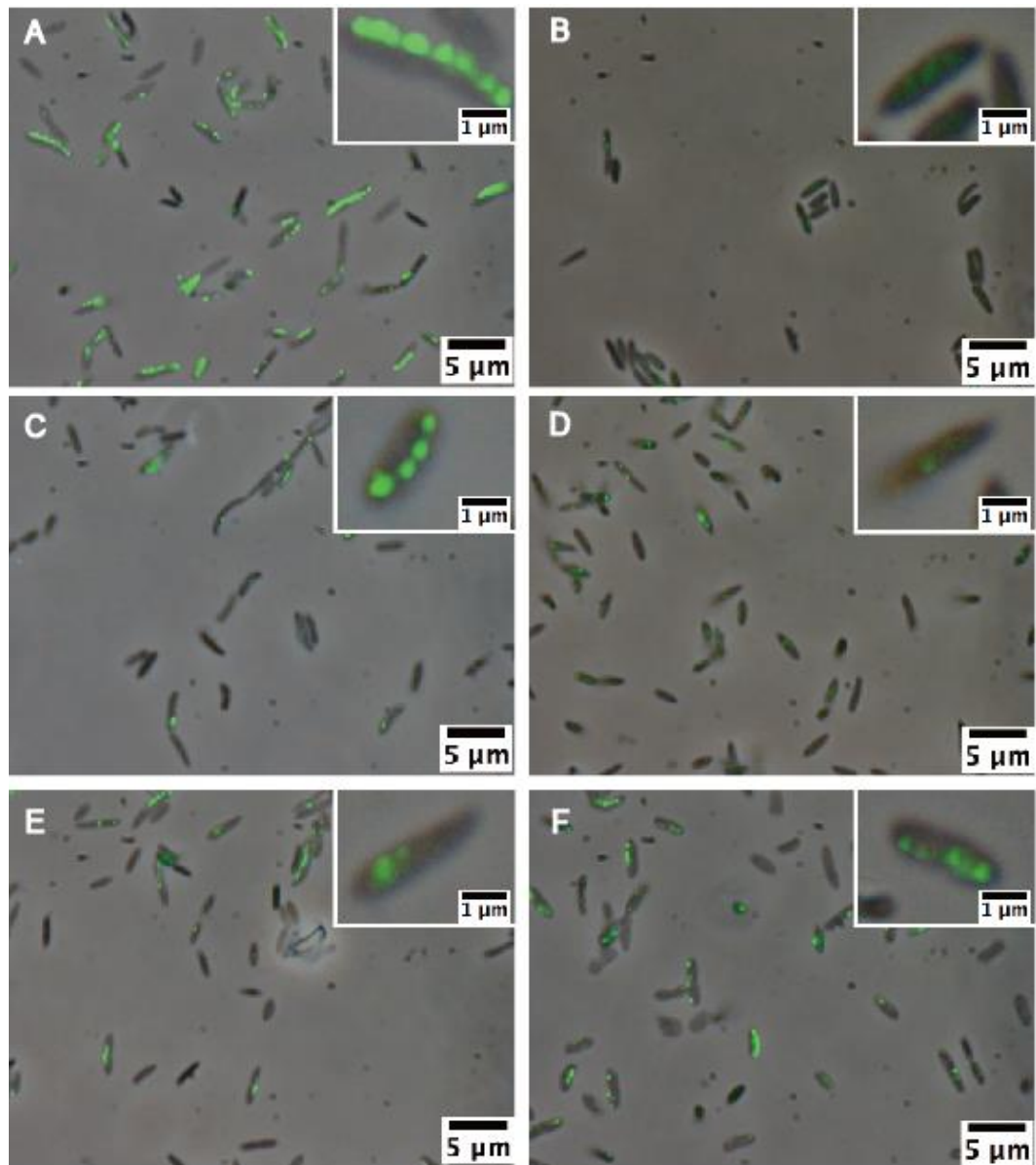


Figure 4.

Fluorescence micrographs with immunolabeling of the strain ATCC53524 by the antibody against CesD protein, merged on the phase-contrast image. Pretreatment of the cell prior to the primary antibody treatment was as follows: (A) lysozyme treatment followed by detergent treatment (the optimized condition in this study), (B) no pretreatment, (C) EDTA treatment, (D) detergent treatment, (E) EDTA treatment followed by detergent treatment, (F) lysozyme treatment. The inset shows the image at a higher magnification.

308 **Table 2.** Summary of the immunolabeling microscopy observations

Pre-treatment after PFA fixation	CesA	CesD
Lysozyme treatment + Detergent treatment	+++	+++
No treatment	—	—
TE* treatment	—	+
Detergent treatment	—	—
TE treatment + Detergent treatment	++	++
Lysozyme treatment	++	++

309 —: Almost no labeling was found

310 +: A small number of the cells were labeled

311 ++: A substantial number of the cells were labeled

312 +++: Most of the cells were labeled

313 *: Tris-EDTA buffer (100 mM Tris-HCl (pH 6.7), 5 mM EDTA), the same buffer used for the
314 lysozyme treatment

315

316 Detergent treatment alone did not allow for the immunolabeling of CesA
317 and CesD, in contrast to the expectation (Figures 3D and 4D). However, EDTA
318 treatment prior to detergent treatment dramatically improved the immunolabeling
319 efficiency for both CesA and CesD (Figures 3E and 4E). This indicates that the
320 permeabilization by the detergent is not sufficient for disturbing the outer
321 membrane of *Acetobacter* to introduce the antibody to the inside of the cell
322 (periplasm and cytoplasm).

323 A substantial number of cells were immunolabeled when treated with
324 lysozyme alone for both CesA and CesD (Figures 3F and 4F). Given that CesD is

325 localized in the periplasm, as shown above, the lysozyme treatment without
326 permeabilizing the inner membrane was sufficient to immunolabel CesD protein.
327 However the substantial immunolabeling of CesA protein from such pretreatment
328 requires a speculative interpretation given that the carboxyl terminal of CesA
329 protein (the epitope of the antibody used in this study) is on the cytoplasmic side
330 and prevents access of the antibody unless the inner membrane is permeabilized.
331 We consider that this observation reflects weak but nevertheless significant cell
332 lysis due to the lysozyme treatment.

333

334 DISCUSSION

335 This study provides evidence that CesA is included in the TC of bacterial
336 cells, which had already been reported for plant cells (Kimura et al. 1999). Based
337 on fluorescence immuno-microscopy, this study also showed that CesD is
338 included in the linear TC, as reported previously (Sunagawa et al. 2013). These
339 observations are not sufficient to conclude that the CesA and CesD proteins are
340 colocalized in the TC. Direct immunolabeling with a fluorescence dye-labeled
341 primary antibody should provide a clearer conclusion for the colocalization of
342 CesA and CesD proteins in the TC. Neverthelss, the linear labeling pattern
343 observed for CesA and CesD in this study is striking enough to propose that CesA
344 and CesD are colocalized in the TC of *Acetobacter*, regardless of whether their
345 interaction is direct or indirect.

346 The structural models for the CesA/CesB complex (Morgan et al. 2016,
347 Morgan et al. 2013, Morgan et al. 2014) and CesD (Hu et al. 2010) also support
348 the functional link between CesA and CesD, given that the former generates
349 cellulose from UDP-glucose and the latter includes cellulose chains in the channel

350 formed by its homo-octamer. It is then proposed that CesD functions downstream
351 of the CesA/CesB complex in the process of cellulose biosynthesis, and that they
352 are spatially close. This hypothesis is consistent with the observation that
353 immunolabeling of CesA and CesD proteins showed a linear pattern in the cells in
354 this study.

355 We also attempted the immunolabeling of CesB and CesC protein in this
356 study although no successful data were obtained. For CesC protein, which is
357 currently the most enigmatic subunit, the reason for the failure is unclear. A
358 possible reason could be related to access of the antibody to the epitope, which is
359 significantly influenced by the stereo arrangement of this subunit in the cell.
360 However, it was unexpected that the immunolabeling of CesB protein did not
361 show a linear labeling pattern as previously observed by SDS-FRL (Kimura et al.
362 2001). It is noticeable that the antibody against CesB used in this study allowed
363 for the linear TC to be labeled by SDS-FRL (Figure 5), despite a relatively high
364 non-specific labeling probably owing to that it is a polyclonal antibody. This
365 result indicates that this antibody is able to label CesB protein in the SDS-treated
366 freeze-replica prepared from the cells fixed by flash-freezing with no chemicals.
367 Therefore, a possible interpretation for this unexpected result is that the PFA
368 fixation might kill the epitope activity of the CesB protein, for example by
369 changing the protein itself and/or its surrounding environment, so as to inhibit
370 binding of the antibody in immunofluorescence labeling.

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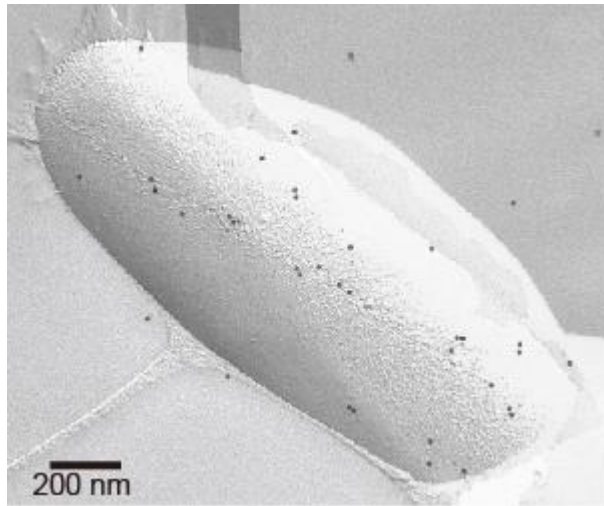


Figure 5.

Electron micrograph of SDS-FRL for the strain ATCC53524 with the antibody against CesB protein. The antibody location was visualized by colloidal gold of a 15 nm diameter. Linear pattern of the labeling was clearly found despite a relatively high non-specific antibody labeling, which is probably due to that the antibody is a polyclonal antibody against peptide.

The antibody against CesA protein, which gave successful immunolabeling as shown in Figure 2A, was also used for SDS-FRL to visualize CesA protein in *Acetobacter* TC. However to date, no labeling for CesA protein was found on the replica despite successful labeling for CesB protein as shown above. This is probably due to that CesA protein in the inner membrane was detached from replica of the outer membrane by solubilization with SDS. Significant improvement will be required for successful SDS-FRL with the antibody against CesA protein.

Compiling the results of this and previous studies, we propose a hypothetical model for the TC of *Acetobacter* as shown in Figure 6. The CesA/CesB complex is embedded in the inner membrane, given that the ligands (UDP-glucose and c-di-GMP) are cytosolic molecules and the product cellulose is extruded outside through the membrane-spanning channel (Morgan et al. 2013).

394 CesC is depicted as the cellulose-translocating channel in the outer membrane
395 according to the currently accepted model (McNamara et al. 2015, Saxena et al.
396 1994). No immunolabeling from the lack of pretreatment, and weak
397 immunolabeling from EDTA treatment alone for CesD protein indicated that
398 CesD is located in the periplasmic space. The result of a biochemical study using
399 marker enzyme assays also support this hypothesis (Iyer et al. 2011). Given that
400 the function of CesD is carried out downstream of CesA as discussed above, CesD
401 protein is located close to the exit of the cellulose-translocation channel of CesA
402 protein in the periplasm, as proposed based on a previous structural analysis of the
403 *Acetobacter* CesA/CesB complex with electron microscopy (Du et al. 2016).

404 Given that one CesA/CesB complex produces one cellulose chain
405 (Morgan et al. 2013), and the CesD oligomer includes four chains in its inner pore
406 (Hu et al. 2010), the model in Figure 6a represents only one CesA/CesB complex,
407 and the other three complexes are not shown for visual clarity. A combination of
408 these molecules could be the functional unit to produce the primary assembly of
409 the polymerized cellulose chains prior to microfibril formation, which has been
410 proposed as a “mini-sheet” in a previous study (Cousins and Brown Jr. 1995). The
411 linear array of this whole complex should be visualized as the linear type TC in
412 *Acetobacter* (Figure 6b).

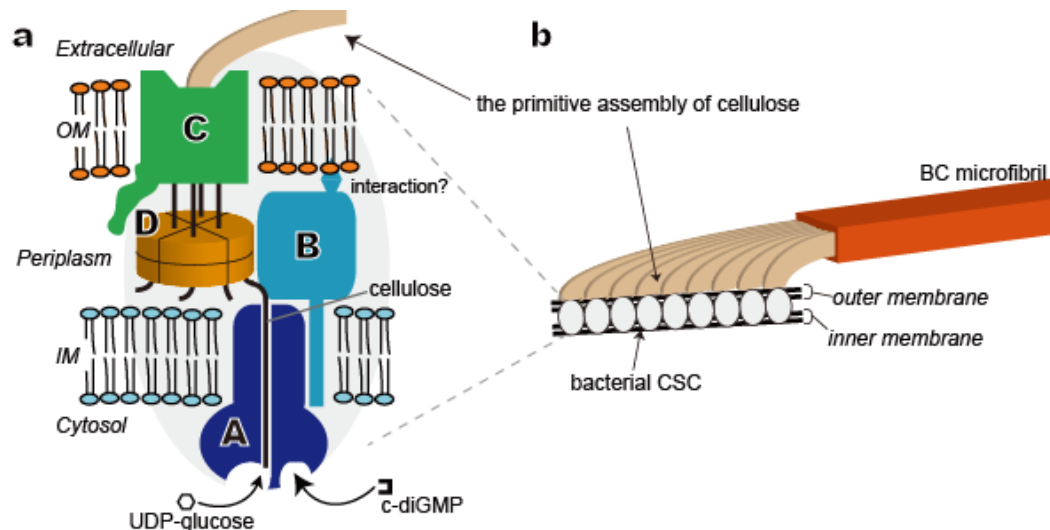


Figure 6.

A schematic model for the cellulose synthase complex of *Acetobacter*. In the schematic diagram of the subunit location in one complex (a), CesA and CesB are depicted as monomers while CesD is illustrated as an octamer through which four cellulose chains pass, as reported previously (Hu et al. 2010). CesC is located in the outer membrane according to the currently accepted model (McNamara et al. 2015, Saxena et al. 1994). The terminal complexes are probably formed by the linear array of these complexes as shown in (b). OM: outer membrane; IM: inner membrane

The SDS-FRL experiment also provided insight about CesB protein.

Given the smoothness of the fractured surface, the linear TC of *Acetobacter* is found in the P-face (the extracellular surface of the inner leaflet of the lipid bilayer) of the outer membrane (Kimura et al. 2001). The successful immunolabeling of CesB protein by SDS-FRL indicates that this protein remains with the replica even after SDS-treatment, indicating that CesB protein significantly interacts with the outer membrane from the periplasmic side. This interaction is likely important for guiding the cellulose chain to the extracellular side and/or the crystallization of cellulose chains into a microfibril. Further SDS-FRL experiment with other antibodies will shed light on the locations of the other subunits.

434 CONCLUDING REMARKS

435 This study demonstrated that CesA, the core catalytic subunit of cellulose
436 synthase, is the molecule included in the linear-type TC or the CSC of
437 *Acetobacter*. Structural analysis of these proteins has recently started providing
438 many insights about the enzymatic mechanism of cellulose synthase as well as
439 other well-known membrane proteins such as ion/water channels and transporters.
440 However, for cellulose synthase, which functions in the assembly of polymer
441 chains into a supermolecular aggregation, the structural analysis of the protein
442 complex at a cellular/subcellular scale is important for understanding the
443 underlying mechanism. Further studies with microscopy will play an important
444 role for shedding light on the mechanism of cellulose chains assembly into the
445 microfibril.

446

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