1      **Structure of the bacterial cellulose ribbon and its assembly-guiding cytoskeleton by**

2      **electron cryotomography**

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17      **Abstract**

18      Cellulose is a widespread component of bacterial biofilms, where its properties of exceptional

19      water retention, high tensile strength and stiffness prevents dehydration and mechanical

20      disruption of the biofilm. Bacteria in the *Gluconacetobacter* genus secrete crystalline cellulose,

21      with a structure very similar to that found in plant cell walls. How this higher-order structure is

22      produced is poorly understood. We used cryo-electron tomography and focused ion beam

23      milling of native bacterial biofilms to image cellulose-synthesizing *G. hansenii* and *G. xylinus*

24      bacteria in a frozen-hydrated, near-native state. We confirm previous results suggesting that

25      cellulose crystallization occurs serially following its secretion along one side of the cell, leading

26  to a cellulose ribbon that can reach several microns in length and combine with ribbons from

27  other cells to form a robust biofilm matrix. We were able to take direct measurements in a near-

28  native state of the cellulose sheets. Our results also reveal a novel cytoskeletal structure, that

29  we name the cortical belt, adjacent to the inner membrane and underlying the sites where

30  cellulose is seen emerging from the cell. We find that this structure is not present in other

31  cellulose-synthesizing bacterial species, *Agrobacterium tumefaciens* and *Escherichia coli* 1094,

32  which do not produce organized cellulose ribbons. We therefore propose that the cortical belt

33  holds the cellulose synthase complexes in a line, to form higher-order cellulose structures such

34  as sheets and ribbons.

35

**Importance**

37  This work's relevance for the microbiology community is two-fold: It delivers for the first time

38  high-resolution  near-native  snapshots  of  the  *Gluconacetobacter*  spp.  (previously

39  *Komagataibacter* spp.) in the process of cellulose ribbon synthesis, in their native biofilm

40  environment. It puts forward a non-characterized cytoskeleton element associated with the side

41  of the cell where the cellulose synthesis occurs. This represents a step forward in the

42  understanding of the cell-guided process of crystalline cellulose synthesis, particularly studied

43  in the *Gluconacetobacter* genus and still not fully understood. Additionally, our successful

44  attempt to cryo-FIB mill through biofilms to image the cells in their native environment will

45  drive the community to use this tool for the morphological characterization of other studied

46  biofilms.

47

**Introduction**

49  Humans rely on cellulose for building material, clothing and fuel[1–3]. More recently the polymer

50  has sparked interest in the biotechnology field as a potential source of biofuel feedstock[4], and

51  in the biomedical industry as a biologically neutral scaffold to promote tissue regeneration[5,6] .

52  Cellulose is a linear polymer of glucose molecules connected with $\beta$1-4 linkages by a

53  glucosyltransferase. Individual linear glucan chains can pack via hydrogen bonding and Van

54  Der Waals interactions in various ways to form different types of celluloses, with different

55  properties[3,7,8]. The most common way glucan chains organize in nature is to form hydrogen-

56  bonded planes stacked into parallel layers via Van Der Waals interactions[9,10]. These stacked

57  layers give rise to cellulose I microfibrils, or "native cellulose", that can then coalesce to form

58  larger arrays. Because glucan chains pack in a regular lattice but cannot sustain this regular

59  pattern over their entire length, cellulose I is considered paracrystalline. Depending on how the

60  lattice is organized, cellulose I can be of the α form, bearing a triclinic unit cell, or β form,

61  bearing a monoclinic unit cell[11,12]. Cellulose Iβ is mainly found in plants, where it is a major

62  structural element of the cell wall[13].

63

64  In the prokaryotic world, cellulose is an important component of bacterial biofilms[14,15], which

65  increase cells' tolerance for a range of biotic and abiotic stresses and enhance surface adhesion,

66  cell cooperation and resource capture[14]. Cellulose-containing biofilms have also been involved

67  in pathogenicity, enabling bacteria to resist antibiotics and disinfection[16,17]. Most cellulose-

68  synthesizing bacteria produce amorphous (non-crystalline) cellulose, but a few genera,

69  including *Gluconacetobacter*, can produce cellulose Iα microfibrils. In *Gluconacetobacter*,

70  these paracrystalline cellulose microfibrils can further aggregate into wide ribbon structures

71  and larger arrays[18], giving rise to thick biofilms that are predominantly pure cellulose I.

72  Bacterial cellulose is synthesized by an envelope-spanning machinery called the Bacterial

73  Cellulose Synthase (BCS) complex, encoded by the BCS gene cluster and first identified in

74  *Gluconacetobacter*[15]. While the components vary, most of the species encode BcsA, a

75  component in the inner membrane that, with BcsB, catalyzes transfer of UDP-glucose to the

76    nascent glucan chain[15,19,20]. BcsD forms a periplasmic ring thought to gather glucan chains from

77    several BcsA/B units[21,22]. BcsA and B are essential for cellulose synthesis *in vivo*, and BcsD is

78    essential for the crystallization of nascent glucan chains[23]. BcsC forms a pore in the OM and

79    very recent work has solved its crystallographic structure[24]. Consistent with previous data

80    relying on sequence homology with the exopolysaccharide secretin components AlgE and AlgK

81    from *P. aeruginosa*, BcsC is found to form an outer-membrane β-barrel pore at its C-terminal

82    end, secreting the nascent elementary cellulose fibrils into the environment[23–27]. It is

83    hypothesized that the elementary cellulose fibrils can aggregate with neighboring elementary

84    fibrils upon secretion to form microfibrils[28,29]. Genes flanking the operon, *cmcAx* (endo-β-1,4-

85    glucanase)*, ccpAx* (unknown function) and *bglxA* (β-glucosidase), are essential for cellulose

86    crystallization and despite knowledge of their enzymatic functions, how they take part in this

87    process is unclear[29–32].

88    In the following report, the terms used to describe the cellulose assembly process are adapted

89    from the ones defined in[29], elaborating on the cell-directed hierarchical model for cellulose

90    crystallization[7,10]. Glucan chains are linear polymers of β-1,4 linked glucose residues

91    synthesized by a single catalytic site of a cellulose synthase. An elementary fibril (also termed

92    mini-crystal in previous work[10,33,34]) is the product of the periplasmic aggregation of multiple

93    glucan chains which is then extruded through a single BcsC subunit into the environment.

94    Microfibrils result from the aggregation of several elementary fibrils, at least three according

95    to earlier work[34], outside the cell. These microfibrils can then crystallize into sheets that stack

96    on each other to form ribbons. The latter terminology differs somewhat with previous usage  in

97    that our definition of a sheet is equivalent to the "bundles of microfibrils", the polymerization

98    step prior to the ribbon, described in [29].

99    Much work has already been done to understand the synthesis of paracrystalline

100    cellulose[18,20,21,23,30–33,35–41] . In particular freeze-fracture/freeze etching electron microscopy

101  (EM) studies have found that the *G. hansenii* BCS complexes are arrayed linearly along the

102  side of the cell[18,33,38,39], and this arrangement seems to determine the extracellular organization

103  of cellulose I into ribbons[18,33,39]. How this linear arrangement is achieved is not known.

104

105  Here we used cryo-electron tomography (cryo-ET) of isolated cells and cryo-Focused Ion Beam

106  (FIB)-milled biofilms to visualize native cellulose production in *G. hansenii* and *G. xylinus*,

107  allowing the morphological characterization of the cellulose ribbons in a near-native state. We

108  identified a novel cytoplasmic structure, which we call the cortical belt. We found that this

109  cortical belt is absent from *Escherichia coli* 1094, which produces amorphous cellulose, and

110  *Agrobacterium tumefaciens*, which produces crystalline microfibrils but not higher-order

111  sheets, suggesting that the cortical belt functions to align BCS complexes to produce cellulose

112  sheets.

113

114  **Results**

115  <u>Cellulose is laid out in stacked sheets on one side of the cells.</u>

116  To visualize bacterial cellulose production, we used cryo-ET to image intact frozen-hydrated

117  *G. hansenii* cells separated from their cellulose biofilm according to the original method from

118  Brown et al. 1976. Previous work showed that newly synthesized cellulose ribbons are visible

119  under the electron microscope at one hour post-separation[38]. To assure that the cells would have

120  enough time to synthesize cellulose ribbons we imaged cells 5 hours (300 minutes) after

121  separation. To confirm cellulose production, we stained cells with mitoTracker Deep Red FM

122  to visualize membranes and Calcofluor-White to visualize cellulose. By confocal imaging, we

123  observed cellulose filamentous structures tens of microns long (Fig. 1A and B, cyan

124  arrowheads). We next plunge-froze cells at the same timepoint and imaged them by cryo-ET.

125  The rod-shaped cells always lay flat on the grids, but their long axis was oriented randomly in

5

126    the grid plane. Of 33 cells imaged, we found putative cellulose ribbons associated with 29

127    (88%), always on one side of the cell, including the top and bottom, and always aligned with

128    the cell's long axis (Fig 1C-E, yellow arrows). To confirm that the ribbon was in fact cellulose,

129    we treated cells with cellulase and observed a large reduction in the occurrence of ribbons in

130    cryo-EM and negative stained images (Supplemental figure 1, yellow arrowheads). Instead, we

131    observed aggregated material we think is likely partially digested cellulose (Supplemental

132    figure 1F, orange arrowheads).

133    The spatial relation between the cellulose ribbons and the OM was examined. In 3 out of the

134    29 tomograms, the cellulose ribbon was observed running beneath or on top of the cell, causing

135    it to be normal to the electron beam thus inherently not well resolved and difficult to assess its

136    spatial relation with the OM[42]. Therefore, data from these 3 tomograms was excluded for these

137    measurements. In the remaining tomograms two distinct configurations were observed: a

138    "tight" configuration in 23 out of 26 tomograms (88%), where the average outer membrane

139    (OM)-to-ribbon distance was $16 \pm 5$ nm (n = 23) (Fig. 1C-H, supplemental video 1,

140    https://figshare.com/s/74891ac625fe8125c60c), and a "loose" configuration in 3 out of 26

141    tomograms (12%), where the average OM-to-closest sheet distance was $99 \pm 49$ nm (n = 3)

142    (Fig. 2), most probably resulting from a mechanical stress resulting in the cellulose ribbon to

143    pull away from the cell. Among the tomograms showing a "tight" configuration, 17 out of 23

144    (65%) displayed multiple clear direct contacts between the OM and the ribbon (Fig. 1F-H, white

145    arrows). Tomograms in the "loose" configuration exhibited ribbons that seemed detached from

146    the OM, with an increased OM-to-closest sheet distance compared to the "tight" configuration

147    (Fig. 2E). All three tomograms presented disorganized aggregates bearing a mesh-like

148    appearance between the OM and the ribbon (Fig. 2A-D, orange asterisks and dashed bracket).

149    These aggregates always connected to the ribbon (Fig. 2A, black lined orange arrows).

150    Throughout the study and in line with previous studies, *G. hansenii* was never seen harboring

151    a flagellum, pilli, curli or any other appendages other than the cellulose ribbons (Supplemental

152    figure 2). Additionally, similar cellulose aggregates have been seen previously by negative

153    staining[18,28], hence we are confident that these structures are cellulose in a disorganized form.

154    These cells and their cellulose structures (the ribbons) were imaged in a near-native (frozen

155    hydrated) state, allowing measurement of their native dimensions. In our description of the

156    cellulose ribbons below, by length we mean the dimension parallel to the long axis of the cell

157    (Fig. 3A). By thickness we refer to the dimension normal to the cell surface (Fig. 3A, black

158    inset). By width we refer to the dimension tangential to the cell surface (Fig. 3B). The cellulose

159    ribbons we observed were very similar to what has been seen previously by negative stain

160    EM[28,38]. Ribbons comprised long flexible stacked sheets, too long to be measured by cryo-ET

161    because they are never entirely in the field of view. Relative to previous morphological work,

162    our flexible sheets equate to what was described as "microfibrillar bundles" in previous

163    studies[10,28,29,34]. However, our observations in a frozen-hydrated state allowed us to visualize

164    them in a sheet-like configuration, therefore we chose to call them sheets instead of bundles.

165    Missing wedge-induced Z-elongation of the cellulose sheets distorts width measurements[43,44].

166    Despite this artefact, we estimated it at $38 \pm 14$ nm (n = 45) (Fig. 3C), which is therefore an

167    overestimate. To see if width increased along the cell, width estimates were performed along

168    the length of the cellulose ribbon in 3 tomograms. Unfortunately, these estimates are heavily

169    influenced by the missing wedge-induced elongation in the Z-dimension, therefore the

170    measurements did not give any conclusive trend in one way or another (increase, decrease or

171    constant width along the cellulose ribbon). These sheets then stack into a ribbon ($2.3 \pm 0.9$

172    sheets on average; n = 24), with a variable inter-sheet distance ($16 \pm 7$ nm; n = 23). Inter-sheet

173    distance was accurately measured peak-to-peak (Fig. 3D), which encompasses 2 halves of the

174    two neighboring sheets' density and the space between them (Fig. 3A, black inset). Because

175    the apparent thickness of single densities in cryo-ET is strongly affected by the defocus applied,

176   individual cellulose sheet thickness measurements will be overestimated. Therefore, we can

177   only say confidently that they are thinner than the inter-sheet distance. Despite careful

178   inspection, although densities could be seen in the periplasmic space, we did not recognize a

179   consistent shape which we could confidently attribute to the BCS machinery. This is likely due

180   to the large cell diameter (~800nm), and the small size and/or flexibility of the BCS complexes.

181

182   <u>Sheets arise from the stacking of microfibrils</u>

183   To visualize earlier stages of cellulose synthesis, we plunge-froze cells at earlier timepoints

184   after separation from the biofilm. A total of 6 and 15 tomograms were acquired at 13- and 20-

185   minutes post-separation, respectively. At 13 minutes (the most quickly we could complete

186   plunge freezing), no cells exhibited a cellulose ribbon, however, disorganized aggregates were

187   observed in the vicinity of 1 out of the 6 tomograms. At 20 minutes post-separation, cellulose

188   ribbons were observed adjacent to the cell in 9 out of 15 tomograms (64% versus 88% (n = 33)

189   at 300 minutes post-separation) (Fig. 4A). Out of these 9 cells harboring an adjacent cellulose

190   ribbon, 3 had it on the top or bottom of the cell and were excluded from the analysis for the

191   same reason explained above. Therefore, the analysis of the OM-ribbon interface was

192   conducted on the remaining 6. The cellulose ribbons observed at 20min post-separation

193   comprised only one cellulose sheet (n = 6), significantly smaller amount than at 300min post-

194   separation (P-value <0.0001, Fig. 4B). Four out of these 6 tomograms (67%) exhibited a "tight"

195   configuration. The average OM-to-closest sheet distance of 14 ± 3 nm (n = 4) was not

196   significantly different from the 300 minutes post-separation "tight" configuration average OM-

197   to-closest sheet distance (P-value > 0.9, Fig. 4C-D, n = 4 and 23 for 20min and 300min post-

198   separation, respectively). The two other tomograms bore ribbons in the "loose" configuration,

199   i.e. at an OM-to-closest sheet distance >40 nm with disorganized aggregates in-between. These

200   "loose" ribbons had an OM-to-closest sheet distance of 43 and 59 nm, respectively. The

8

201     disorganized aggregates visible at 20 minutes post-separation emanated perpendicularly from

202     the OM to connect to the nascent cellulose sheet. They were thinner than the ones observed at

203     300 minutes post-separation and rod-shaped (Fig. 4E-F, red arrowheads). Average density

204     profiles normal to the direction of the cylindrical-shaped densities were traced to estimate their

205     diameter (Fig. 4G). We again emphasize the inherent overestimation of such measurements due

206     to defocus. The average estimates on the two cells, $11 \pm 2$ nm (n = 12) and $6.5 \pm 1$ nm (n = 4),

207     respectively (Fig. 4G), therefore establish upper limits of the true diameter. These estimates are

208     also less than the above-measured inter-sheet distances (Fig. 4H). Because elementary fibrils

209     are thought to be between 1.5 and 6 nm in thickness[18,38,39], we hypothesize these structures are

210     microfibrils composed of several elementary fibrils. The variability of the microfibril diameter

211     measurements between cells (Fig. 4G, Cells #1 and #2) suggests these structures can contain a

212     varying number of elementary fibrils more-or-less tightly packed together. This configuration

213     is reminiscent of what was seen in previous studies of microfibrils coming out of clusters of

214     pores[28,38] and likely represents an early stage of cellulose sheet formation that has been

215     mechanically disturbed. Sheets at 20 minutes post-separation had an estimated width of $25 \pm 8$

216     nm (n = 6) (Fig. 4I), smaller than those at 300 minutes, although the difference did not appear

217     significant (P-value = 0.26).

218     These results show that 1) the microfibrils emanating from the OM have roughly the same

219     thickness as the cellulose sheet, 2) sheet width seems to increase over time and 3) the number

220     of cellulose sheets comprising a ribbon increases over time.

221

222     <u>A novel cytoplasmic structure is associated with cellulose production</u>

223     We next examined the interior of *G. hansenii* cells during cellulose synthesis. These cells had

224     extensive cytoplasmic vesicles in the center and at the periphery of the cell (Supplemental

225     figure 3), which is a rare and largely uncharacterized aspect of bacteria[45]. The most notable

226  feature we observed was another ribbon-like structure closely associated with the inner

227  membrane (24 ± 4 nm from it; n = 19, for an example peak-to-peak measurement see Fig. 3D)

228  and several hundred nanometers in length (Fig. 5A, purple arrows). We found it in 90% of cells

229  with a cellulose ribbon (n = 29), always on the same side as, and underlying, the nascent

230  cellulose      sheet      (Fig.      5B-C,      supplemental      video      2

231  https://figshare.com/s/74891ac625fe8125c60c). This cytoplasmic structure is not a tube but

232  rather a stack of sheet-like structures, 47 ± 23 nm wide (n = 10), parallel to the inner membrane

233  and spaced (peak-to-peak) by 15 ± 5 nm (n = 7) (Fig. 5D-F). We refer to it here as the "cortical

234  belt". Interestingly, in tomograms acquired in shaking conditions in SH media supplemented

235  with cellulase, although the cellulose ribbons had vanished, the cortical belt was observed

236  (Supplemental figure 1F, purple arrows).

237

238  <u>Structural hallmarks of crystalline cellulose synthesis are also present in intact biofilms</u>

239  It is possible that separating bacteria from the cellulose mat for whole cell cryo-ET imaging

240  could have altered structures associated with cellulose synthesis. We therefore imaged *G.*

241  *hansenii* cells *in situ* in young cellulose biofilms grown on gold grids. We imaged biofilms after

242  3 or 6 hours before plunge-freezing in hope of visualizing any change in the ordering of the

243  fibers or the aspect of the cells over the course of biofilm growth. To access cells within the 5-

244  to 10-micron thick biofilm, we used cryo-FIB milling to generate thin (~200 nm) lamellae

245  suitable for imaging by cryo-ET (Fig. 6A-C). In a total of 19 analyzed tomograms (9 and 10

246  tomograms for 6h and 3h biofilms, respectively, Table 1), we observed fields of living and dead

247  bacteria encased in a matrix of bundled cellulose ribbons at both time points (Fig. 6D-E and

248  supplemental video 3 https://figshare.com/s/74891ac625fe8125c60c). Overview tomograms

249  (low magnification with low total dose) and high-resolution composite images of the lamellae

250  allowed extraction of positional information of the cells in relation to the biofilm. There were

251  $0.10 \pm 0.02$ and $0.27 \pm 0.04$ cells/um$^2$ and 15% and 28% of the volume of the lamellae was

252  occupied by cells at 3 and 6h time points, respectively (Fig. 6F) (n = 6 and 4 lamellae,

253  respectively). This approximate 2-fold increase in cell density from a 3-hour to a 6-hour biofilm

254  suggests that cell division is occurring during biofilm growth.

255  Dead cells can be easily differentiated from living cells (Fig. 6D, red asterisks) by the wavy

256  aspect of their envelope, sometimes presenting punctures and by the appearance of their cytosol.

257  Living cells typically have ribosome-rich and nonribosomal regions (bacterial chromosome)

258  while dead cells have coagulated cytosols with large electron-dense aggregates and very low

259  ribosome counts. The live-to-dead cell ratio was calculated at $0.9 \pm 0.1$ in both 3- and 6-hour

260  biofilms, revealing no increase in the proportion of dead cells between these two timepoints

261  (Fig. 6G). Because lamellae give access to the native organization and layering of the cells

262  within the biofilm, the depth of dead/living cells within the biofilm was assessed by measuring

263  their distance from the leading edge of the lamella (see methods). No trend between cell depth

264  within the biofilm and state of the cells was detected (Fig. 6H).

265  In all 19 tomograms (combining 3h and 6h lamellae), we observed numerous cellulose ribbons

266  surrounding the cells (Fig. 7A, yellow arrowheads). In 5 out of the 19 tomograms (26%), a

267  cellulose ribbon was closely appended to the cell's OM, as we previously had seen in separated

268  cells (Fig. 7B-C, dark-lined yellow arrowhead). Among those 5 tomograms, 4 showed a cortical

269  belt  adjacent  to  the  cellulose  ribbon  (Fig.  7B-D  and  supplemental  video  3

270  https://figshare.com/s/74891ac625fe8125c60c). The OM-to-cellulose ribbon distance ($19.2 \pm$

271  $8$ nm, n = 4) and inner membrane to cortical belt distance ($22 \pm 2$ nm, n = 4) were very similar

272  to those measured before in separated cells. In 5 out of the 10 tomograms in 3h biofilm lamellae,

273  disorganized cellulose aggregates were observed connected to well-ordered ribbons just as in

274  the separated cells, whereas this was never observed in the 6h biofilms. This suggests that

275  crystallization is disrupted more often in early biofilm growth (Fig. 7E-G, orange dashed

11

276  lining). Because *Gluconacetobacter* cells are thick, electron transmittance in the central region

277  of the cytoplasm is very low when imaging whole cells, making it difficult to visualize this

278  area. Reducing sample thickness to approximately 200 nm by cryo-FIB-milling allowed us to

279  observe these central regions with greater contrast and visualize the extensive vesicle network

280  deep inside the cell (Fig. 7E, white arrowheads) while losing the ability to capture the full extent

281  of the cellulose sheet stacking because of the lamellar sampling.

282

283  <u>The cortical belt is specific to bacterial species that produce crystalline cellulose ribbons</u>

284  To see whether the cortical belt is specific to *G. hansenii*, we imaged another species of

285  *Gluconacetobacter*, *G. xylinus* (also referred to as *Komagataibacter sucrofermentans* BPR-

286  2001), by cryo-ET at 300 minutes post-separation. *G. xylinus* is a species isolated from cherry,

287  originally called *Acetobacter xylinum* bearing the ability to produce an increased amount of

288  cellulose in shaking culture conditions[35]. G. *hansenii* and *xylinus* have diverged quite

289  substantially and differ in their GC content, and *G. hansenii* has its bcsA and bcsB genes fused

290  and harbors no gene clusters associated with acetan metabolism, commonly found in other

291  Gluconacetobacter species[46]. In our hands, we also observed that *G. xylinus* biofilms seem to

292  grow more slowly and are stiffer in comparison to *G. hansenii* biofilms. Four out of 8 cells

293  (50%) exhibited an extracellular cellulose ribbon along the cells' long axis (Supplemental

294  figure 4A). The cellulose ribbons observed had 2 sheets of cellulose, with an estimated average

295  width of $27 \pm 16$ nm (n = 5). All four cells also possessed a cortical belt (Supplemental figure

296  4A-B, purple arrows), with similar dimensions to those in *G. hansenii.* The average distance

297  from the cortical belt to the inner membrane was $24 \pm 4$ nm (n = 4). In one instance, the cortical

298  belt also contained three stacked layers spaced (peak-to-peak) by 9 nm (Supplemental figure

299  4C). Aside from *Gluconacetobacter*, other bacterial species produce different types of

300  cellulose. For instance, *Escherichia coli* 1094 can make amorphous cellulose[47] and

301 *Agrobacterium tumefaciens* makes paracrystalline cellulose microfibrils during plant

302 infection[48]. Neither of these species are known to make cellulose ribbons, though. We asked

303 whether structures similar to the cortical belt observed in *Gluconacetobacter* were present in

304 these species. Our lab had previously imaged *A. tumefaciens* for other studies, and therefore

305 cryo-tomograms of *A. tumefaciens* were already available. We confirmed by confocal

306 microscopy that *A. tumefaciens* produces cellulose in the same growth conditions as had been

307 used for the earlier experiments (Fig. 8A), and then screened the available tomograms for the

308 presence of cellulose. As the purpose of the previous studies had not been cellulose synthesis

309 observation, relatively few (65 out of 1,854 tomograms) showed distinct cellulose fibers in the

310 vicinity of the cells (Fig. 8B-C, yellow arrowheads, supplemental video 4

311 https://figshare.com/s/74891ac625fe8125c60c). These fibers did not adopt any preferential

312 orientation and ran in all directions around the cell. They also had a decreased width ($14 \pm 5$

313 nm, n = 52 fibers measured in 5 tomograms) compared to *G. hansenii* cellulose sheets (P-value

314 <0.0001), confirming that *A. tumefaciens* does not elaborate wide cellulose sheets nor ribbons

315 but rather simpler structures of crystalline cellulose, presumably bundles of microfibrils. In the

316 65 cellulose-producing cells, we never observed a cortical belt structure. Two notable features

317 were however observed: 1) a polar outer-membrane flattening in 28 cells with a thickening of

318 the OM (43% out of the 65 cells presenting cellulose, Fig. 8B, cyan arrow) and 2) polar

319 amorphous aggregates in 24 cells (37% out of the 65 cells presenting cellulose), (Fig. 8B,

320 orange dashed lining).  19 cells exhibited all three described features, the polar flattening, the

321 amorphous aggregates and the cellulose fibers. We suspect these polar amorphous aggregates

322 to be the unipolar polysaccharides (UPP) described in previous work and shown to allow the

323 attachment of *A. tumefaciens* to biotic and abiotic surfaces in the early stages of biofilm

324 formation[49]. The very close proximity of the putative UPP to the polar flattening suggests the

325 latter could hold the UPP-secreting complexes.

326

327    We confirmed that *Escherichia coli* 1094 grown in minimal medium produces cellulose (Fig.

328    8D). The cells aggregated, making it difficult to image single cells by cryo-ET, so instead we

329    FIB milled through bacterial mats, producing approximately 200 nm-thick lamellae. To identify

330    cellulose structures, we also imaged lamellae from cultures grown in minimal medium

331    supplemented with cellulase. In 3 of the 5 tomograms of untreated cells, we observed

332    amorphous fibrous material (Fig. 8E, orange asterisk), that was not visible in 2 tomograms of a

333    cellulase-treated culture (Fig. 8F). None of the cells imaged in either condition contained a

334    cortical belt (n = 13 untreated and 5 cellulase-treated cells), suggesting that it is unique to

335    bacteria producing higher-order paracrystalline cellulose structures, *i.e.* sheets.

336

337    **Discussion**

338    Here we characterized bacterial cellulose synthesis in two *Gluconacetobacter* species and

339    compared it to two other species by cryo-ET. We identified a novel cytoplasmic structure

340    associated with the production of cellulose I ribbons in *Gluconacetobacter* spp. We also

341    performed cryo-FIB milling followed by cryo-ET on native biofilms.

342

343    Cryo-ET confirms the need of a tight interaction between the nascent sheet and the OM

344    The cell-directed hierarchical model proposes linearly arranged 3.5-nm diameter pores on the

345    surface of the cell[39], each extruding an elementary fibril[28,33]. The arrangement of these pores in

346    lines allows the crystallization of the elementary fibrils upon secretion and integration into a

347    cellulose sheet parallel to the long axis of the cell[7,50,51]. Our results agree with this model.

348    Indeed, we observed that when the gap between the nascent sheet and the OM exceeds

349    approximately 40 nm, disorganized aggregates occur (Fig. 2). Along with previous work that

350    observed similar events[28], we hypothesize that these aggregates are microfibrils failing to

14

351  integrate into an ordered sheet. Furthermore, it has been shown that the addition of compounds

352  which bind directly to cellulose drastically alters the assembly of the sheets and leads to the

353  formation of similar aggregates[18,28,51]. It appears as though preventing the nascent microfibrils

354  from interacting with each other upon secretion prevents them from forming one organized

355  sheet. Conversely, a confined spacing between the nascent sheet and the OM promotes proper

356  crystallization of the nascent microfibrils. This proximity could be maintained either by a

357  previously synthesized sheet preventing the nascent one from separating too far from the OM,

358  or by specialized cellulose binding enzymes situated in the outer-leaflet of the OM, such as

359  CmcAx, which has the ability to bind cellulose[52].

360

361  <u>Cryo-ET sheds light on the buildup of a microfibril</u>

362  Many values have been reported for the elementary fibrils' dimension, mainly through direct

363  observation by negative staining electron microscopy[18,33,38]. The most favored hypothesis is an

364  approximately 1.5-nm thick elementary fibril (thoroughly discussed in [28]). Very recently, the

365  characterization of the structure of the BcsC subunit (the OM pore) describes a 1.5 nm inner-

366  diameter pore with a very narrow constriction caused by a mobile gating loop, restricting the

367  channel to a 0.2 nm bottleneck[24]. It is however not known to what extent this gating loop can

368  open the pore. Therefore, two hypotheses arise: 1) one BcsC pore can accommodate a 1.5 nm

369  elementary fibril through an opening of the gating loop or 2) it can accommodate a smaller

370  elementary fibril, perhaps only a single glucan chain. In the latter case the building of the

371  elementary fibril would then take place upon secretion of the glucan chains in the environment.

372  While negative staining has provided high-resolution views of cellulose ribbons [28,38], observing

373  them in a frozen-hydrated state enables more accurate measurements of their dimensions and

374  observation of their interaction with the OM. This is particularly important for extracellular

15

375    polysaccharides, which have been shown to collapse and undergo drastic conformational

376    changes upon dehydration, staining and embedding[53].

377    We were able to image in two tomograms, microfibrils extruded perpendicularly to the OM and

378    integrating to form a thin parallel sheet (Fig. 4E-F). A possible interpretation of why these

379    events are rare is that they result from an accidental mechanical separation of the nascent sheet

380    from the OM, revealing early forms of cellulose bundling such as thin microfibrils. As

381    explained earlier, precise measurement of the thickness of densities is difficult in cryo-ET since

382    it is influenced by the defocus applied during imaging (causing overestimation of the true

383    thickness). Despite this uncertainty, our measurements are done in a near native state. We

384    estimated these microfibrils to be less than 11 nm in diameter (Fig. 4G-H), in line with previous

385    work which measured microfibril thicknesses from 3 to 12 nm  in cellulose sheets splayed apart

386    by cellulase treatments[54]. If we assume an elementary fibril is 1.5 nm in diameter and that it

387    can go through a single BcsC subunit, an 11 nm diameter cylindrical microfibril (maximal

388    thickness estimation) would comprise 53 elementary fibrils. This would require a cluster of 53

389    BcsC subunits. Previous reports have stated the cellulose extrusion pores    cluster in linear

390    bunches of 2 to 4 pores[7,33]. Accommodating both observations would require that there is more

391    than one BcsC subunit per extrusion pore. For example, if each 3.5 nm diameter extrusion

392    pore[39]  maximally held 5 BcsC subunits, a cluster of 11 extrusion pores could produce an 11

393    nm diameter microfibril (Fig. 9). In this case, each extrusion pore holding multiple BcsC

394    subunits would produce a crystalline aggregate of elementary fibrils which would pack with its

395    neighboring aggregates to form a microfibril.

396

397    <u>Cryo-ET sheds light on the assembly of a cellulose sheet</u>

398    We found that ribbons were stacks of sheets that likely interact loosely with one another since

399    the inter-sheet distance varied from 7- to 31-nm. This loose stacking corroborates previous

400   observations[34]. Previous measurements done by negative staining had estimated cellulose sheet

401   width to range from 40 to 600 nm[28,38,54], wider than our measurements ranging from 11 to 69

402   nm (Fig. 4I). These variations have been attributed to the cell strain, growth conditions and

403   intercellular variation[28,38,39]. We found that the thickness of cellulose sheets is similar to the

404   diameter of the microfibrils. Therefore, our data suggest that microfibrils lie down in rows to

405   create the width of the sheet. This was also suggested in[7].

406

407   While the number of sheets produced by a single cell increased with time, the main dimension

408   of growth appears to be ribbon length, as suggested by previous work and our fluorescence data

409   showing cellulose ribbons several cell lengths long (Fig. 1A-B)[29]. Wider sheets occur in later

410   time points (Fig. 4I), suggesting that sheet width also grows with time. However, in the current

411   model, sheet width is correlated with the number of extrusion pores, hence to cell length[7,39]. It

412   is possible that at 300min post-separation, cells are longer and possess more extrusion pores,

413   therefore producing wider sheets. As mentioned earlier, our attempt to observe this by

414   monitoring sheet width along its length failed. The magnification employed to acquire the data

415   would only allow us to capture partial lengths of the cells and their cellulose ribbon. We think

416   that upon the segments we captured, the sheet width increase or decrease, probably in the range

417   of 10 to 20 nm, was unlikely to be observe because of the estimates being heavily influenced

418   by the missing wedge.

419

420   <u>Cryo-ET on *G. hansenii* cells allowed the visualization of a novel cytoskeletal element, the</u>

421   <u>cortical belt</u>

422   Negative stain, cryo-fracture and immuno-EM studies have shown that cellulose extrusion

423   pores in *Gluconacetobacter* align in a line on one side of the cell [28,39,55], but what causes this

424   alignment is unknown. Here, we identify a novel cytoplasmic structure in two species of

425  *Gluconacetobacter* that spatially correlates with the nascent cellulose ribbon (Fig. 1C-E and

426  Fig. 5). This structure, which we term the cortical belt, is found at a fixed distance from the

427  inner membrane ($24 \pm 4$ nm) and remains intact upon cellulase treatment in shaking conditions

428  (Supplemental figure 1F, purple arrow), suggesting that it is stable even in the absence of the

429  cellulose ribbon and in turbulent culture conditions.

430  We observed the cortical belt in both *Gluconacetobacter* spp. imaged but not in other bacteria

431  that produce less-ordered forms of cellulose, including *Escherichia coli* 1094, which

432  synthesizes amorphous cellulose[47], and *Agrobacterium tumefaciens,* which synthesizes

433  cellulose I microfibrils[56] (Fig. 8). This suggests the cortical belt is a peculiar cytoskeletal

434  filament only found in the *Gluconacetobacter* genus. Its striking spatial colocalization with the

435  extracellular cellulose ribbons leads us to propose that the cortical belt functions in the

436  formation of cellulose ribbons. The periplasmic BcsD and its interacting partner CcpAx[22,31], as

437  well as two cell wall-related enzymes, have been shown to be involved in the crystallization

438  process of the ribbons [29,31,57]. It is possible that the cortical belt interacts with one or more of

439  these components to guide the positioning of the BCS complexes. Unfortunately, as stated

440  earlier, we failed to identify any repeated density above the cortical sheet that could be

441  associated to the secreting complexes. However, the thick cells and crowded periplasm

442  obscured and very likely masked relevant densities. Moreover, it is unknown whether these

443  secreting complexes are channels spanning the periplasmic space, given the structures of the

444  individual components[19–21,24,58]. Their predicted position in the membranes shows very small

445  portions protruding in the periplasm. We therefore think the BCS complexes are too

446  small/flexible for particle picking and sub-tomogram averaging in such a crowded environment.

447  If the cortical belt is responsible for scaffolding the BCS complexes, it represents a novel

448  prokaryotic cytoskeletal element, i.e. "a cytoplasmic protein filament and its associated

449  superstructures that move or scaffold material within the cell"[59]. Other bacterial cytoskeletal

18

450     elements have been observed to form belt-like structures, including bactofilins[60], or to stack,

451     like the CTP synthase[61], although with different dimensions. We hope that future work will

452     identify the component(s) that form the cortical belt, shedding more light on the molecular

453     processes involved in the organization and clustering of the BCS complexes in *G. hansenii*.

454

455     The cortical belt reveals another similarity between cellulose synthesis in *Gluconacetobacter*

456     and land plants.

457     Historically, the first plant cellulose synthase genes were identified by cDNA homology with

458     the *G. xylinum* acsA (bcsA) gene[62]. Later on, phylogenetic studies highlighted an early

459     divergence between cyanobacterial and plant cellulose synthases[63,64]. A large number of

460     cellulose I synthesizing organisms have in common that the synthase complexes arrange in

461     specific patterns, determining the final architecture of the cellulose structures[7]. A simple row

462     in systems like *Gluconacetobacter* spp. or certain charophytes and chlorophytes[65] and

463     hexameric rosette structures called Cellulose Synthase Complexes (CSC) in land plants. In

464     both, the extrusion of a crystalline form of cellulose exerts a force believed to be able to propel

465     the CSCs in plants[66,67] and the whole cell in *Gluconacetobacter*[29,38]. Our work uncovers an

466     additional similarity, the involvement of a cytoskeletal element, the cortical belt, to guide the

467     synthase complexes. In land plants CSCs have been shown to interact indirectly with underlying

468     cortical microtubules, mediating trans-membrane cross-talk[68–70], guiding and regulating CSC

469     velocity[71–73]. While CSCs were shown to be motile in land plants, they are believed to be static

470     in *Gluconacetobacter*[28], perhaps held in place by the cortical belt, in order to transfer the

471     propelling force to the whole cell.

472

473     Insights from FIB-milling native biofilms

474 Cryo-FIB milling through native biofilms offers the possibility of observing bacteria in the

475 context of their original biofilm environment and retrieving high resolution morphological and

476 positional information about the cells relative to one another and relative to the biofilm layers.

477 Visualization of the density and organization of the extracellular matrix and its interaction with

478 the cells is also rendered possible by cryo-FIB milling. This is especially important since in

479 nature most bacterial species are found in complex interacting communities, in the form of

480 homogeneous or heterogeneous communities that organize in biofilms[14].

481 Milling the *Gluconacetobacter* biofilms to 200 nm revealed numerous cytosolic vesicles of

482 variable shapes and sizes. Although we were not able to connect the presence of these numerous

483 vesicles with the process of cellulose production, cytosolic vesicles in bacteria are uncommon

484 but have already been observed several times in *M. xanthus*, *A. tumefaciens* and *E. coli* for

485 example[45]. Their detailed structure, function and biogenesis are not known. The cortical belt

486 was also visible, as in the isolated cells. The cellulose ribbons aligned with each other to form

487 larger arrays 2-3 µm wide (Fig. 6D, yellow arrowheads and supplemental video 3

488 https://figshare.com/s/74891ac625fe8125c60c), showing the propensity of these structures to

489 interact with each other. This propensity was previously characterized by live imaging of the

490 cellulose biosynthesis and crystallization process in *Gluconacetobacter,* which showed that the

491 bacterial cells preferentially follow already established tracks, *i.e.* previously synthesized

492 cellulose ribbons[29]. The occurrence of disorganized cellulose clusters in biofilms grown for 3h

493 but not 6h, suggests that such aggregates are either 1) digested by enzymes, likely CmcAx,

494 reported to have an endoglucanase capable of digesting amorphous cellulose[74] and to be present

495 on the surface of *G. hansenii* or released in the environment[30,52] or 2) diluted by a gradual

496 increase in well-ordered ribbons over time.

497 Cell death in biofilms, with the fraction of dead cells measured at 10% in our biofilms, is a

498 well-known phenomenon[14], caused by programmed cell death mechanisms, cannibalistic

499    behaviors such as already described in *B. subtilis*[75] or nutrient/oxygen depletion[76,77]. We did

500    not observe a preferential location of dead cells at the bottom of the biofilm, ruling out anoxic

501    conditions being the primary cause of cell death. This could be because the thickness of the

502    biofilm, between 1.5- and 3-um according to the cell depth distribution (Fig. 6H), is too small

503    to have a significant oxygen gradient, as suggested by studies that measured total anoxia being

504    reached generally between 70- and 80-um depth[77–79]. Processing thicker biofilms in the range

505    of tens of microns would allow visualization of the effects of nutrient/oxygen gradients on cell

506    distribution and physiology. For now, plunge freezing such as performed in this study can only

507    properly vitrify samples less than ~10 microns thick[80]. Moreover, milling thicknesses above 8-

508    10 microns becomes labor intensive and technically difficult. A possible course of action for

509    further studies would be to perform high-pressure freezing on thicker biofilms and then produce

510    thin sections either by cryosectioning, hybrid cryosectioning/FIB-milling methods such as

511    described in[81–83] or following a cryo-lift out procedure[83].

512

513

**Conflicts of interest**

515    The authors declare that there are no conflicts of interest.

516

21

523    the help and scientific advice given during this study and also to Candace Haigler for sharing

524    her thoughts and her precious experience on the not so common *Gluconacetobacter* spp.

525

526    **Methods**

527    <u>Cell culture</u>

528    *Gluconacetobacter hansenii* (ATCC 23769) was cultured as previously described [37] in SH

529    medium: 2% glucose, 0.5% bactopeptone, 0.5% yeast extract, pH 6. For solid medium, 2.5%

530    bacto-agar was added. Cells were separated from the cellulose biofilm by mechanical disruption

531    as previously described[38]. Briefly, the bacterial cellulose biofilm developing at the air-media

532    interface was picked up with a single-use sterile inoculating loop and transferred to fresh

533    medium, where it was vigorously shaken and then removed. In preparation for freezing, cells

534    were pelleted by centrifugation for 10 minutes at 2500rcf at 20C and resuspended in 0.5mL of

535    SH media. The culture was incubated for the desired length of time at 30ºC without shaking

536    before plunge freezing. For cellulose digestion, 0.2g/L cellulase (Worthington, purified exo-

537    and endo-glucanases, #LS002598) was added.

538

539    *Gluconacetobacter xylinus* (ATCC 700178/BPR2001) was cultured as described above in

540    Fructose–Peptone–Yeast Extract (FPY) media: 2% fructose, 1% bactopeptone, 0.5% yeast

541    extract and 0.25% $K_2HPO_4$.

542

543    *Escherichia coli* 1094 was cultured in Lysogeny Broth (LB) and induced for cellulose

544    production in minimal medium: 0.2% $(NH_4)_2SO_4$, 1.4% $KH_2PO_4$, 0.1% $MgSO_4$, 0.5%

545    $FeSO_4.7H_2O$, 0.4% glucose, 0.01% thiamine, pH 7. A saturated overnight LB culture was

546    diluted 1:50 into 3mL of minimal medium with or without 0.2g/L cellulase (Worthington,

547    purified exo- and endo-glucanases, #LS002598). Cultures were incubated at 37ºC with shaking

548   at 220rpm. When the medium transitioned from turbid to clear and white flakes appeared

549   (cellulose and bacteria), the induction of cellulose synthesis is considered successful.

550

551   *Agrobacterium tumefaciens* was cultured as described in previous work[84]. Briefly, *A.*

552   *tumefaciens* C58 was cultivated in liquid AB medium (glucose 0.2%, NH4Cl 18.7mM, MgSO4

553   2.5uM, KCl 2mM, CaCl2 0.07mM, FeSO4 0.01mM, K2HPO4 8.4mM, NaH2PO4.7H2O

554   4.16mM, pH 7) at 30C overnight. Induction was done by pipetting 100uL of overnight culture

555   and spreading onto AB induction plates (glucose 0.2%, NH4Cl 18.7mM, MgSO4 2.5uM, KCl

556   2mM, CaCl2 0.07mM, FeSO4 0.01mM, K2HPO4 8.4mM, NaH2PO4.7H2O 4.16mM, Bactagar

557   1.7%, Acetosyringone 100uM, pH 5.8). Plates were then incubated for 3 days at 20C. Cells

558   were resolubilized by scraping a small amount from the plate with an inoculation loop and

559   resuspending it in 100uL of liquid induction AB medium.

560   The following strains are the ones included in the tomogram analysis: **NT1** is a C58 strain

561   without the pTiC58 (tumor inducing) plasmid; **A139** strain is NT1REB(pJK270) + pJZ041.

562   NT1REB is a "bald strain", no flagellin mutant, derived from NT1. The pJK270 is pTiC58 with

563   the transposed NPTII gene for kanamycin resistance. The pJZ041 plasmid carries a GFP tagged

564   VirB8 gene, a component of the T4SS (Aguilar et al. 2011); **JX148** strain is a C58 derived

565   mutant of the rem gene. The strain is non motile; **AD348** is a GV3101(pMP90) strain with its

566   whole VirB system deleted. GV3101 is a pTiC58 free, rifampicin resistant C58 strain and

567   pMP90 is a helper pTiC58 without the T-DNA; **AD1484** is a AD348 variant, transformed with

568   pAD2079 containing the whole VirB system.

569

570

571

572   <u>Confocal microscopy</u>

23

573 Cellulose was stained with Calcofluor-white (Sigma-Aldrich, #18909) at a concentration of

574 0.001% and cell membranes were stained with MitoTracker Deep Red FM (Thermo-Fisher,

575 #M22426) at a concentration of 0.5ug/uL. Stack acquisition was done on a Zeiss LSM880 Airy

576 Scan microscope. Airy scan acquisitions were performed in super-resolution mode with Z-step

577 set at the optimal optical sectioning. The Mito-Tracker Deep Red FM channel was set as the

578 following: excitation at 633 nm, use of the 488/561/633 main beam splitter and a band-pass

579 570-620 + long-pass 645 filter. The Calcofluor White channel was set as the following:

580 excitation at 405 nm, use of the 405 main beam splitter and a band-pass 420-480 + band-pass

581 495-550 filter. Airy scan processing was performed on the fly by the in-built algorithm of Zeiss

582 Black.

583

584 Sample preparation for cryo-EM

585 For isolated cells, Quantifoil Cu R2/2 Finder grids (*Quantifoil Micro Tools GmbH*) were glow-

586 discharged at 15mA for 1min. The grids were pre-incubated with fiducial marker solution

587 prepared as follows: 50µL of 10nm colloidal gold (*Ted Pella, Inc*) mixed with 50uL of 5%

588 BSA, vortexed 1 min and centrifuged at 15,000rcf for 15 min, supernatant discarded, and pellet

589 resuspended in 40µL of PBS buffer. 3µL were deposited on each grid, left for 1 minute then

590 back-blotted with Whatman paper. Cells were plunge frozen with a Vitrobot Mark IV (*Thermo

591 Fisher Scientific*) with 100% humidity at 30ºC and back-blotted for 3 to 5s.

592

593 For native biofilms, Quantifoil gold R2/2 Finder grids were placed in 35mm glass bottom petri

594 dishes (*MatTek Corporation* #P35G-1.0-2.0C) containing 1mL of SH media inoculated with a

595 2-day old biofilm. The dishes were sealed with Micropore tape (*3M*) and incubated without

596 shaking at 30ºC for 3 to 6 hours. Plunge-freezing was done at 22C, 50% humidity, either with

597 manual blotting on both sides of the grids (first back-blotted then front-blotted) or using the

24

598  automatic blotting function of the Vitrobot with a blot time of 5-6s, blot force of 15 and drain

599  time of 2s.

600

601  For *E. coli* 1094, after 4 hours of incubation in minimal media, the medium should turn from

602  turbid to clear with white flakes. $OD_{600}$ of the cultures was monitored using the culture (always

603  turbid) where cellulose induction was performed in the presence of cellulase to keep the cells

604  from aggregating. It was then used as a reference to concentrate the cells to high $OD_{600}$ (10-

605  20), in order to form bacterial mats on the EM grids, for control and cellulase conditions.

606  Plunge-frozen was done at 20C, 100%, either with manual back-blotting for 5-7s and a drain

607  time of 1s or using the automatic blotting function of the Vitrobot with a wait time of 10s, blot

608  time of 5-6s, blot force of 3 and drain time of 1s.

609

610  <u>FIB milling</u>

611  Grids were clipped in Autogrid holders (*Thermo Fisher*) machined with a notch to allow FIB

612  milling closer to the edge of the grid. Autogrids were placed in a custom-built shuttle and

613  inserted into a Versa 3D dual-beam FIB/SEM microscope with FEG (*FEI*) equipped with a

614  PP3000T cryo-transfer apparatus (*Quorum Technologies*). They were maintained at -175ºC at

615  all times by a custom-built cryo-stage[85]. To reduce sample charging and protect the sample

616  from curtaining during milling, the grids were sputter-coated with platinum at 15 mA for 60

617  seconds. Thin lamellae were generated with the $Ga^+$ ion beam at 30 kV at angles ranging from

618  10 to 17 degrees. Rough milling was done at high currents, ranging from 0.3 nA to 100 pA until

619  the lamellae measured 1 micron in thickness under the FIB view. Current was then

620  progressively brought down to 10 pA for the final milling steps until the measured thickness

621  was between 100-200 nm. Final polishing of the back end of the lamella is also done at 10pA

622     where the sample is tilted +0.5 to 1° to homogenize the lamella thickness. During the whole

623     procedure, imaging with the SEM beam was done at 5 kV and 13 pA.

624

625     Electron cryo-tomography

626     Tomography of whole cells and FIB-milled lamellae was performed on either a Titan Krios or

627     Tecnai G2 Polara transmission electron microscope (*Thermo Fisher*) equipped with 300 kV

628     field emission gun, energy filter (*Gatan*) and K2 or K3 Summit direct electron detector (*Gatan*).

629     The Krios is equipped with a Volta phase plate (*Thermo Fisher*) [86]. Tilt-series acquisition was

630     done with SerialEM [87] with a 2-3˚ tilt increment for a total range of ±60˚ or ±50˚, defocus of -

631     4, -6 or -8 µm, and total dose up to 180 e$^-$/Å$^2$. Volta phase plate imaging was performed in

632     Figures 1, 2, 5 and 7A-B with a defocus of -2µm and a measured phase shift of 0.5 π/rad before

633     tilt series acquisitions.

634

635     Low magnification tomography on the biofilm lamellae was performed at 6500 magnification

636     (14 Å$^2$ pixel size) with a -10 or -15 µm defocus and a total dose between 5 and 10 e$^-$/Å$^2$.

637     Tomography of FIB-milled lamellae was done exclusively on the Titan Krios. Because samples

638     were thinner, the total dose was limited to ~80 e$^-$/Å$^2$.

639

640     Data processing

641     Tomograms were reconstructed using the IMOD software (http://bio3d.colorado.edu/imod/) [88].

642     Alignment was done on 1k x 1k binned tilt-series with fiducial-based alignment. Aligned stacks

643     were low-pass filtered (0.35, σ = 0.05) to eliminate high-frequency noise. Weighted back

644     projection reconstruction was performed and the "SIRT-like filter" was used with 20 iterations.

645     Segmentation was also done using IMOD and drawing tools developed by Andrew Noske

646     (http://www.andrewnoske.com/student/imod.php). To better distinguish features during the

647    segmentation steps, tomograms were filtered with the 3D non-linear anisotropic diffusion filter

648    in IMOD. The cell contours and cortical belt were segmented manually on a Cintiq 21uX tablet

649    (*Wacom*) and cellulose was segmented using a semi-automated thresholded method. 1) A

650    denoising Non-linear Anisotropic Diffusion filter was applied (included in the *etomo* package,

651    http://bio3d.colorado.edu/imod/) on the tomogram; 2) Precise boundary models are drawn

652    around the structures to be thresholded; 3) Thresholding segmentation is performed with

653    3Dmod using the isosurface function and the previously drawn contours are used as a mask.

654    When the contours are precisely following the contours, this technic allows to raise the

655    isosurface threshold without picking up background noise.

656    Measurements for all distances between elements (cellulose sheet – outer-membrane, width of

657    the cellulose ribbon, cortical belt – inner-membrane) were taken by generating normalized

658    density profile plots and measuring the distances between the density peaks of the

659    corresponding sub-cellular features (Fig. 3). This was automated with a custom script, *sideview-*

660    *profile-average*, written by Davi Ortega (https://www.npmjs.com/package/sideview-profile-

661    average).

662

663    Estimation of the cell depth in the native biofilm lamellae was calculated as follows: 1) using

664    the two parallel walls of the milled trench, a perpendicular line is traced at the leading edge of

665    the lamella (where the platinum meets the frozen material); 2) Lines are drawn from the center

666    of the cells to the leading edge perpendicular line (Fig. 6H, red line in top view of lamella); 3)

667    The distance from the cell center to the limit of the platinum on the leading edge, which is the

668    surface of the sample, is measured. The real depth is then calculated using the following

669    equation: opposite side (real depth) = tan (*a*) x adjacent side (distance measured, d in Fig. 6H).

670    The angle *a* is the angle between the grid surface and the FIB gun during the milling process,

671    which can be accurately measured during reconstruction with 3dmod.

672

673 <u>Statistical analysis</u>

674 All statistics were performed with GraphPad Prism software

675 (https://www.graphpad.com/scientific-software/prism/). All datasets were first analyzed for

676 normality using the Shapiro-Wilk test and homoscedasticity (equal standard deviations). If

677 dataset is normal, appropriate parametric tests were performed and if not, appropriate non-

678 parametric tests were performed. Detailed statistical tests are listed in order of appearance in

679 the manuscript.

680

681 **Figure 2E:** n = 3 and 23 for the "loose" and "tight" configuration respectively. Two tailed P-

682 value = 0.0008, Mann-Whitney test.

683 **OM-to-closest-sheet distance in 20min vs 300min post separation cells:** n = 4, 2, 23 and 3

684 for 20min "tight", 20min "loose", 300min "tight" and 300min "loose" configurations,

685 respectively. Kruskal Wallis test followed by Dunn's multiple comparison test was performed.

686 20min "tight" vs 20min "loose", 300min "tight" and "loose" showed adjusted P-values of 0.12,

687 >0.99 and 0.024, respectively. 20min "loose" vs 300min "tight", "loose" and 300min "tight" vs

688 300min "loose" showed adjusted P-values of 0.23, >0.99 and 0.032, respectively.

689 **Figure 4A**: n = 6, 15, 33 for 13-, 20- and 300-minutes, respectively.

690 **Figure 4B**: n = 6 and n = 21 tomograms for 20- and 300-minutes post-separation, respectively.

691 Two tailed P-value < 0.0001, One sample Wilcoxon signed rank test against a theoretical value

692 of 1 (number of sheets observed at 20-min post-separation).

693 **Figure 4H**: n = 12 and 4 microfibril thickness measurements performed on two separate

694 tomograms (Cell #1 and #2, left side of the graph). N = 47 measurements for inter-sheet

695 distances performed on 23 tomograms. ANOVA followed by Tukey's multiple comparison test

696    was performed. Cell #1 vs Cell #2, Cell #1 vs 300-min inter-sheet distances and Cell #2 vs 300-

697    min inter-sheet distances showed adjusted P-values of 0.073, 0.15 and 0.0015, respectively.

698    **Figure 4I**: n = 6 and 45 sheets measured at 20- and 300-minutes post-separation. Welch's t test

699    (parametric t-test without equal SD assumption) showed a P-value of 0.23.

700    **Figure 6F**: n = 6 and 4 for biofilms let to grow for 3h and 6h, respectively. Unpaired T-test

701    showed a two-tailed P-value of 0.0011.

702    **Figure 6G**: n = 6 and 4 for biofilms let to grow for 3h and 6h, respectively. Unpaired T-test

703    showed a two-tailed P-value of 0.2720.

704    **Figure 6H**: n = 49, 46, 4 and 11 for live and dead cells in 3h and 6h biofilms, respectively.

705    Mann-Whitney tests were performed on live vs dead cells in 3h and 6h biofilms conditions,

706    showing two-tailed P-values of 0.82 and 0.54, respectively.

707    ***G. hansenii* cellulose sheet width versus *A. tumefaciens* cellulose fibrils width:** n = 52, 45

708    and 6 width measurements on A. tumefaciens, G. hansenii 20-min and 300-min post-separation,

709    respectively. Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple

710    comparisons test was performed. T-20min vs t-300min, t-20min vs A. tumefaciens and t-

711    300min vs A. tumefaciens showed adjusted P-values of 0.25, 0.11 and <0.0001, respectively.

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713    **Bibliography**

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1002   **Figures**

1003   **Figure 1 | Interactions between the bacterial envelope and the cellulose ribbon: the "tight"**

1004   **configuration**

1005   (**A**) Confocal-Airy scan optical slices show representative examples of *G. hansenii* cells in red

1006   (MitoTracker Deep Red FM) displaying the cellulose ribbon on their side in cyan (Calcofluor-

1007   white). (**B**) Enlarged view indicated by white dashed rectangle in (A). The cellulose structure

1008   is clearly seen closely appended to one side of the cell (cyan arrowheads). (**C**) 9-nm thick

1009   tomographic slice showing the typical *G. hansenii* cell harboring the cellulose ribbon on its

1010   right side (yellow arrows). White arrowheads point to ribosomes and red arrows point to

1011   cytosolic vesicles. Here and below, IM: Inner-membrane; OM: Outer-membrane; S: Storage

1012   granule; CB: Cortical belt. (**D**) Manual segmentation of the cell shown in (C). (**E**) Rotated

1013   segmented volume shown in (D) showing the very close contact between the cellulose ribbon

1014   (yellow) and the outer membrane (green). (**F-H**) Transverse 9-nm thick tomographic slices

1015   through the bacterial envelope of the cell shown in (C) at the levels indicated by the blue, black

1016   and red dashed lines, respectively. Two cellulose sheets (yellow arrows) are seen. One interacts

1017   with the OM all along (white arrow). Our working model is that integration of the cellulose

1018   fibers into the sheet occurs immediately upon secretion.

1019

1020 **Figure 2 | Interactions between the bacterial envelope and the cellulose ribbon: the "loose"**

1021 **configuration**

1022  (**A**) 9-nm thick tomographic slice showing a cell where aggregates of disorganized cellulose

1023 (orange asterisks) occur between the ribbon (yellow arrows) and the OM. Note the cortical belt

1024 (CB) cannot be seen in this slice. Black line orange arrows indicate points of contact between

1025 the cellulose sheet and the disorganized aggregates. Red arrows point to vesicles. (**B**) Manual

1026 segmentation of the tomogram in (A) showing these disorganized aggregates in 3-D. (**C-D**)

1027 Transverse 9-nm thick tomographic slices through the envelope of the cell shown in (A) at the

1028 levels indicated by the blue and pink dashed lines highlighting the distance between the two

1029 cellulose sheets (yellow arrows) and the OM and the presence of the disorganized clusters

1030 (orange dashed brackets). (**E**) Plot showing the OM-to-closest sheet distance in the two types

1031 of configuration. n = 3 and 23 for the "loose" and "tight" configuration respectively. Two tailed

1032 P-value = 0.0008, Mann-Whitney test.

1033

1034 **Figure 3 | Cellulose sheet dimensions**

1035 (**A-B**) Longitudinal and transverse schematic depiction defining the different dimensions

1036 measured, namely OM-to-sheet distance, sheet width and inter-sheet distance. Identical

1037 terminology is used for the measurements of the cortical belt. (**C**) Transverse 12-nm thick slice

1038 of the bacterial envelope of the cell shown in (D) at the level indicated by the blue dashed line.

1039 The yellow arrows highlight the two stacked sheets. On the right, the average density profile

1040 along the red line demonstrates how the cellulose sheet widths were estimated. Vertical axis is

1041 length in nm along the red line and horizontal axis is the normalized electron density. (**D**) 12-

1042 nm thick tomographic slice showing the typical organization of the bacterial envelope on the

1043 side where cellulose sheets (yellow arrows) are being synthesized. The average density profile

42

1044    on the right taken along the red line shows the CB-IM, IM-OM OM-sheet and inter-sheet

1045    distances (green dashed lines).

1046

1047    **Figure 4 | The cellulose ribbon is a composite structure made of stacked sheets**

1048    (**A**) Percentages of cells exhibiting disorganized aggregates (blue) and cellulose ribbons (red)

1049    at 13-, 20- and 300minutes post-separation. While disorganized aggregate occurrence is steady,

1050    there is an increase in the occurrence of cellulose ribbons over time. n = 6, 15, 33 for 13-, 20-

1051    and 300-minutes, respectively. (**B**) Number of cellulose sheets composing the ribbons as a

1052    function of time after cell separation. n = 6 and n = 21 tomograms for 20- and 300-minutes

1053    post-separation, respectively. Two tailed P-value < 0.0001, One sample Wilcoxon signed rank

1054    test against a theoretical value of 1 (number of sheets observed at 20-min post-separation). (**C**)

1055    Composite image composed of 10-nm thick tomographic slices spaced by 24 nm in Z, of a cell

1056    20 minutes post separation in the "tight" configuration. The cellulose ribbon is thin (yellow

1057    arrows), composed of one sheet immediately adjacent to the OM. Limits of the two original

1058    images are indicated by the red dashed line. (**D**) 11 nm thick tomographic slice of a cell 300

1059    minutes post-separation. The cellulose ribbon (yellow arrows) is large and composed of

1060    multiple sheets. (**E**) Nascent cellulose sheet 20 minutes post-separation (yellow arrow). Putative

1061    microfibrils can be seen coming out perpendicularly from the outer membrane (red

1062    arrowheads). (**F**) Corresponding manual segmentation of (E). (**G**) Enlarged view of the blue

1063    boxed region in (E). Below is the average density profile showing the estimation of the diameter

1064    of one putative microfibril (red line). (**H**) Estimated diameters of microfibrils observed at 20-

1065    minutes post-separation in the two cells where they are visible (left vertical axis) as in (E) and

1066    the inter-sheet distances measured in the 300-minutes post-separation cellulose ribbons (right

1067    vertical axis). n = 12 and 4 microfibril thickness measurements performed on two separate

1068    tomograms (Cell #1 and #2, left side of the graph). N = 47 measurements for inter-sheet

43

1069   distances performed on 23 tomograms. ANOVA followed by Tukey's multiple comparison test

1070   was performed. Cell #1 vs Cell #2, Cell #1 vs 300-min inter-sheet distances and Cell #2 vs 300-

1071   min inter-sheet distances showed adjusted P-values of 0.073, 0.15 and 0.0015, respectively. (**I**)

1072   Sheet width estimations at 20- and 300-minutes post separation. n = 6 and 45 sheets measured

1073   at 20- and 300-minutes post-separation. Welch's t test (parametric t-test without equal SD

1074   assumption) showed a P-value of 0.23.**Figure 5 | The cortical belt lies below the cellulose**

1075   **ribbon in the cytoplasm**

1076   (**A**) 9-nm thick tomographic slice showing a representative cortical belt (purple arrows) just

1077   inside the IM and proximal to the cellulose ribbon on the outside of the cell (yellow arrows).

1078   (**B**) Manual segmentation of the tomogram shown in (A) highlighting the cellulose ribbon and

1079   the cortical belt. (**C**) Same segmentation rotated 90° about the long axis of the cell shows how

1080   the cortical belt and the cellulose ribbon follow the same trajectory. (**D**) 9-nm thick tomographic

1081   slice taken from the same tomogram as in figure 2, showing one out of several cases where the

1082   cortical belt presented stacked layers (red dashed box). (**E**) Enlarged view of the red dashed

1083   boxed region in (D) showing the arrangement of the stacked layers. On the right is a density

1084   profile displayed normal to the cortical belt to measure the inter-layer distance (15 nm). (**F**)

1085   Transverse 9-nm thick tomographic slice of the cell region shown in (D), at the level indicated

1086   by the blue dashed line, highlighting stacked layers of the cortical belt. The cellulose ribbon

1087   can be seen at a distance (yellow arrowheads) with disorganized aggregates in between (orange

1088   dashed brackets and asterisk).

1089

1090   **Figure 6 | FIB-milling through native *G. hansenii* biofilms**

1091   (**A**) cryo-SEM overview of a 6-hour biofilm (outlined in red) grown on a gold quantifoil grid.

1092   (**B**) cryo-SEM view of a thick biofilm area (boxed in blue in (A)). Wrinkles in the biofilm are

1093   typical of a biofilm a few microns thick. (**C**) Milled lamella (boxed in yellow) from the green

1094    boxed region shown in (A). (**D**) 23-nm thick tomographic slice of a low mag tomogram taken

1095    on the lamella shown in (C). Living (when frozen) and dead cells are visible (green and red

1096    asterisks, respectively) and large cellulose arrays can be seen filling the gaps between the cells

1097    (yellow arrowheads). (**E**) Manual segmentation of the tomogram shown in (D). (**F**) Fraction of

1098    the lamella volume occupied by the cells was assessed for each lamella. n = 6 and 4 for biofilms

1099    let to grow for 3h and 6h, respectively. Unpaired T-test showed a two-tailed P-value of 0.0011.

1100    (**G**) Live cell ratio in 3h and 6h biofilms. n = 6 and 4 for biofilms let to grow for 3h and 6h,

1101    respectively. Unpaired T-test showed a two-tailed P-value of 0.2720. (**H**) Violin boxplots

1102    reporting the absolute depth of the live and dead cells within the biofilms grown for 3 and 6

1103    hours. The dashed red lines indicate the first and third quartiles and solid red line represents

1104    median. This shows that while the biofilms get thicker with time, the ratio of live-to-dead cells

1105    appears constant through depth and time. Method of calculation is detailed on the left of the

1106    panel and in the methods section. Lamella is drawn in blue, with the platinum coated leading

1107    edge represented in gray. n = 49, 46, 4 and 11 for live and dead cells in 3h and 6h biofilms,

1108    respectively. Mann-Whitney tests were performed on live vs dead cells in 3h and 6h biofilms

1109    conditions, showing two-tailed P-values of 0.82 and 0.54, respectively.

1110

1111    **Figure 7 | Lamellae of native biofilms also reveal numerous vesicles and the cortical belt**

1112    (**A-B**) Two tomographic slices of a *G. hansenii* cell from a biofilm grown for 6h surrounded by

1113    cellulose ribbons (yellow arrowheads). The cortical belt is visible in (B) (purple arrow) and

1114    seems to follow the trajectory of the cellulose sheet proximal to the OM (dark lined yellow

1115    arrowhead). (**C**) Manual segmentation of the tomogram displayed in (A) and (B) showing the

1116    juxtaposition of the cortical belt (purple to red) and the nascent cellulose ribbon (yellow). (**D**)

1117    Enlargement of the boxed region in (B) showing the layered cortical belt. (**E**)  Tomographic

1118    slice of a cell surrounded by cellulose ribbons (yellow arrowheads) from a biofilm grown for

45

1119    3h and harboring numerous vesicles in its cytosol (white arrowheads). Disorganized aggregates

1120    (orange dashed lines) are visible at this timepoint. (**F-G**) Tomographic slices showing

1121    additional examples of disorganized cellulose aggregates (orange dashed lines) surrounded by

1122    cellulose ribbons (yellow arrowheads) visible in 3h biofilms. Scale bars = 100 nm. All

1123    tomographic slices are 11-nm thick.

1124

1125    **Figure 8 | The cortical belt is not found in other cellulose-synthesizing species**
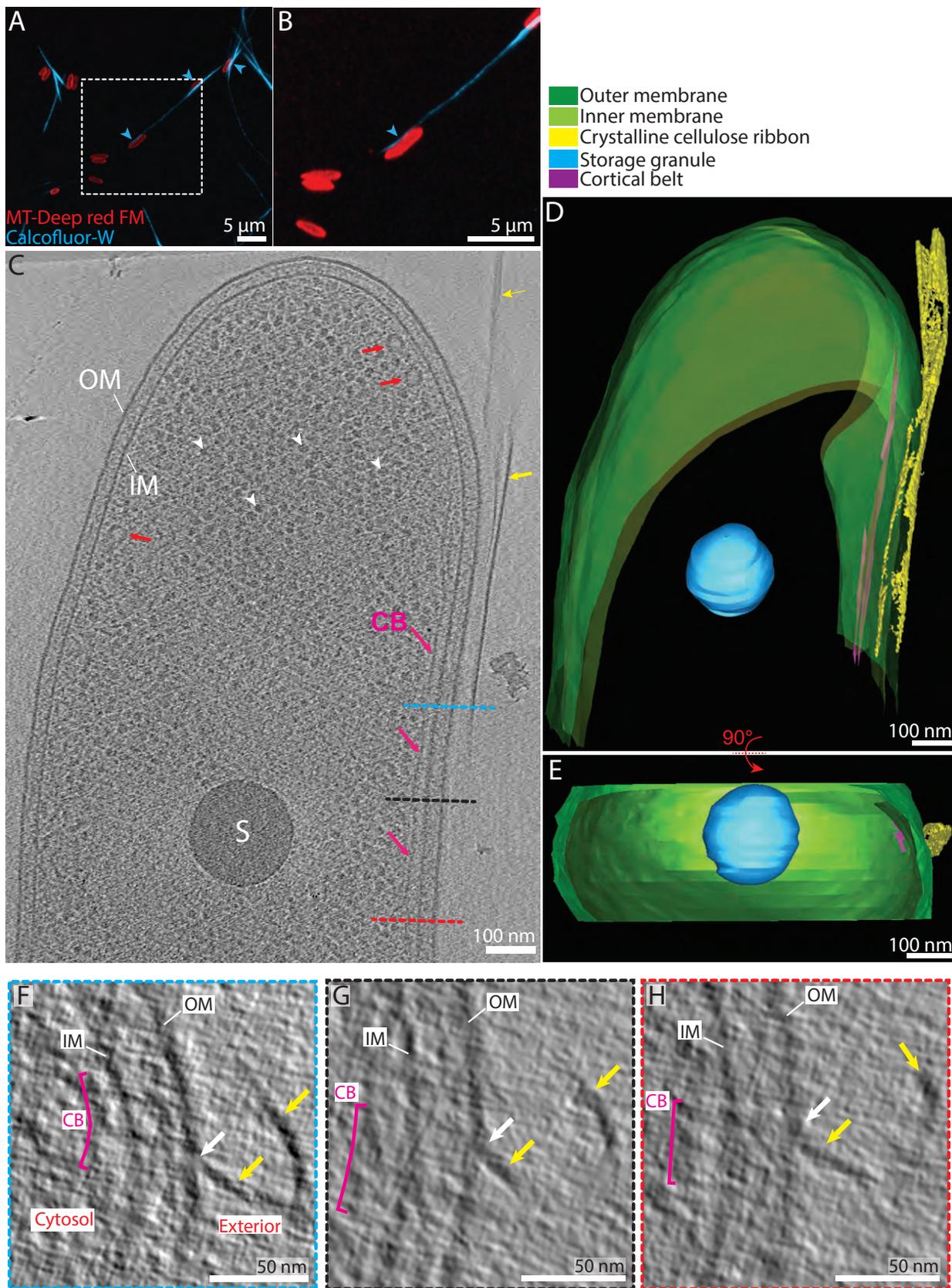
1126    (**A**) Maximum projection of *A. tumefaciens* cells synthesizing cellulose. Cells are stained with

1127    Mito Tracker Deep Red (red) and cellulose with Calcofluor-white (cyan). (**B**) 10-nm thick

1128    tomographic slice of a typical *A. tumefaciens* cell with cellulose microfibrils around (yellow

1129    arrowheads). No cortical belt can be seen in the cells. A polar flattening can be seen at the lower

1130    pole (cyan arrow) with an amorphous aggregate (orange dashed lines). These aggregates are

1131    most probably the UniPolar-Polysaccharide (UPP) synthesized specifically by *A. tumefaciens*.

1132    (**C**) Manual segmentation of the tomogram in (B) showing the organization of the cellulose

1133    microfibrils around the cell, the absence of the cortical belt and the putative UPP.   (**D**) 50-nm

1134    optical slice of an induced *E. coli 1094* cellulose biofilm. Cells are stained with mitoTracker

1135    Deep Red (red) and cellulose with Calcofluor-white (cyan). (**E**) 6-nm tomographic slice of a

1136    lamella tomogram of bacterial mat showing three *E. coli 1094* cells and an amorphous cellulose

1137    aggregate between them (orange asterisk). (**F**) 6-nm tomographic slice of a lamella through a

1138    bacterial mat treated with cellulase, showing multiple cells. No cellulose was visible in this

1139    condition. No cortical belt can be seen in the cells in either condition.

1140

1141    **Figure 9 | Updated cell-directed hierarchical model**

1142    Top (left) and side (right) view of a *G. hansenii* cell showing the different aggregation steps

1143    leading to a cellulose sheet, how microfibrils contribute to sheet width and the role of the

1144    cortical belt. In this model, clusters of 11 extrusion pores are depicted (green circles), the real

1145    numbers and distribution are unknown. Each extrusion pore is presented as comprising 5 BcsC

1146    subunits each (red circles), the actual number is not known. Inset in blue is a magnified view

1147    of the line of 11 extrusion pores, each hypothesized to extrude an aggregate of multiple

1148    elementary fibrils (yellow dashed lines). All aggregates then coalesce to form a microfibril of

1149    increasing thickness as it incorporates an increasing number of elementary fibril aggregates.

1150    These microfibrils then stack together, contributing to the width of the cellulose sheet. Adapted

1151    from the cell in figure 4E-G.

Legend:
- Outer membrane
- Inner membrane
- Crystalline cellulose ribbon
- Storage granule
- Cortical belt

A. MT-Deep red FM / Calcofluor-W — 5 µm
B. 5 µm
C. OM, IM, CB, S — 100 nm
D. 90° — 100 nm
E. 100 nm
F. OM, IM, CB, Cytosol, Exterior — 50 nm
G. OM, IM, CB — 50 nm
H. OM, IM, CB — 50 nm

Outer membrane
Inner membrane
Crystalline cellulose ribbon
Cortical belt
Disorganized cellulose aggregates

A

Cell surface
Long axis of the cell

Sheet thickness

Intersheet distance

$^a$ OM-to-closest sheet distance
$^b$ Microfibril thickness

Length

B

Long axis of the cell
Cell surface
Sheet thickness

90°

Width

Transverse view

C

density (arbitrary unit)

IM
OM

CB

50 nm

49 nm

Cellulose sheet width

D

CB
OM
IM

100 nm

density (arbitrary unit)

Cellulose ribbon

CB
20 nm
IM
26 nm
OM
32 nm
9 nm
7 nm
12 nm

Inter-sheet distances

A

Cellulose ribbons
Disorganized aggregates

Fraction of cells (%)

13min    20min    300min

B

# cellulose sheets / ribbon

20min    300min

C

OM
IM
CB

100 nm

+ 20 min

D

OM
IM

100 nm

+ 300 min

E

100 nm

F

Bacterial envelope
Cellulose

100 nm

G

50 nm

11 nm

density (arbitrary unit)

0  2  4  6  8  10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

H

Microfibril diameter (nm)

Intersheet distance (nm)

Cell #1    Cell #2    t-300min

I

Sheet width (nm)

20min    300min

A

IM

OM

CB

100 nm

B

100 nm

C

90°

CB

100 nm

D

100 nm

Outer membrane
Inner membrane
Cellulose ribbon
Cortical belt

E

CB 1
CB 2
CB 3
IM
OM

15 nm
15 nm
19 nm
31 nm

50 nm

F

IM
OM

*

50 nm

**Figure 5 | The cortical belt lies below the cellulose ribbon in the cytoplasm**
(**A**) 9-nm thick tomographic slice showing a representative cortical belt (purple arrows) just inside the IM and proximal to the cellulose ribbon on the outside of the cell (yellow arrows). (**B**) Manual segmentation of the tomogram shown in (A) highlighting the cellulose ribbon and the cortical belt. (**C**) Same segmentation rotated 90° about the long axis of the cell shows how the cortical belt and the cellulose ribbon follow the same trajectory. (**D**) 9-nm thick tomographic slice t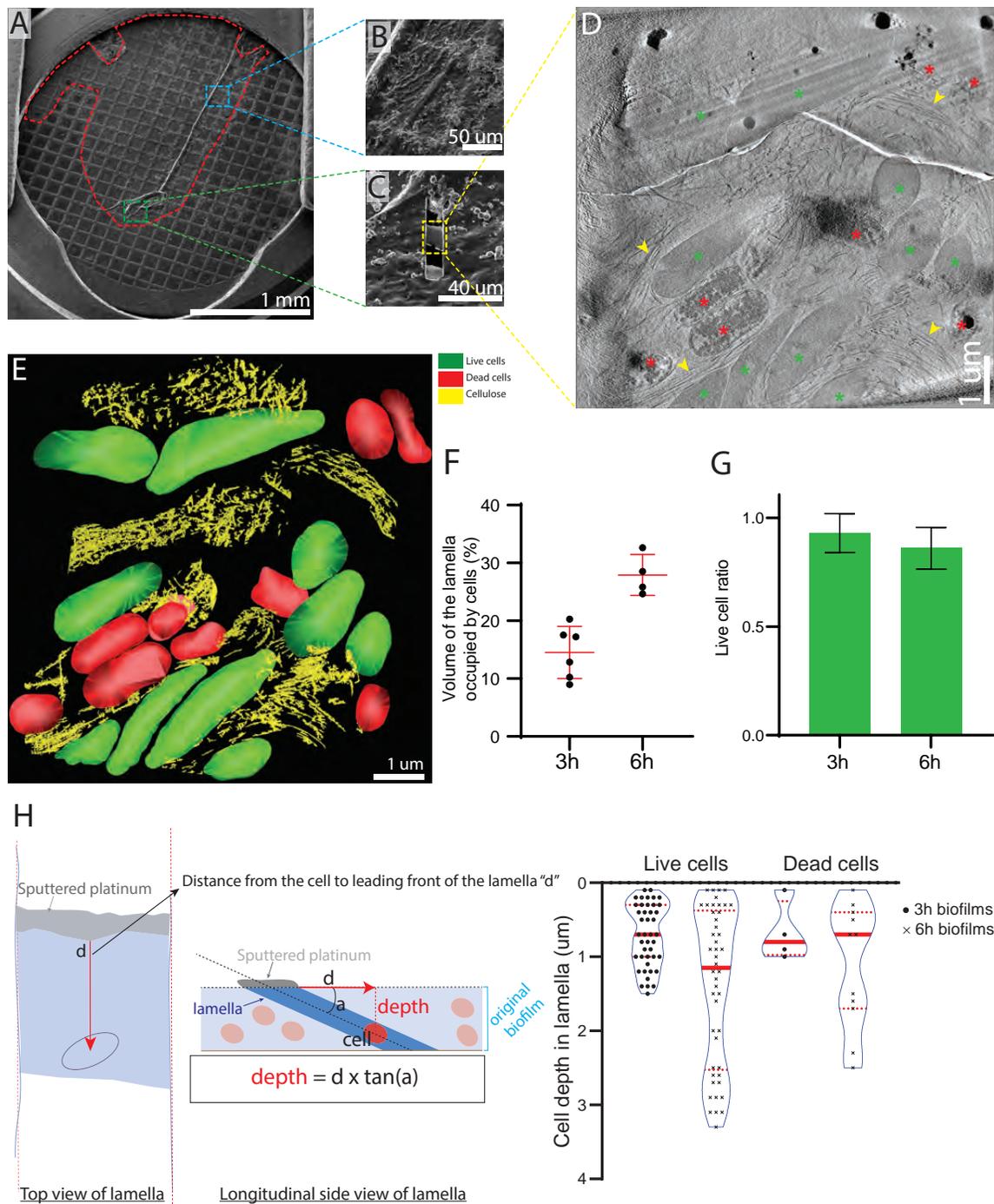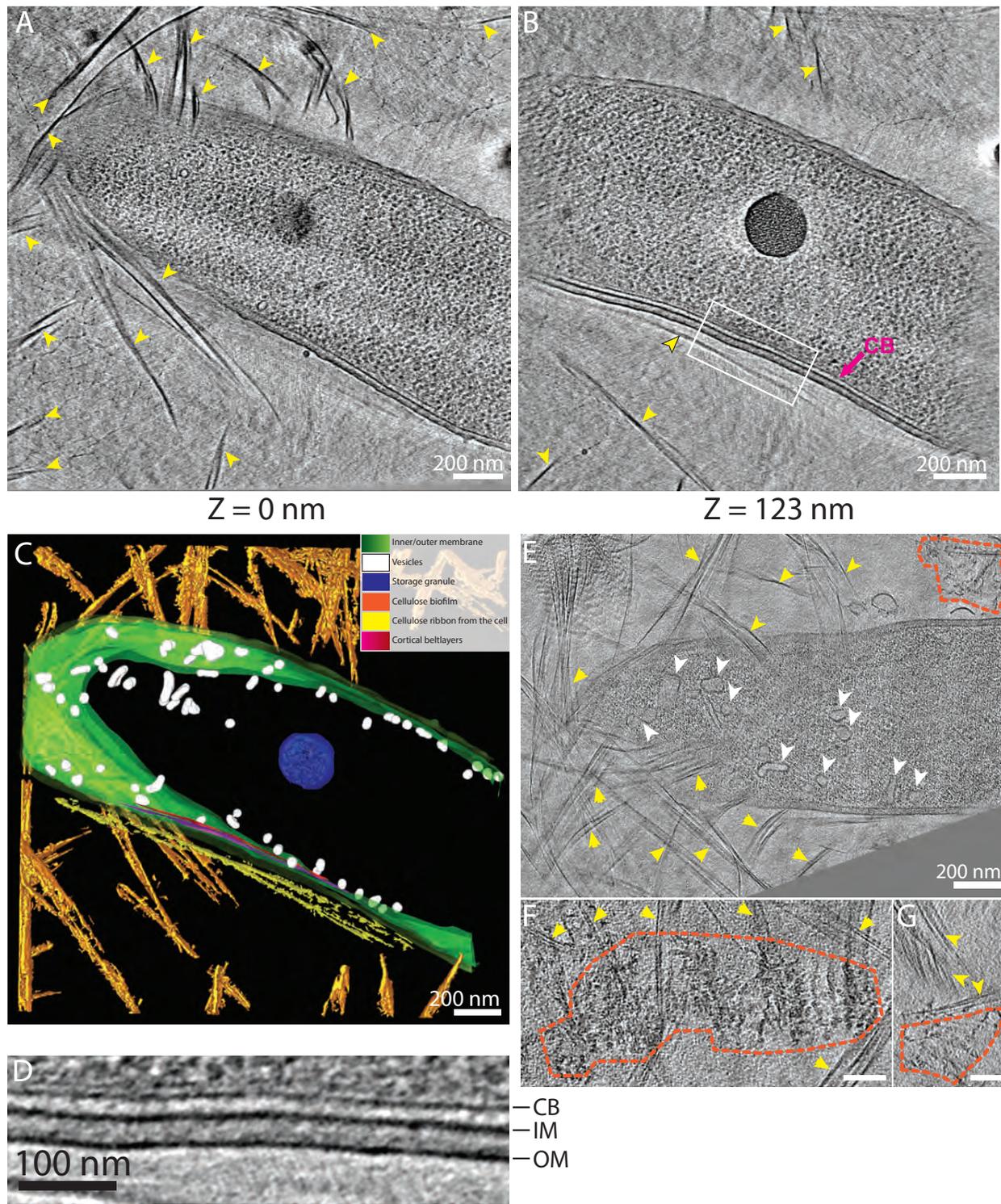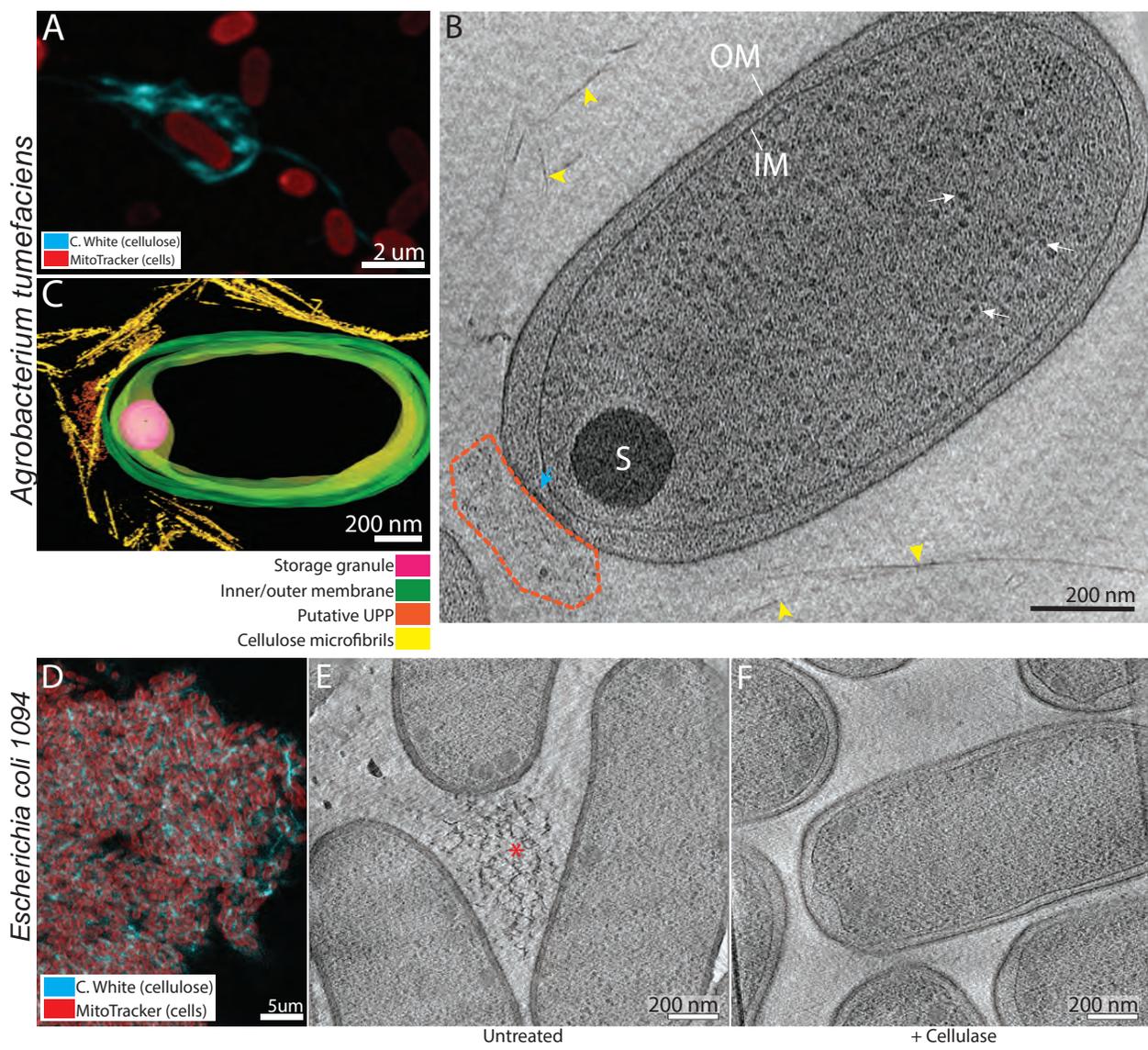aken from the same tomogram as in figure 2, showing one out of several cases where the cortical belt presented stacked layers (red dashed box). (**E**) Enlarged view of the red dashed boxed region in (D) showing the arrangement of the stacked layers. On the right is a density profile displayed normal to the cortical belt to measure the inter-layer distance (15 nm). (**F**) Transverse 9-nm thick tomographic slice of the cell region shown in (D), at the level indicated by the blue dashed line, highlighting stacked layers of the cortical belt. The cellulose ribbon can be seen at a distance (yellow arrowheads) with disorganized aggregates in between (orange dashed brackets and asterisk).

A

B
50 um

C
40 um

D

1 mm

Live cells
Dead cells
Cellulose

E
1 um

F

Volume of the lamella occupied by cells (%)

40
30
20
10
0
            3h        6h

G

Live cell ratio

1.0
0.5
0.0
            3h        6h

H

Distance from the cell to leading front of the lamella "d"

Sputtered platinum

d

Sputtered platinum

d
a
lamella
depth
cell
original biofilm

depth = d x tan(a)

Top view of lamella        Longitudinal side view of lamella

Live cells        Dead cells

Cell depth in lamella (um)

0
1
2
3
4

• 3h biofilms
× 6h biofilms

Z = 0 nm

Z = 123 nm

Inner/outer membrane
Vesicles
Storage granule
Cellulose biofilm
Cellulose ribbon from the cell
Cortical beltlayers

100 nm

— CB
— IM
— OM

*Agrobacterium tumefaciens*

A
C. White (cellulose)
MitoTracker (cells)
2 um

C
200 nm

Storage granule
Inner/outer membrane
Putative UPP
Cellulose microfibrils

B
OM
IM
S
200 nm

*Escherichia coli 1094*

D
C. White (cellulose)
MitoTracker (cells)
5um

E
200 nm
Untreated

F
200 nm
+ Cellulase

Forward movement of cell

Top view of the extrusion pores

BcsC subunits
Cortical belt
Elementary fibrils aggregates
Extrusion pore

IM  OM

~10 nm diameter microfibrils

Cellulose sheet

Lengthening over time

Width

90°

Forward movement of cell

Side view of the extrusion pores

IM
OM

Cellulose sheet

Lengthening over time

Thickness

Elementary fibril aggregates

Microfibril

IM  OM

Cellulose sheet

| Whole cell tomography | | | | | |
|---|---|---|---|---|---|
| Species | Condition/strain | Lamellae | Tomograms | VPP | Source |
| *G. hansenii* (ATCC 23769) | Untreated - 13min post sep | N/A | 6 | - | ATCC |
| | Untreated - 20min post sep | N/A | 14 | - | |
| | Untreated - 300min post sep | N/A | 33 | 24 | |
| | Cellulase 0.2g/L | N/A | 4 | - | |
| *G. xylinus* (ATCC 700178) | Untreated - 5h post sep | N/A | 8 | - | ATCC |
| E. Coli 1094 | Non cellulose induced | N/A | 1 | - | gift from Jean Marc Ghigo (Institute Pasteur) |
| Caulobacter crescentus | NA1000 (WT)[3] | N/A | 1 | - | Zhuo Li |
| *A. tumefaciens* C58[1, 3] | WT[2] | N/A | 47 | - | gift from Patricia Zambrisky (UC Berkeley) to Elitza Tocheva |
| | A139[2] | N/A | 10 | - | gift from Patricia Zambrisky (UC Berkeley) to Elitza Tocheva |
| | AD348[2] | N/A | 1 | - | gift from Anath Das (University of Minnesota) to Debnath Ghosal |
| | AD1484[2] | N/A | 1 | - | gift from Anath Das (University of Minnesota) to Debnath Ghosal |
| | JX148[2] | N/A | 4 | - | gift from Patricia Zambrisky (UC Berkeley) to Elitza Tocheva |
| | NT1[2] | N/A | 2 | - | gift from Patricia Zambrisky (UC Berkeley) to Elitza Tocheva |
| | | | | | |
| **Tomography on milled lamellae** | | | | | |
| Species | Condition/strain | Lamellae | Tomograms | | Source |
| *G. hansenii* (ATCC 23769) | Native biofilm - untreated | 12 | 33 | 3 | ATCC |
| *E. coli* 1094 induced for cellulose synthesis | Untreated | 2 | 6 | - | gift from Jean Marc Ghigo (Institut Pasteur) |
| | Cellulase 0.2g/L | 1 | 2 | - | |

[1] C58 or ATCC 33970 is the wild type *A. tumefaciens*

[2] All these strains have a C58 background.

[3] These strains were imaged for other purposes, but used here as well