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1 **Teichoic acids in the periplasm and cell envelope of *Streptococcus***
2 ***pneumoniae***

3

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22

23 **Abstract**

24 Teichoic acids (TA) are linear phospho-saccharidic polymers and important constituents of the
25 cell envelope of Gram-positive bacteria, either bound to the peptidoglycan as wall teichoic acids (WTA)
26 or to the membrane as lipoteichoic acids (LTA). The chemical composition of TA varies greatly but the
27 presence of both WTA and LTA is highly conserved, hinting at an underlying fundamental function
28 that is distinct from their numerous specific roles in diverse organisms. We report here the observation
29 of a periplasmic space in the Gram-positive *Streptococcus pneumoniae* by cryo-electron microscopy of
30 vitreous section. The thickness and appearance of this region change upon deletion of genes involved
31 in the attachment of teichoic acids, supporting the role of TA in the maintenance of a periplasmic space
32 in Gram-positive bacteria as their main universal function. Consequences of these mutations were
33 further examined by super-resolved microscopy (dSTORM), following metabolic and fluorophore
34 coupling by click-chemistry in pulse and pulse-chase experiments. This novel labeling method also
35 enabled the titration the actual amount of TA per cell and to determine the ratio of WTA to LTA, to
36 follow the change of TA length during growth phases, and to discover that a mutant devoid of LTA
37 accumulates the membrane-bound TA precursor, that may compensate the absence of LTA. Most
38 importantly, the possibility to follow TA during cell fractionation led to the discovery that LTA
39 containing membranes sediment at low centrifugal forces, allowing easy separation.

40

41 **Significance**

42 The existence of a periplasmic space in Gram-positive has long been debated. The finding that
43 compromising the attachment of teichoic acids changes the appearance and thickness of the periplasm
44 in the pneumococcus indicates a role of these polymers in the maintenance of this space between the
45 membrane and the cell wall. Using metabolic labeling and electrophoresis showed that LTA-containing
46 membranes are easily sedimented. This finding casts doubts on previous results, since most LTA were
47 likely unknowingly discarded in these studies. Our method of TA analysis opens a new era in the
48 investigation of these important and poorly known bacterial polymers and their role in the periplasmic
49 space of Gram-positive organisms.

50

51

52 Introduction

53 Teichoic acids (TA) are major components of the cell envelope of Gram-positive bacteria. TA
54 are complex linear polysaccharides attached either to the plasma membrane (lipoteichoic acids, LTA)
55 or to the peptidoglycan (PG) (wall teichoic acids, WTA) (1–3). Although generally non-essential, LTA
56 and WTA participate in numerous cellular processes such as cell wall elongation and hydrolysis, cell
57 division, transport processes, cation homeostasis, resistance to antimicrobial peptides, and interactions
58 with the environment or the host.

59 In studies of Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus*
60 *gallinarum* and *Streptococcus gordonii*) by cryo-electron microscopy of vitreous sections (CEMOVIS
61 (4)), a region between the cytoplasmic membrane and the cell wall was proposed to be a periplasmic
62 space (5–7). A most interesting speculation is the possible role of TA in maintaining this periplasmic
63 space, so that it would be isotonic with the cytoplasm ([Fig. 1A](#)) (8).

64 In most organisms, LTA and WTA have different compositions and are assembled and exported
65 by different pathways (1, 3). TA of streptococci of the *mitis* group, such as *S. pneumoniae*, are particular
66 in two ways: LTA and WTA are products of the same biosynthetic pathway and are therefore of very
67 similar composition, and they are uniquely decorated by phosphocholine residues ([Fig. 1B](#)) (2).

68 The role of TA in the pneumococcus is also critical because 13 to 16 different proteins (choline-
69 binding proteins) are anchored to the cell envelope by the interaction of their choline-binding domain
70 to phosphocholine residues (9). The functions of the choline-binding proteins are diverse, several of
71 them are acting on the cell wall itself as hydrolases such as LytA and LytB, others are virulence factors
72 mediating interactions with the host and its immune system.

73 The pneumococcus has a strict nutritional requirement for choline, which is solely incorporated
74 in its TA (10). However, numerous derivatives can substitute for choline, as long as the hydroxyl group
75 is present and the amine substituents are not too bulky (11). Nutritional shift between choline and
76 ethanolamine had been used in early studies of the localization and composition of nascent cell wall
77 (12). More recently, we have used the incorporation of alkyne- and azido-choline (aCho) derivatives to
78 label TA with clickable-fluorophores and investigate the spatial and temporal relationship between TA
79 and PG assembly by fluorescence microscopy (13, 14).

80 The pneumococcal repeating unit of TA consists of a pseudo-pentasaccharide ((→4)-6-O-P-Cho-
81 α -D-GalpNAc-(1→3)-6-O-P-Cho- β -D-GalpNAc-(1→1)-Rib-ol-5-P-(O→6)- β -D-Glcp-(1→3)-
82 AATGalp-(1→)) (SI [Fig. S1](#)) (15). The glucose (D-Glcp) and the ribitol (Rib-ol) are joined by a
83 phosphodiester bond. The two N-acetylgalactosamine (D-GalpNAc) are substituted on position 6 with
84 a phosphocholine, as mentioned above. The phosphocholine is sometimes lacking on the proximal
85 GalpNAc of some subunits, as well as from both GalpNAc from the terminal unit. Subunits are linked
86 together by α -(1→6) bonds. In LTA, the acetamido-4-amino-6-deoxygalactopyranose (AATGalp) of

87 the first subunit is β -(1→6)-linked to the glucose of a mono-glucosyl-diacyl-glycerol (DGlcp-DAG)
88 (16). In WTA, the first AATGalp is thought to be α -linked via a phosphodiester to position 6 of the PG
89 N-acetyl muramic acid, as in WTA of other species, although this labile linkage has never been
90 experimentally observed in *S. pneumoniae* (15, 17).

91 Like other surface polysaccharides such as the PG or the capsule, the basic unit is intracellularly
92 synthesized at the plasma membrane onto the carrier lipid undecaprenyl pyrophosphate by a succession
93 of glycosyl- and phosphotransferases (2). The basic unit precursor is then thought to be flipped across
94 the membrane by the flippase TacF (*spr1150*) to allow elongation of the TA polymers at the cell surface
95 by the polymerase TarP (*spr1222*) (18–20). Once on the external surface of the membrane, the
96 polymerized precursors can undergo two fates ([Fig. 1B](#)). A phosphotransferase can anchor the polymer
97 onto the PG to yield WTA. Alternatively, a glycosyltransferase can transfer the polymer to DGlcp-DAG
98 to form LTA. The phosphotransferase attaching WTA is thought to be LytR (*spr1759*) (21), although
99 LytR and the two homologous enzymes Cps2A (*spr 0314*) and Psr (*spr1226*) that form the LCP (LytR-
100 Cps2A-Psr) family may exhibit some redundancy (22, 23). The glycosyltransferase producing LTA was
101 found to be TacL (*spr1702*), since the purification procedure normally yielding LTA produced no
102 detectable TA compounds from a Δ *tacL* strain (24).

103 Some fundamental aspects of the pneumococcal biology have recently been proposed to rely on
104 shifting the ratio of WTA to LTA. Notably, competence, the process allowing incorporation of DNA
105 from the extracellular environment, was found to be accompanied by an increase of the amount of WTA
106 dependent on the presence of the phosphotransferase LytR (21). In contrast, autolysis, which occurs
107 during the stationary phase of planktonic cultures and is mostly driven by the PG amidase and choline-
108 binding protein LytA, was shown to depend on the amount of WTA, modulated by the
109 phosphodiesterase WhyD that releases TA from the PG (25).

110 In this work, using CEMOVIS (cryo-electron microscopy of vitreous thin section), we report the
111 existence of a periplasmic space in *S. pneumoniae* WT, Δ *tacL* and Δ *lytR* strains. The observed
112 differences in the appearance and thickness of the periplasmic space supports a participation of TA to
113 its maintenance. The sites of TA incorporation in the cell wall were examined by the metabolic
114 incorporation of aCho and subsequent secondary fluorescent labeling that allowed imaging by super-
115 resolved microscopy, to reveal consequences of the absence of TacL and LytR. In addition, the
116 discovery of the unexpected and previously unreported sedimentation property of the LTA containing-
117 membranes of *S. pneumoniae* allows the simple separation and analysis by gel electrophoresis of labeled
118 LTA and WTA in different strains and conditions. This novel simple method of TA analysis will prompt
119 the reassessment of previous findings, notably regarding the ratio of LTA to WTA in various cellular
120 processes.

121

122 Results

123 Cryo-electron microscopy analysis of *S. pneumoniae* cell envelope

124 CEMOVIS is a cellular cryo-electron microscopy method that previously provided evidence for
125 a periplasmic space in several Gram-positive bacteria (7, 26). To investigate the contribution of TA to
126 the architecture of the cell envelope of *S. pneumoniae*, we applied the CEMOVIS (SI [Fig. S2](#)) to the
127 WT (wild-type) strain and two mutants thereof thought to be deficient in the attachment of TA to the
128 cell wall (Δ lytR) or to the plasma membrane (Δ tacL) (21, 24).

129 As in other bacterial species, CEMOVIS reveals the existence of a periplasm in WT *S.*
130 *pneumoniae*, as evidenced by a light region observed between the plasma membrane and the PG ([Fig.](#)
131 [2](#)). Within this light region, a single dark line runs parallel to the membrane. This observation in *S.*
132 *pneumoniae* supports the idea that a periplasmic space exists in Gram-positive bacteria, and that this
133 space contains organized material, which was previously described as a granular layer (7).

134 If a Gram-positive periplasm filled with TA exists, it must contain LTA, which are anchored in
135 the plasma membrane, and possibly WTA protruding from the inner face of the PG layer (8). This
136 hypothesis predicts that variations in LTA and/or WTA incorporation should modify the architecture of
137 the cell envelope. Strikingly, the granular layer present in the WT strain completely disappears in Δ lytR
138 and Δ tacL cells, ([Fig. 2](#)). In the absence of TacL, the thickness of the periplasmic space is significantly
139 reduced by $(34 \pm 5)\%$ (SE) compared to that of the WT strain ([Table 1](#)), supporting the idea that LTA
140 occupy the periplasmic space. Similarly, the absence of LytR induces a significant reduction of the
141 periplasm thickness ($(29 \pm 5)\%$ (SE), [Table 1](#)), suggesting that WTA also participate in the maintenance
142 of this region. The thickness of the periplasmic spaces in Δ tacL and Δ lytR strains does not differ
143 significantly from each other. In order to increase the signal-to-noise ratio of our cryo-EM images, we
144 took advantage of the exceptional grid adhesion of one vitreous ribbon of Δ tacL cells (SI [Fig. S2](#)) to
145 perform CETOVIS (cryo-electron tomography of vitreous sections). The reconstructed cryo-electron
146 tomogram confirmed the absence of the granular layer throughout the entire volume of a Δ tacL cell
147 section (SI [Figs S2A-B](#)).

148 Interestingly, CEMOVIS data obtained from the WT, Δ lytR and Δ tacL strains also showed that
149 the thickness of the PG layer itself is affected when WTA or LTA assembly is impaired ([Fig. 2](#), [Table](#)
150 [1](#)). Indeed, we observed a significant reduction of the cell wall thickness of $(17 \pm 5)\%$ (SE) or $(36 \pm$
151 $4)\%$ (SE) in the absence of TacL or LytR, respectively. Consistent with the fact that WTA are covalently
152 linked to the PG, the reduction in the cell wall thickness is more pronounced in Δ lytR cells compared
153 to Δ tacL cells. In contrast to the CEMOVIS method, transmission electron microscopy analysis of
154 sectioned stained freeze-substituted resin-embedded samples (SI [Fig. S3C](#)) did not reveal measurable
155 differences between the cell wall of the WT and mutant strains. The differing appearance of Gram-

156 positive cell envelope using the CEMOVIS and freeze-substitution methods, and the difficulty of
157 evidencing the periplasmic space with the latter, was observed and discussed before (26).

158 **Cellular localization of TA insertion**

159 Intrigued by the effect of the absence of TacL or LytR on the ultrastructure of the cell envelope,
160 we examined how the deletion of *tacL* or *lytR* impacts the localization of TA assembly in *S. pneumoniae*
161 cells. To localize newly synthesized TA, an excess of aCho was added for 5 min to exponentially
162 growing cell cultures. Cells were then fixed prior to secondary labeling by strain-promoted azide-alkyne
163 cyclo-addition (SPAAC) click reaction with the DBCO-linked fluorophore Alexa Fluor 647 (DBCO-
164 AF647), following a protocol adapted from (27). As expected, TA insertion occurs at the division site
165 (SI [Fig. S4](#)). Although the morphology of $\Delta tacL$ cells is altered, with pointy poles and some expanded
166 and shrunk cells, enriched aCho incorporation was also detected at midcell. In the $\Delta lytR$ strain, in
167 contrast to the WT parental and $\Delta tacL$ strains, the 5-min incubation with aCho yielded only a weak
168 midcell labeling in few cells (SI [Fig. S4](#)). The weak labeling of $\Delta lytR$ cells may reflect one or a
169 combination of the following: TA are less abundant, cells incorporate less aCho into TA, cells grow
170 more slowly, so that the 5 min pulse represents a shorter fraction of their cell cycle.

171 The photophysical properties of AF647 are amenable to direct stochastic optical reconstruction
172 microscopy (dSTORM), which allowed the reconstruction of super-resolved images as presented in
173 [Fig. 3](#), showing the localization of fluorescent TA after a 5-min labeling pulse experiment.
174 Corresponding images in conventional fluorescence and bright field mode are shown in SI [Fig. S5](#). In
175 the three strains, TA were mostly inserted at the division site, where the PG is assembled (28). However,
176 some localizations were also detected elsewhere at the cell surface in all three strains. This heterogenous
177 labeling was similar in all three strains, rendering the weak labeling at the division site of $\Delta lytR$ cells
178 difficult to identify. This is in contrast to what is observed with PG insertion, where very little signal is
179 observed outside of the cell division zone ([Fig. 3](#) and Trouve et al. 2021). The localization patterns of
180 newly incorporated TA observed in these experiments are mostly that of WTA, since this distribution
181 is preserved in sacculi preparations (SI [Fig. S5B](#)). The bright spots that were often seen in sacculi likely
182 correspond to aggregated material that is trapped inside unbroken sacculi.

183 While there is no discernible difference between the WT and $\Delta tacL$ cells in the distribution of
184 TA labeled during a 5-min pulse, the evolution of this distribution following a chase, when growth is
185 pursued for up to 35 min (about one WT generation time) without aCho, is markedly different between
186 the two strains ([Fig. 3](#)). In the WT strain, the separated banded pattern expected from the insertion of
187 unlabeled TA at the division site is observed, mirroring the patterns produced by PG labeling. After 35
188 min of chase, most WT cells show polar or equatorial labelings following completion of their division.
189 In contrast, in $\Delta tacL$ cells, labeled TA can be seen over the whole new cell surface that is generated
190 during the pulse and the first 15 min of chase. An unlabeled region is visible at midcell in $\Delta tacL$ cells

191 only after 35 min of chase. The chased pattern of labeled PG in the $\Delta tacL$ strain is not affected and is
192 similar to that in WT.

193 In dSTORM imaging of the $\Delta lytR$ strain, the patterns are difficult to discern and many cells show
194 no specific midcell labeling. When visible, the labeled area extends during the chase, as described above
195 for the $\Delta tacL$ strain.

196

197 **Separation of labeled LTA and WTA by low-speed centrifugation**

198 In previous studies, unlabeled TA from the pneumococcus have been analyzed by polyacrylamide
199 gel electrophoresis in the presence of SDS (SDS-PAGE). Purified WTA were revealed in gel by an
200 Alcian blue staining procedure, whereas LTA from crude membrane fractions were revealed by
201 immune-blotting with anti-phosphocholine antibodies (e. g. (Flores-Kim et al. 2019; 2022)). The
202 standard preparative purification of TA from *S. pneumoniae* starts with the sedimentation of
203 mechanically broken cell walls, which contain the WTA, with the concomitant detergent solubilization
204 of LTA (e.g. (24)). Alternatively, for analysis of LTA by immuno-blotting for example, protoplasts
205 prepared by PG enzymatic digestion would be osmotically lysed, cellular debris would be sedimented
206 by low-speed centrifugation and discarded, and LTA-containing membranes would finally be collected
207 by ultracentrifugation (e. g. (25, 29)). On the other hand, WTA can be released from cell wall fragments
208 by thorough digestion of the PG (e. g. (17, 24)) or alkaline hydrolysis (e. g. (25, 29)).

209 Since we were able to fluorescently label TA, we thought it would allow simpler in-gel analysis.
210 WT cells were cultured over two generations in C-medium devoid of choline but supplemented with
211 200 μM aCho. Cells having thus metabolically incorporated aCho were lysed by overnight digestion of
212 the cell wall with lysozyme, mutanolysine and recombinant LytA. The aCho was coupled to a DBCO-
213 fluorescent Alexa dye (AF488) by strain-promoted azide-alkyne click reaction during the lysis
214 incubation. The lysates were then cleared of debris by a low-speed centrifugation at 10,000 g , and
215 ultracentrifugation of the supernatant at 100,000 g was subsequently performed to separate the
216 membrane from the soluble fraction. Since at this stage the TA were fluorescently labelled, we were
217 surprised to find more fluorescence in the low-speed pellet than in the pellet resulting from the
218 ultracentrifugation. Analysis of the soluble fraction (the supernatant) by SDS-PAGE showed the pattern
219 expected from labeled WTA migrating as a set of poorly resolved bands with low electrophoretic
220 mobility, as in typical Alcian blue-stained gels ([Fig. 4A](#)). The pattern expected from labeled LTA, a
221 ladder of well-resolved bands with a greater electrophoretic mobility than the WTA, was observed
222 chiefly in the low-speed pellet. Centrifugation of cells lysates with labeled TA at different speeds
223 confirmed that 2 min at 20,000 g were sufficient to sediment most of the LTA ([Fig. 4A](#)). The nature of
224 high-mobility species and other details on the fractionation and the electrophoresis are commented in
225 the SI (Notes on the fraction of LTA and WTA, SI [Fig. S6](#); Notes on the electrophoresis, SI [Fig. S7](#)).

226 Coomassie blue staining of electrophoresis gels of the supernatant and pellets of low-speed
227 centrifugation showed that the pellet and supernatant have different protein profiles (SI [Fig. S8A](#)). After
228 chloroform extraction, iodine-stained thin layer chromatography showed that the low-speed pellet
229 contained most of the lipids (SI [Fig. S9A](#)) and negative-stain transmission electron micrograph of the
230 low-speed pellet revealed membranous material (SI [Fig. S9B](#)). Taken together, these observations
231 indicate that the LTA-containing membrane fraction of lysed *S. pneumoniae* cells sediments when
232 submitted to low relative centrifugal force.

233

234 **NMR analysis of LTA prepared after low-speed centrifugation**

235 To determine that the low-speed sedimentation of LTA-containing membranes was not an artifact
236 of the labeling method, we applied the same separation method to unlabeled cells followed by the the
237 extraction procedure previously reported (24) for NMR analysis. The ^{31}P NMR spectrum showed the
238 presence of the typical TA phosphorus peaks from internal and terminal units (SI [Fig. S10](#)). Subsequent
239 treatment with hydrazine to remove acyl chains improved the spectra as expected (16). Natural
240 abundance ^{13}C , ^1H -HSQC spectrum showed the presence of the expected sugar species, including the
241 glucosyl-glycerol from the lipid anchor of LTA (A and GRO peaks, SI [Fig. S10](#)).

242

243 **Titration of cellular TA**

244 The fluorescent labeling of TA by click chemistry and separation of LTA and WTA allowed the
245 titration of the different species in cells. For this, we incubated varying quantities of the aCho-labeled
246 cell lysate with fixed amounts of DBCO-AF488. Samples were analyzed by gel electrophoresis and the
247 relative amount of the various fluorescent species was determined by densitometry and plotted against
248 the concentration of cells. The gels and quantifications are shown in [Figs 4B-C](#) and SI [Figs S11A-B](#)
249 for two concentrations of DBCO-AF488. For intermediate amounts of lysate, the concentrations of
250 clickable groups were too low to reach reaction completion during the incubation time. Only with the
251 highest amount of lysate could the fluorophore be nearly consumed. However, for low amounts of
252 lysate, when the fluorophore is in large excess, the reaction is also expected to have neared completion.
253 Therefore, the fraction of unreacted DBCO-AF488 was linearly extrapolated to zero from the
254 measurements at low lysate concentrations, to obtain the amount of lysate that would titrate the
255 fluorophore in an infinite reaction time. Thus, $1.9\ \mu\text{M}$ DBCO-AF488 would titrate the metabolized
256 aCho from $(0.24 \pm 0.01) \times 2.8 \cdot 10^8\ \text{cells} \cdot \text{mL}^{-1}$ and $3.7\ \mu\text{M}$ would titrate aCho from $(0.45 \pm 0.01) \times$
257 $2.8 \cdot 10^8\ \text{cells} \cdot \text{mL}^{-1}$ ([Figs 4B-C](#) and SI [Figs S11A-B](#)), which is equivalent to $(17 \pm 2) \cdot 10^6$ incorporated
258 aCho molecules per cell. Since about 55% of labeled choline are incorporated into TA, the rest being
259 found in the form of phosphocholine, there are about $(9.4 \pm 1.1) \cdot 10^6$ labeled TA choline per cell. Over
260 two generations grown in the exclusive presence of labeled choline, at most 75% of TA choline residues
261 are expected to be labeled, as measured previously (13). The total amount of TA choline residues per

262 cell can therefore be estimated to be about $(12.5 \pm 1.5) \cdot 10^6$. As the major species of TA consists of 6
263 repeating units (16, 17), with each unit comprising about two choline residues, the number of TA
264 molecules per cell is about 10^6 .

265 Although the electrophoretic mobility of the longest species of LTA overlaps that of the shortest
266 WTA species, the major species are well separated ([Figs 4B-C](#) and SI [Figs S11A-B](#)) and allowed their
267 relative quantification. Labeled choline residues incorporated into LTAs were found to account for 15
268 ± 5 % of the TA choline residues. Assuming a similar size distribution of LTA and WTA, LTA and
269 WTA represent roughly 15 and 85% of pneumococcal TA, respectively.

270

271 **Evolution of TA along *S. pneumoniae* growth phases**

272 Using TA labeling and sedimentation separation, we examined whether changes in the
273 distribution of TA could be detected during growth. WT and $\Delta lytA$ cells were grown with aCho over
274 two generations. LytA is the main autolysin of *S. pneumoniae*, and culture of cells lacking this PG
275 hydrolase show an extended stationary phase with minimal lysis. Cells were harvest during the
276 exponential phase of growth, at the onset of the stationary phase, and when the WT cells started to lyse.
277 Remarkably, longer species of LTA can be observed at later stages of growth ([Figs 5A-B](#) and SI [Fig.](#)
278 [S12A](#)). Assuming that the most abundant form consists of 6 repeating units, the 5- and 7-unit species
279 are the next most abundant species, and traces of the 8- and 9-unit species can be detected during the
280 exponential phase of growth. Later during the culture, although the 6-unit species remains the most
281 abundant and the 5-unit is detected at level comparable as earlier in the culture, longer species become
282 more abundant with up to 17 identifiable units. Surprisingly, the size distribution appears to be bimodal
283 with a first group of species consisting of 5 to 11 units, and a second group of longer forms where the
284 most abundant one contains 15 units. When considering the electrophoretic pattern, it should be
285 considered that the signal of the different species is proportional not only to their amount, but also to
286 their length, since longer species harbor more labeled choline residue. Therefore, long species are less
287 abundant than they appear.

288 There was no significant difference between the strains with and without LytA ([Figs 5A-B](#) and
289 SI [Fig. S12A](#)). The $\Delta lytA$ did not lyse spontaneously, but a similar amount of TA with the same
290 distribution was obtained from the lysing $lytA^+$ culture. Thus, although the WT cells lyse, their WTA
291 and LTA are not degraded and remain unchanged in the medium.

292 We also monitored the fate of the labeled species during a chase period of further growth
293 following a 5-min labeling pulse of WT cells, mirroring the dSTORM observations. No modification
294 of the pulse-labeled TA could be detected during the chase time, whether the growth, labeling pulse and
295 chase were performed in BHI or C-medium and supplemented with yeast extract ([Fig. 5C](#) and SI [Fig.](#)
296 [S12B](#)).

297

298 **LTA and the role of TacL**

299 WTA and LTA were examined in a strain devoid of TacL, which is thought to be responsible for
300 the attachment of LTA to membrane glycolipids ([Fig. 6A](#)). Labeled TA were found in the pellet, and
301 when analyzed by gel electrophoresis were indistinguishable from LTA. After double checking that the
302 strain was indeed deleted for the *tacL* gene, we hypothesized that the sedimented TA could be
303 membrane-bound precursors still attached to the undecaprenyl-pyrophosphate lipid carrier. In support
304 of this idea, pulse-labeled LTA-like bands disappeared during a chase ([Fig. 6B](#)), as would be expected
305 if these compounds were precursors being transformed into WTA.

306 The pyrophosphate linkage in the PG precursor lipid II is labile at low pH and high temperature
307 (30). Reasoning that the same should be true for the TA precursor, we incubated the sedimented fraction
308 from the WT and $\Delta tacL$ strain at 110°C at either pH 8 or pH 4.2. The TA sedimented from the $\Delta tacL$
309 lysate were degraded at both pH, whereas those from the parental strain were still detected ([Fig. 6C](#)).
310 This observation suggested that the previously reported failure to isolate TA from the membrane
311 fraction of a $\Delta tacL$ strain could be due to the process that involves boiling at pH 4.7 (24). To test this
312 hypothesis, we submitted resuspended pellets of labeled TA from the WT and $\Delta tacL$ strains to the initial
313 steps of the reported isolation procedure for LTA: boiling in SDS at pH 4.7, lyophilization and ethanol
314 wash of the lyophilizate followed by butanol extraction and retention of the aqueous fraction (24).
315 Although material was lost at each step, LTA from WT cells survived the procedure unscathed, whereas
316 pelleted TA from the $\Delta tacL$ strain was lost at the first step, which involves boiling at low pH ([Fig. 6D](#)).
317 In that case, labeled material with a lower electrophoretic mobility appeared as a smear like that of
318 solubilized WTA.

319 To confirm that TA sedimented from a lysate of cells devoid of TacL are membrane bound
320 through a pyrophosphate, we incubated these TA with recombinant colicin M from *Pseudomonas*
321 *aeruginosa*. Colicin M are toxins that kill bacteria by hydrolyzing the ester bond between the
322 undecaprenol and the pyrophosphate of the PG precursors lipid I and II (31). Colicin M from *P.*
323 *aeruginosa* was shown to be the most active *in vitro* and to display some activity on undecaprenyl-
324 pyrophosphate without saccharide, thus being less stringent regarding its substrates than other colicin
325 M (32). *P. aeruginosa* colicin M could indeed hydrolyze labeled sedimented TA from $\Delta tacL$ cells,
326 whereas it was inactive on labeled LTA from WT cells ([Fig. 6E](#)). The colicin M hydrolytic activity was
327 inhibited by chelation of divalent cation by EDTA, as expected (32).

328 The combined results show that TacL is indeed the glycosyltransferase responsible for the
329 attachment of TA to the membrane glycolipids. However, in the absence of TacL, membrane-bound
330 TA remain present in abundance in the form of undecaprenyl-pyrophosphate-attached precursors.

331 To evaluate if the membrane-bound TA precursors accumulating in the $\Delta tacL$ strain are on the
332 inside or outside of the plasma membrane, cells were grown in C-medium in the presence of 0.2 μM
333 aCho, and the secondary labeling with DBCO-AF488 was performed by a 2 h incubation at room
334 temperature either before, or after cell lysis. Samples were analyzed by gel electrophoresis ([Fig. 6F](#)).
335 LTA in the WT strain or membrane-bound precursors in the $\Delta tacL$ strain were equally labeled before
336 and after lysis, like WTA. In contrast, cytoplasmic azido-phosphocholine was only labeled after lysis,
337 demonstrating that the DBCO-AF488 probe is membrane impermeant.

338

339 **LytR is the main WTA phosphotransferase**

340 WTA and LTA were also observed in strain $\Delta lytR$ which is devoid of one of the three
341 phosphotransferases involved in the attachment of surface polysaccharides (the LCP enzymes),
342 although WTA are less abundant and LTA appear to be longer ([Fig. 6A](#)). In the WT strain, labeled
343 choline in WTA in this particular experiment was found to represent $\sim 80\%$ of the total labeled cholines
344 in TA, whereas in the $\Delta lytR$ strain, this proportion was decreased to $\sim 51\%$. Relative to LTA, the amount
345 of WTA is decreased about four-fold in the absence of LytR.

346 In C-medium, the $\Delta lytR$ strain grew more slowly and reached a lower cell density than its parental
347 strain (SI [Fig. S13](#)). While the WT cells were harvested during their exponential phase of growth, this
348 could not be achieved with $\Delta lytR$ cells that were collected at the end of their growth phase to obtain
349 sufficient material for analysis. The longer LTA observed in the $\Delta lytR$ strain may therefore result from
350 the cells entering the stationary phase (like the WT strain in [Fig. 5A](#)), rather than from the absence of
351 LytR.

352

353 Discussion

354 Multiple roles have been found for TA in various bacteria. These varied functions, however, fail
355 to explain why the presence LTA and WTA with different compositions is conserved in Gram-positive
356 organisms. An underlying common function based on the attributes shared by TA in different species
357 must lie at the root of the conservation of these cell wall polymers through evolution. The hypothesis
358 proposed by Erickson posits that TA are necessary to maintain a periplasmic space at an osmotic
359 pressure that counter balances that of the cytoplasm (8). TA being maintained for this reason may then
360 have been coopted by evolutionary process to carry out additional functions, like evolutionary spandrels
361 (33). This attractive hypothesis is however difficult to test, not least because data supporting the
362 existence of a periplasmic space in Gram-positive bacteria are scarce.

363 Here, our CEMOVIS images of *S. pneumoniae* ([Figs 1](#) and [SI S3A-B](#)) reveal the presence of a
364 region of lower electron density between the membrane and the PG, as reported previously with other
365 Gram-positive organisms and defined as periplasmic space (5–7). About halfway between the PG and
366 the membrane, this periplasmic space appears to be partitioned by a thin layer of more electron dense
367 material, termed the granular layer in a previous study of *S. gordonii* (7). By contrast, freeze-substitution
368 and osmium staining produced thin section of cells with no visible periplasm ([SI Fig. S2C](#)). Since high-
369 pressure frozen cells are directly sectioned for CEMOVIS but undergo numerous subsequent treatments
370 for freeze-substitution and contrasting, it is tempting to consider that the CEMOVIS images yield more
371 truthful representations of the cellular architecture. The thinning of the periplasmic space and
372 disappearance of the granular layer in strains with diminished amounts of either WTA (Δ lytR) or LTA
373 (Δ tacL) support Erickson’s hypothesis that a periplasmic space is maintained by the physical properties
374 of WTA emanating from the PG layer and LTA springing from the membrane (8). In this framework,
375 it is also striking that the appearance of the cell envelope of *S. gordonii* (7) and *S. pneumoniae* are so
376 similar, despite the fact that the composition of their TA are very different (2, 34, 35), lending credence
377 to a fundamental function of TA stemming from their general physico-chemical properties, rather than
378 from specific molecular interactions.

379 Erickson listed four physical mechanisms of the polyelectrolyte brush model of TA that could
380 explain the formation of a periplasmic space where TA chains closely packed on a surface of the plasma
381 membrane generate a pressure when pushed against another TA covered surface (the PG layer). These
382 four contributing factors are: “(1) excluded volume, where the chains cannot occupy the same space;
383 (2) electrostatic repulsion within and between chains; (3) reduced entropy of the chains as they are
384 confined to a narrow cylinder; (4) reduced entropy of the counterions as they are concentrated near the
385 anionic charges” (8). Although generally applicable, the electrostatic repulsion experienced by most
386 anionic TA cannot apply to TA from *S. pneumoniae*, since these are zwitterionic and polyampholytes.
387 Indeed, the phosphocholine side chains carry both a positive and a negative charge at physiologically
388 relevant pH (zwitterionic), while the main chain repeated unit also carry one negative chain from its

389 phosphodiester linkage and one positive charge from the amino-group of its AATGalp ring
390 (polyampholyte character). Being globally neutral, pneumococcal TA may not attract counterions,
391 which therefore would not contribute to the osmolality and pressure of the periplasmic compartment.
392 Consequently, the excluded volume and reduced entropy of TA due to confinement, of the neutral brush
393 polymer theory (36), could be the main drivers of the maintenance of a periplasmic space in streptococci
394 of the *mitis* group. Since *S. pneumoniae* and *S. gordonii* display similar periplasmic spaces when
395 observed by CEMOVIS, despite having TA with different electrostatic properties, future comparisons
396 of these species in different pH, osmolality and ionic strength regimen may provide clues about the
397 physico-chemical interactions of these polymers.

398 The brush polymer model of TA function in forming a periplasmic space applies only if they are
399 present at a density at which volume exclusion occurs. Our metabolic labeling method using aCho that
400 can be titrated with a clickable fluorescent probe has allowed to evaluate the amount of TA chains per
401 cell to about 10^6 . Since WTA may be distributed throughout and on both sides of the PG layer, let us
402 concentrate on the LTA which are less abundant but are all expected to be in the periplasmic space with
403 about $1.5 \cdot 10^5$ chains per cell. A single pneumococcal cell could be approximated by a sphere with a
404 diameter of 1 μm and surface area of $3 \cdot 10^{-12} \text{ m}^2$. Pneumococcus exists mostly as diplococci, so let us
405 assume an average surface area per cell of $5 \cdot 10^{-12} \text{ m}^2$. The surface density of LTA would therefore be
406 $3.3 \cdot 10^{16} \text{ molecule} \cdot \text{m}^{-2}$ or one LTA chain per 30 nm^2 . This density value is lower than that of one chain
407 per 5 nm^2 calculated for LTA from *Staphylococcus aureus* based on the ratio of LTA to membrane
408 lipids (8). Although both density values are rough estimates, they require volume exclusion to
409 accommodate TA chains at the membrane surface and are therefore compatible with the polymer brush
410 model.

411 The morphogenesis and cell division of Gram-positive bacteria rely on the carefully orchestrated
412 assembly of the cell envelope, which includes the membrane, the PG layer and the TA. To gain a better
413 understanding of the incorporation of TA in the cell wall, TA were metabolically labeled with aCho
414 that allowed the subsequent grafting of fluorescent probes. Cells labeled during short pulses of
415 incubation could be observed by dSTORM, revealing the regions of TA insertion in unprecedented
416 details. In parallel, labeled TA, after short pulses or continuous growth in labeling media, could be
417 analyzed by gel electrophoresis after cell wall digestion by PG hydrolases. As expected from an earlier
418 study with conventional microscopy (Bonnet et al. 2018), TA are incorporated in the cell wall at the
419 division site ([Fig. 3](#) and SI [Figs S4](#) and [S5](#)). Patterns after a 5-min labeling pulse are very similar to
420 those obtained when the PG is pulse-labeled (28) and do not differ in the absence of the
421 glycosyltransferase TacL. In contrast, the absence of the phosphotransferase LytR markedly reduce the
422 fluorescence signal. These observations are consistent with the role attributed to TacL of anchoring the
423 less abundant LTA, and to LytR of attaching the more abundant WTA. The electrophoretic analysis of
424 labeled TA confirmed that WTA are less abundant in the ΔlytR strain ([Fig. 6](#)). The amount of LTA

425 appeared comparable in WT and Δ lytR cells, but LTA seem to contribute little to the dSTORM
426 localization patterns, since cell wall sacculi that lacks LTA show patterns identical to that of whole cells
427 (SI [Fig. S5B](#)).

428 The notable difference with TA compared to PG is the presence of some heterogenous surface
429 localization after a labeling pulse. This surface signal is not due to non-specific binding of the
430 fluorophore to the surface, since it is not observed in labeling studies of the PG (28). Since the same
431 level of heterogenous surface labeling was observed with the three strains investigated, the most likely
432 reason is some non-specific adsorption of aCho at the cell surface. It is also possible that some LTA or
433 membrane-bound precursors, which are not covalently bound to the cell wall, can diffuse from the
434 division site across the plasma membrane during the pulse and prior the fixation. Finally, it is possible
435 that hitherto unidentified choline-containing compounds at the cell surface are also labeled.

436 When imaging was performed after a chase time during which cells are allowed to grow in the
437 absence of probes following the labeling pulse, patterns of labeled TA in Δ tacL and Δ lytR cells appeared
438 different from those produced when the PG was labeled ([Fig. 3](#)). In WT cells, in contrast, TA and PG
439 labeling patterns were similar throughout the chase time. Although the appearance of pulse labeled
440 Δ tacL cells resemble that of WT cells, the labeling patterns are strikingly different after a chase period.
441 In Δ tacL cells, all the cell wall generated during the chase is labeled, resulting in a wide contiguous
442 fluorescent region, whereas it is not labeled in WT cells, producing separated bands. It is most likely
443 that in WT cells, production of WTA and LTA are competing pathways as the two polymers share the
444 same composition, that rapidly consumes the undecaprenyl-linked polymerized TA precursors, whereas
445 these precursors accumulate in the Δ tacL strain ([Figs 6B-E](#)). The slower turnover of the undecaprenyl-
446 linked TA in the Δ tacL strain allows the cell wall incorporation of labeled WTA during the chase,
447 several minutes after the aCho has been removed from the medium. This phenomenon could be
448 observed in an electrophoretic analysis of a pulse-chase labeling experiment of the Δ tacL strain, where
449 membrane bound TA precursors disappear over a 15-min course ([Fig. 6B](#)). Patterns are difficult to
450 discern in the Δ lytR strain due to the weak labeling signal, however, they appear to resemble those of
451 Δ tacL cells.

452 In streptococci of the *mitis* group, TA serve as moorings for the attachment of a variety of surface
453 proteins termed choline-binding proteins, because the interaction occurs between the choline residues
454 of TA and protein domains made of repeated choline-binding motifs. The functions of these proteins
455 are varied, playing roles in virulence, competence, or cell wall metabolism (9). The localization of some
456 choline-binding protein can be highly restricted, such as that of the autolysin LytA, which is found at
457 mid-cell to hydrolyze the PG to promote lysis, or the other cell wall hydrolase LytB that localizes to the
458 new poles to complete cell separation (37, 38). Since the choline-binding domains of these proteins
459 participate in their localization (37, 39), it probably requires that TA at different places of the cell
460 surface display some compositional or size differences, that could arise from some maturation process.

461 To investigate this possibility, we analyzed metabolically labeled TA at different stages of cell culture
462 ([Figs 5A-B](#) and SI [S12A](#)). Interestingly, longer LTA are present when the culture enters its stationary
463 phase. One can speculate that the same may be true with WTA, although this could not be determined
464 in our experiment. Surprisingly, when pulse labeling is performed at the onset of stationary phase, the
465 LTA that are synthesized during that phase of the culture do not differ from those produced earlier
466 during the exponential phase, despite the observation of longer LTA are present in late phase culture
467 ([Figs 5A-B](#) and SI [S12A](#)). How could these long LTA arise? Since their length distribution is bi-modal
468 and the longer (12-16 units) are about twice the size of the short ones (5-7 units), it is tempting to
469 imagine that they may arise from the coupling of pre-existing TA.

470 The finding that undecaprenyl-attached TA accumulate in the *ΔtacL* strain to a level that is lower,
471 but comparable to that of LTA in a WT strain raises the question of whether these precursors can fulfill
472 the function of mature LTA, at least partially. The observation by CEMOVIS that the cell envelope
473 architecture is altered in *ΔtacL* cells, with a thinner periplasmic space devoid of granular layer ([Fig. 1](#)),
474 indicates that if some functional replacement occurs, it is not complete. It has been proposed previously
475 that the physical properties of the undecaprenyl chain underlie the localization of the PG precursor lipid
476 II at the sites of cell wall assembly, which occurs at sites of maximal membrane curvature (40). The
477 same reasoning would restrict the presence, and thus the function, of the undecaprenyl-linked TA
478 precursors to sites of cell wall assembly were they can be incorporated into WTA or true LTA. In the
479 absence of *TacL*, undecaprenyl-linked TA accumulate to a greater extent, so that their turn over is
480 slower than in a WT strain, resulting in the incorporation of labeled WTA during a chase after the
481 labeling pulse ([Fig. 3](#)).

482 The propensity of *S. pneumoniae* membrane to sediment at low centrifugal speed, discussed in
483 the SI, is not without experimental consequences in the study of TA. A number of recent studies have
484 sought to investigate the possible role of changes in the relative proportion of WTA and LTA in
485 physiological processes such as the autolysis or the competence (21, 25, 29). However, it is likely that
486 most of the LTA-containing membranes were unknowingly discarded in these experiments during a
487 clarification step of cell lysates, casting doubts on the reported ratio of WTA to LTA and the associated
488 conclusions. The metabolic labeling and electrophoretic analysis methods reported here will certainly
489 help to reappraise several aspects of the biology of TA in *S. pneumoniae*.

490

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508

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520 Supervision ; Writing – review and editing.

521

522 MN and EB contributed equally to this work. MN performed optical microscopy. EB, BG and
523 ChM carried out electron microscopy. MN, CB, AC and AZ carried out biochemical experiments. CL
524 performed NMR experiments. CF and CG designed and produced mutant strains. CD and AZ developed

525 labeling and analysis methods; MB and YSW synthesized aCho. CM and AZ jointly supervised this
526 work.

527

528 **Competing interests**

529 The authors declare no competing interests.

530

531

532 **Materials and methods**

533 **Bacterial strains and culture conditions**

534 *S. pneumoniae* strains are listed in [Table 2](#). Glycerol stocks of WT, *ΔlytA*, *ΔtacL* and *ΔlytR* cells
535 were used to inoculate Bacto™ BHI broth (BD), C-medium (27). Cultures were grown at 37 °C in a
536 static incubator with a 5% CO₂ atmosphere. Two successive dilutions of the cultures were performed
537 to ensure that steady-state growth was reached. To characterize growth phases, cells were grown in 2.5
538 mL of BHI or C-medium in 24-well plates sealed with a transparent film. Cultures were inoculated at
539 an OD₆₀₀ of 0.03 (or 0.12 for the *ΔlytR* strain in C-medium). In a BMG Fluostar Omega plate reader at
540 37°C, turbidity was measured at 595 nm every 20 min after 5 s agitation.

541 **High-pressure freezing**

542 Cells at an OD₆₀₀ of 0.2-0.3 were collected by centrifugation. Pellets were supplemented with or
543 without 20% dextran in phosphate-buffered saline (PBS). Pellets supplemented with dextran were
544 drawn into copper tubes. Pellets without dextran were dispensed in 3-mm type A gold/copper platelets,
545 covered with the flat side of a 3-mm type-B aluminum platelet (Leica Microsystems). The samples were
546 vitrified by high-pressure freezing using an HPM100 system (Leica Microsystems) in which cells were
547 subjected to a pressure of 210 MPa at -196°C.

548 **CEMOVIS and CETOVIS**

549 Copper tubes were trimmed at <-140°C in an EM UC7 Cryo-ultramicrotome equipped with a
550 micromanipulator (Leica Microsystems). Ultrathin sections were produced with a 35° diamond cryo-
551 knife (Diatome) at a nominal thickness of 70 nm and at a nominal cutting feed of 50 mm·s⁻¹. Sections
552 were transferred onto Quantifoil carbon-covered 200-mesh copper grids using the micromanipulator
553 and an antistatic device for favoring the attachment of the ribbon to the grid. Grids were transferred to
554 a Gatan cryoholder kept at -170°C and inserted into a TF20 cryo-electron microscope (FEI) equipped
555 with a tungsten field emission gun. The accelerating voltage was 200 kV. Specimens were irradiated
556 with a low electron dose. Images were recorded with a CMOS CETA camera at a nominal magnification
557 ranging from x5,000 to x29,000 (pixel size 8.1 to 3.7 Å). No peculiar image processing was performed
558 on the micrograph. For CETOVIS, tilt series were acquired from -50° to + 50° with an increment of
559 2.5° according to the dose symmetric Hagen scheme. Images were acquired at a defocus of -10 μm at
560 nominal magnification x29,000 (pixel size 3.7 Å). Tilt series were aligned, and tomogram reconstructed
561 using the IMOD software package (41), using the patch tracking method, followed by weighted back
562 projection and SIRT like filter for purposes of representation.

563 **Freeze substitution and ultramicrotomy at room temperature**

564 The freeze substitution and ultramicrotomy protocols were derived from (Bauda et al., 2023).
565 Briefly, following high pressure freezing, the vitrified pellets were freeze-substituted at -90°C for 80 h
566 in acetone supplemented with 2 % OsO₄ (AFS2; Leica Microsystems). The samples temperature was

567 increased slowly to -60°C ($2^{\circ}\text{C}\cdot\text{h}^{-1}$). The temperature was further raised to -30°C ($2^{\circ}\text{C}\cdot\text{h}^{-1}$) after 8 to 12
568 h, and finally to 0°C within 1 h. The temperature was decreased to -30°C within 30 min, and rinsed in
569 pure acetone 4 times. The samples were infiltrated with progressively higher concentrations of resin
570 (Embed812, EMS) in acetone, while the temperature was gradually increased to 20°C . Pure resin was
571 supplemented at room temperature. Following polymerization at 60°C for 48 h, 70-nm thick sections
572 were produced using a Leica UC7 ultramicrotome and placed onto formvar carbon-coated 200-mesh
573 copper grids (Agar Scientific). Uranyl acetate (2% during 5 min) was used to stain the sections, followed
574 by a 5 min incubation in lead citrate. After rinsing in water, the sections were imaged using a Tecnai
575 G2 spirit BioTwin (FEI) microscope operating at 120 kV, at nominal magnifications of 11,000 to
576 $\times 23,000$ (pixel size of 5.6 to 2.8 Å), with an Orius SC1000B CCD camera (Gatan).

577 **Conventional and super-resolved fluorescence microscopy**

578 Cultures in exponential phase (25 mL, OD_{600} of 0.3) were centrifuged at 3220 g at room
579 temperature for 15 min. The bacterial pellets were resuspended in $1/25^{\text{th}}$ original volume of pre-warmed
580 BHI supplemented with either 2 mM azido-D-Ala-D-Ala for PG labeling or 1.5 mM aCho for labeling
581 of the TA, and incubated for 5 min at 37°C water bath (the “pulse” period).

582 Cells were then pelleted (10,000 g, 1 min) and washed with 1 mL BHI before being resuspended
583 in 20 mL of pre-warmed BHI without probe. Immediately, 2 mL of this resuspension were pelleted
584 (10,000 g, 1 min) to prepare “pulse-only” samples. The rest of the bacterial culture was allowed to
585 continue growing at 37°C (“chase” period). At 5 min, 15 min and 35 min into the “chase” period, 2 mL
586 were pelleted as described earlier. The pellets were resuspended in PBS containing 2%
587 paraformaldehyde for overnight fixation at 4°C .

588 For the preparation of sacculi, the “pulse” period was conducted as above. After cells were
589 washed and resuspended in fresh medium, they were allowed to grow further for 15 min. The chased
590 bacterial cultures (19 mL) were centrifuged (3220 g, 5 min) and the pellets were washed with 1 mL of
591 10% sodium dodecyl sulfate (SDS) (10,000 g, 1 min). Cells were then resuspended in 1 mL of 10%
592 SDS and incubated at 100°C for 1 h, vortexed every 15 min. Sacculi were washed twice with 500 μL of
593 80 mM Tris-HCl pH 7 and incubated in 500 μL of a solution containing 20 $\mu\text{g}\cdot\text{mL}^{-1}$ DNase and 20
594 $\mu\text{g}\cdot\text{mL}^{-1}$ RNase, 20 mM MgCl_2 and 80 mM Tris-HCl pH 7 at 37°C with agitation for 1 h, prior to
595 addition of Proteinase K (final concentration 200 $\mu\text{g}\cdot\text{mL}^{-1}$), 2 mM CaCl_2 , 80 mM Tris-HCl pH 7, and
596 further incubation overnight. Finally, sacculi were washed once with 200 μL of water.

597 For coupling of fluorophores, cells or sacculi were pelleted (10,000 g, 1 min) and resuspended
598 into 50 μL (for fixed cells) or 150 μL (for sacculi) of PBS containing 30 μM DBCO-AF647
599 (JenaBiosciences). After 45 min at room temperature, samples were washed 3 times with 300 μL PBS.
600 For dSTORM imaging, fluorophore-labeled cells or sacculi were resuspended in a solution containing
601 100 mM β -mercaptoethylamine and an oxygen-depleting system consisting of a GLOX enzyme mix

602 (40 $\mu\text{g}\cdot\text{mL}^{-1}$ catalase, 0.5 $\text{mg}\cdot\text{mL}^{-1}$ glucose oxidase) in 75 mM Tris-HCl pH 8, 25 mM NaCl, and 10%
603 glucose.

604 Samples were mounted between a microscopy slide and a high-precision coverslip pre-treated
605 with UV light for 20 min. To coax the cells or sacculi into lying on the slide along their longitudinal
606 axis, a heavy weight was applied on top of the cover slip as described previously (27). The edges of the
607 cover slip were sealed with colorless nail polish.

608 Observations were made with an Abbelight SAFe 360 commercial STORM microscope. For
609 fluorescence signal acquisition, the field was first excited with a 640-nm scanning laser at low power
610 (1%) to capture a low-resolution conventional fluorescence image of the AF647 signal. The laser
611 intensity was then steadily raised to 100%. ASTER technology, implemented in the microscope,
612 provides for a homogeneous sample excitation. In our experiments, the gaussian beam at the power
613 density of 10 $\text{kW}\cdot\text{cm}^{-2}$ (average, FWHM 50 μm , power 200 mW, measured at the sample) scans the
614 region of interest of 160² μm^2 resulting in 800 $\text{W}\cdot\text{cm}^{-2}$ of average effective power density. AF647 dye
615 molecules went through cycles of fluorescent state and dark state. When most AF647 fluorophores had
616 displayed this transition (estimated visually by the frequency and density of fluorescence blinking, a set
617 of 15,000 consecutive frames were taken with 50-ms exposure time. A bright-field image of the field
618 was captured afterwards.

619 The 15,000-frame sets were processed with the FIJI plugin ThunderSTORM, which localizes the
620 center of a Gaussian function fitted to each individual fluorescence signal and returns a data table
621 containing the localization coordinates of all labeled molecules. Correction of drifts occurring during
622 each set of acquisition and reconstruction of the final super-resolution images were performed in the
623 same software.

624 **TA labeling, cell lysis and fractionation for electrophoretic analysis**

625 TA were labeled by growing cells WT or mutant strains in C-medium in the presence of 200 μM
626 aCho to an OD₆₀₀ of 0.5. Cells were harvested, washed and resuspended to an OD₆₀₀ of 15 in
627 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM MgCl₂ containing 0.36 $\text{mg}\cdot\text{mL}^{-1}$ lysozyme, 0.32 $\text{mg}\cdot\text{mL}^{-1}$
628 1 mutanolysine, 0.36 $\text{mg}\cdot\text{mL}^{-1}$ RNase, 0.36 $\text{mg}\cdot\text{mL}^{-1}$ DNase, and 25 μM DBCO-AF488. After lysis
629 overnight on wheel at room temperature cells were centrifuged for 2 min at 1,250, 5,000 or 20,000 g.
630 The pellets were resuspended in the same volume. An aliquot of the 20,000 g supernatant was incubated
631 at 37°C for 2 h with 10 kU of calf intestinal alkaline phosphatase.

632 Samples were then analyzed by electrophoresis on polyacrylamide gel (12%, acrylamide:bis-
633 acrylamide 29:1) in the presence of 1% SDS with a Tris/tricine buffer system. The gels were imaged
634 by trans-illumination with UV light without saturation on a BioRad Chemidoc imager.

635 **Lipid analysis**

636 R800 cells were grown to an OD₆₀₀ of 0.5 in BHI and harvested by centrifugation for 15 min at
637 3220 g. Cells were washed twice with 1 mL 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM MgCl₂ and

638 resuspended in 1 mL of the same solution containing 0.36 mg·mL⁻¹ lysozyme, 0.32 mg·mL⁻¹
639 mutanolysine, 0.36 mg·mL⁻¹ RNase and 0.36 mg·mL⁻¹ DNase. After overnight incubation at room
640 temperature. Half of the sample was centrifuged for 2 min at 20,000 g, and the pellet was resuspended
641 in the initial volume of the same buffer. The pellet, supernatant and whole 500 µL fractions were each
642 extracted twice with 500 µL CHCl₃. The organic fractions were dried and redissolved in 40 µL
643 CHCl₃/methanol (80:20) prior to analysis by thin layer chromatography on 0.2 mm silica plates
644 (Macherey-Nagel Xtra SIL G) developed with CHCl₃/methanol/acetic acid (80:15:8) and stained with
645 iodine vapour.

646 **Negative stain electron microscopy of pellets**

647 Negative Stain-Mica-carbon Flotation Technique (MFT)-Valentine procedure was applied (42).
648 Samples were absorbed to the clean side of a carbon film on mica, stained and transferred to a 400-
649 mesh copper grid. The images were taken under low dose conditions (<10 e⁻·Å⁻²) with defocus values
650 between 1.2 and 2.5 µm on a Tecnai 12 LaB6 electron microscope at 120 kV accelerating voltage using
651 CCD Camera Gatan Orius 1000. The stain was Sodium Silico Tungstate Na₄O₄₀SiW₁₂ at 1% in distilled
652 water (pH 7-7.5).

653 **Isolation of membrane-bound TA for NMR analysis**

654 WT cells grown in BHI to OD₆₀₀ 0.8 were harvested by centrifugation (20 min, 4,500 g) and
655 resuspended in 10 mL of 50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM MgCl₂ with 10 µg·mL⁻¹ each of
656 RNase and DNase, 9 µg·mL⁻¹ mutanolysine, 50 µg·mL⁻¹ lysozyme. After overnight incubation on a
657 rotating wheel at 20°C, the lysate was centrifuged for 10 min at 15,000 g. The pellet was washed thrice
658 with 5 mL water and thrice with 3 mL 100 mM ammonium acetate pH 4.2. The pellet was resuspended
659 in 2.5 mL of the same buffer and 2.5 mL of water-saturated butanol. After vigorous mixing for 30 min,
660 phases were separated by centrifugation at 3,220 g for 10 min. The butanol phase was reextracted twice
661 with 2.5 mL water. The aqueous phases were combined and extracted with 8 mL and then 2 mL CHCl₃.
662 The resulting combined chloroform phases was reextracted with 4 mL water. All water phases were
663 combined and lyophilized. One half of the sample was dissolved in 200 µL of D₂O for NMR analysis.
664 The other half of the sample was dissolved in 500 µL hydrazine 64% and incubated at room temperature
665 for 4 h prior to addition of 500 µL acetone. The sample was dried under nitrogen flow, dissolved in 500
666 µL acetone and dried again. After dissolution in 200 µL D₂O and removal of insoluble material by
667 centrifugation at 20,000 g for 10 min, the hydrazinolized sample was analyzed by NMR.

668 **NMR analysis**

669 NMR experiments were recorded on 600 and 700 MHz spectrometers equipped with a 5 mm TCI
670 probe at 50°C. ¹³C-¹H HSQC (constant time) and 2D ¹³C-edited ¹H-¹H total correlated spectroscopy
671 (TOCSY) were used to confirm ¹H-¹³C assignment. ³¹P 1D experiment was recorded with ¹H decoupling
672 during acquisition and a recycling delay of 1 second. Data were processed with TopSpin 3.5 (Bruker)
673 and analyzed with CcpNmr 2.5.

674

675 **Titration of cellular TA**

676 A preculture of WT cells in BHI was washed twice with C-medium without choline prior to
677 inoculating a C-medium culture containing 200 μM aCho at an OD_{600} of 0.06. After incubation at 37°C,
678 cells were harvested when the OD_{600} reached 0.265, that is after more than two generations in C-
679 medium.

680 Cells from 10 mL culture were resuspended in 160 μL of 50 mM Tris-HCl pH 8, 150 mM NaCl,
681 8 mM MgCl_2 containing 0.4 $\text{mg}\cdot\text{mL}^{-1}$ lysozyme, 0.3 $\text{mg}\cdot\text{mL}^{-1}$ mutanolysine, 0.3 $\text{mg}\cdot\text{mL}^{-1}$ recombinant
682 pneumococcal LytA, and 0.4 $\text{mg}\cdot\text{mL}^{-1}$ each of RNase and DNase. The suspension was left on wheel
683 overnight at room temperature to allow complete lysis.

684 Considering that a cell suspension at an optical OD_{600} of 1 contains about $3.33\cdot 10^8$ cells mL^{-1} , the
685 cell concentration of the lysate was about $5.52\cdot 10^8$ mL^{-1} . The concentration of the stock solution of
686 DBCO-AF488 was determined by its absorbance at 494 nm using $\epsilon = 73000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ to be $93 \pm 5 \mu\text{M}$
687 (sd).

688 The cell lysate and dilutions thereof were mixed with an equal volume of DBCO-AF488 in 50
689 mM Tris-HCl pH 8, 150 mM NaCl to final concentrations of 3.7 ± 0.2 and $1.9 \pm 0.1 \mu\text{M}$ of DBCO-
690 AF488 and a maximum of $2.8\cdot 10^8$ cells mL^{-1} . After 24 h of incubation at room temperature, 1 μL of
691 100 mM aCho were added to block unreacted DBCO-AF488 and incubation was continued for 2 h.

692 Samples were then analyzed by electrophoresis on polyacrylamide gel (17%, acrylamide:bis-
693 acrylamide 29:1) in the presence of 1% SDS with a Tris/tricine buffer system. The gels were imaged
694 by trans-illumination with UV light without saturation on a BioRad Chemidoc imager. Band intensities
695 were quantified using ImageJ.

696 **TA characterization at different stages of cell culture**

697 WT and ΔlytA cells were grown for over two generations in chemically defined medium (C-
698 medium) devoid of choline but supplemented with 200 μM aCho over two generations. Cells were
699 harvest during the exponential phase of growth at an OD_{600} of 0.38, at the onset of the stationary phase
700 at the OD_{600} of 0.75, and when the WT cells started to lyse (OD_{600} 0.46). TA were labeled and cell were
701 lysed, LTA and WTA were separated by centrifugation and analyzed by electrophoresis as described
702 above.

703 **TA pulse and pulse-chase labeling**

704 Exponentially growing WT cells in C-medium were harvested and resuspended at an OD_{600} of 8
705 in the same medium containing 200 μM aCho and no choline. After 0, 2, 5 and 10 min, 150 μL aliquots
706 were withdrawn, immediately centrifuged for 30 s at 10,000 g and resuspended in the same volume of
707 50 mM Tris-HCl pH 8, 150 mM NaCl. The washing procedure was repeated once prior to lysis,

708 secondary fluorescent click-labeling and fractionation as above. Pellets containing LTA were
709 resuspended in one-eighth volume for concentration. TA were analyzed by gel electrophoresis as above.

710 **Hydrolysis of sedimented TA from *ΔtacL* cells**

711 Three procedures were tested. First, pellets of labeled lysates of WT or *ΔtacL* cells were
712 resuspended in solutions of 150 mM NaCl buffered with either 50 mM Tris-HCl pH 8 or ammonium
713 acetate pH 4.2, and incubated for 3 h at room temperature or 110°C.

714 Secondly, the LTA isolation procedure of Hess and colleagues (24) was applied. Labeled pellets
715 resulting from 10 mL of culture of *ΔtacL* or 7 mL of WT cells were dissolved in 200 μL of 50 mM
716 sodium citrate pH 4.7, 4% SDS and incubated at 100°C for 45 min. After withdrawal of a 20 μL aliquot
717 for analysis, the solution was lyophilized. The solid was washed four times with ethanol and
718 resuspended in 180 μL of 50 mM sodium citrate pH 4.7. A second 20 μL aliquot was withdrawn, and
719 the solution was extracted with 150 μL water-saturated butanol. The organic phase was back extracted
720 with 100 μL water. The aqueous phases were combined, lyophilized, redissolved in 160 μL of 50 mM
721 sodium citrate pH 4.7, and a 20 μL aliquot was taken for analysis. To the 20 μL aliquots, 2 μL of 3 M
722 Tris-HCl pH 8.45 were added prior to analysis by electrophoresis.

723 Thirdly, recombinant colicin M from *P. aeruginosa* (generous gift from T. Touzé) prepared as
724 described previously (43), was added at various concentrations to resuspended pellets and the mixtures
725 were incubated overnight at room temperature.

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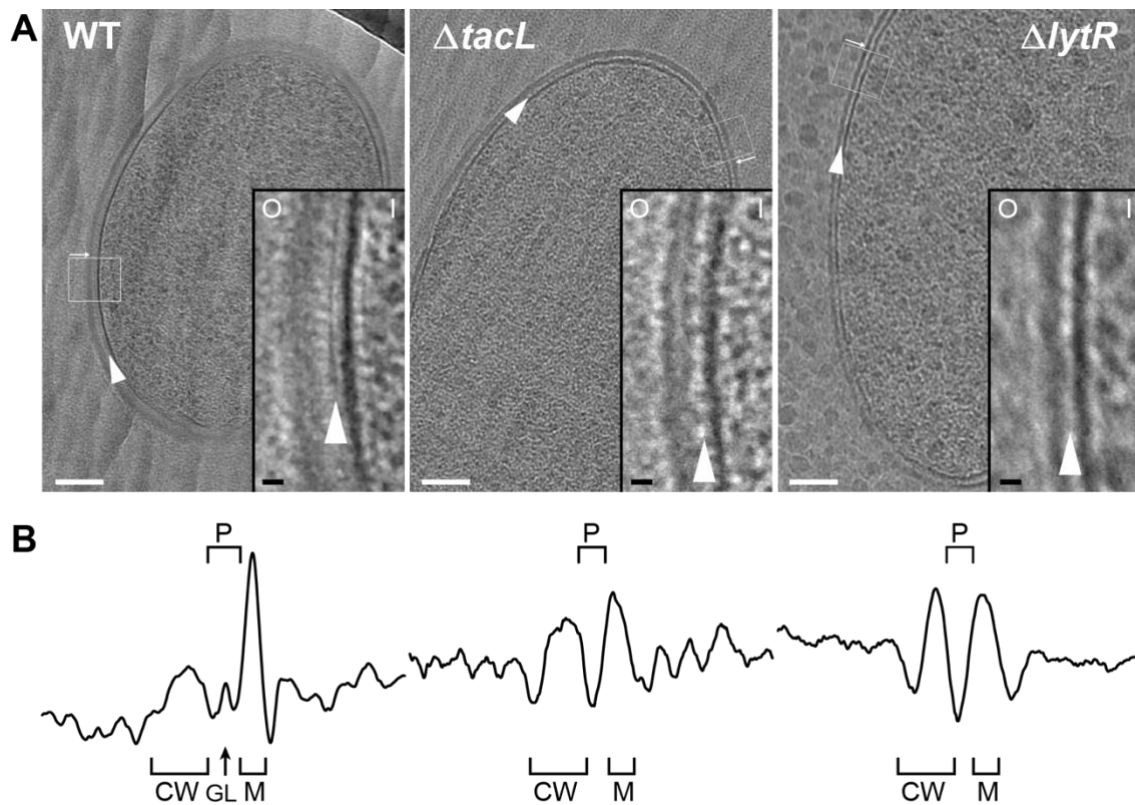
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835

840 **Figure 1.** Models of the functions and structures of TA in *S. pneumoniae*. **(A)** TA (multicolored rods)
841 attached to the membrane (M) or to the peptidoglycan (PG) exclude each other to maintain a
842 periplasmic space (PS). Additional functions arise from the presentation of various choline-binding
843 proteins (blue). **(B)** Polymerized TA attached to undecaprenyl (UndPr)-pyrophosphate are transferred
844 at the cell surface onto PG by the phosphotransferase LytR to form WTA; or onto DGlc p -DAG by
845 TacL to form LTA. The enzymes are depicted by their AlphaFold models. The most abundant TA
846 species have 6 repeating units ($n = 4$).

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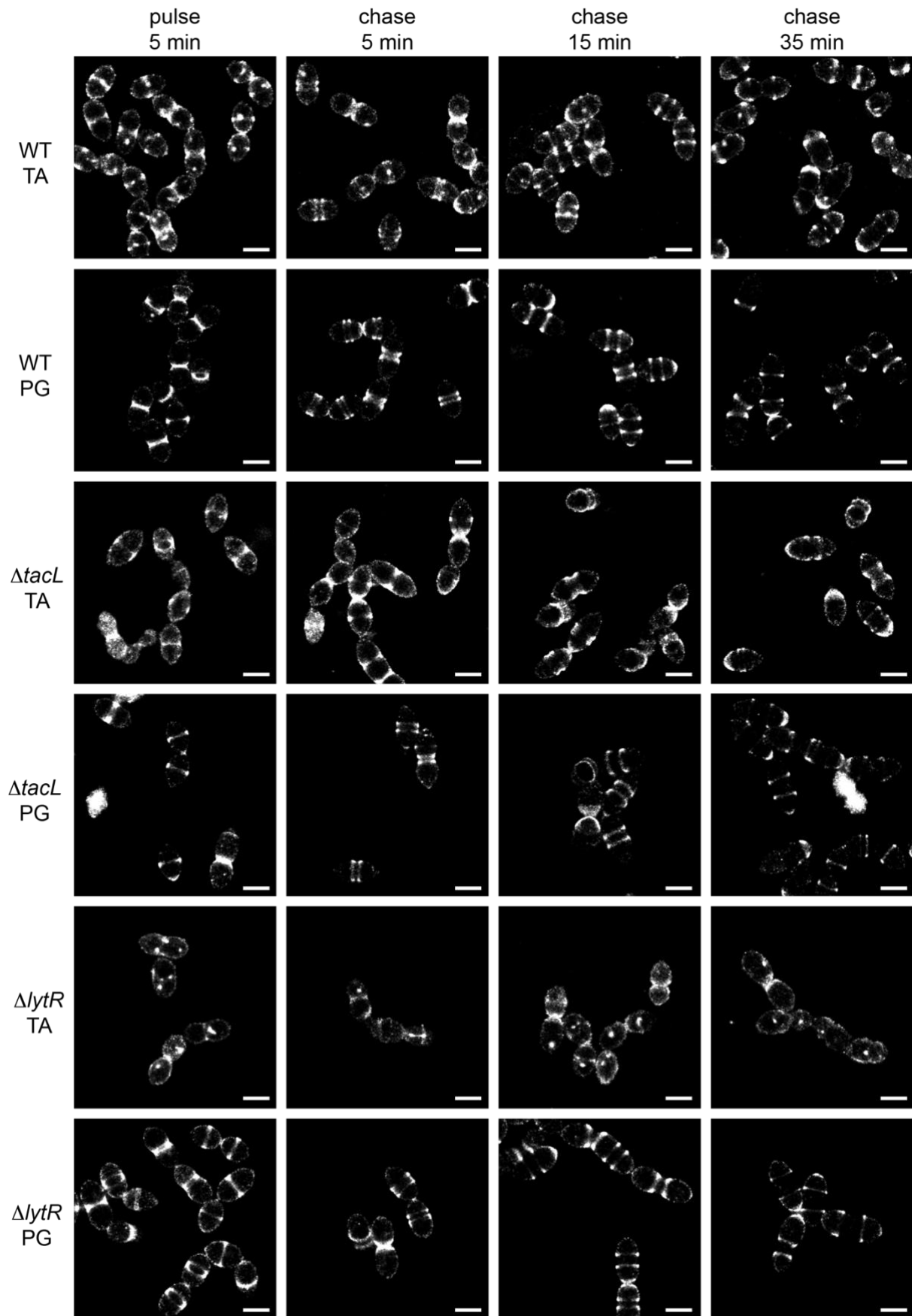


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850 **Figure 2.** Electron micrographs of WT, $\Delta tacL$ and $\Delta lytR$ strains. (A) CEMOVIS micrographs allowing
851 full view (white scale bar, 100 nm) and zoom (black scale bar, 10 nm) of WT and mutant strains. O and
852 I signal the outer and inner sides of the cell envelope. A periplasmic space can be observed in all three
853 strains. A granular layer is seen as a thin black line in the periplasmic space (arrowheads) of WT cells,
854 whereas it is not observed in the $\Delta lytR$ and $\Delta tacL$ strains. (B) Corresponding pixel intensity profile of
855 the cell envelope is shown for each strain. The intensity profiles were measured perpendicularly to the
856 cell surface in regions boxed in white, and show variations in the density of the envelope ultrastructure.
857 CW, cell wall; P, periplasm; GL, granular layer; M, membrane.

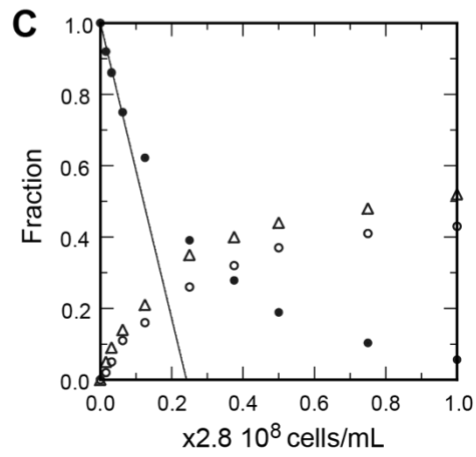
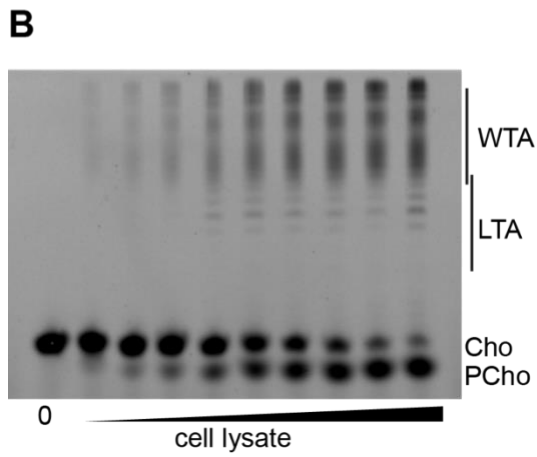
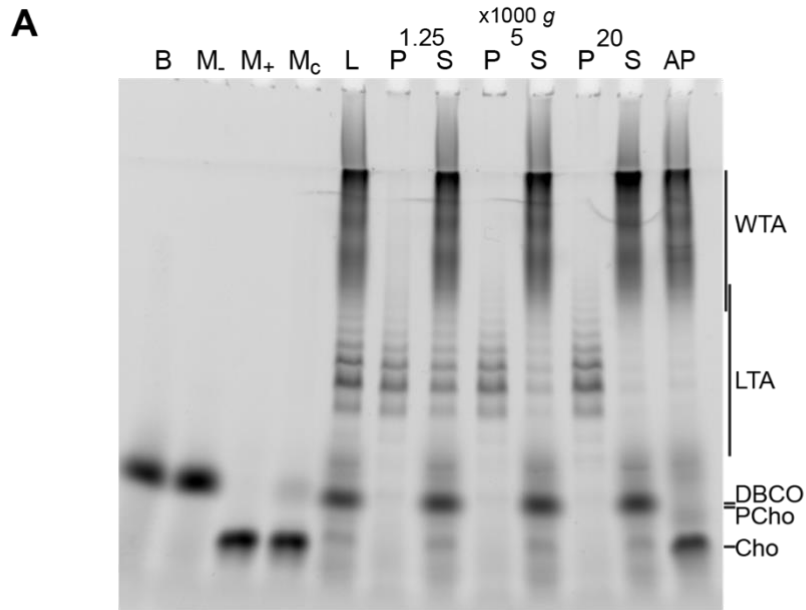
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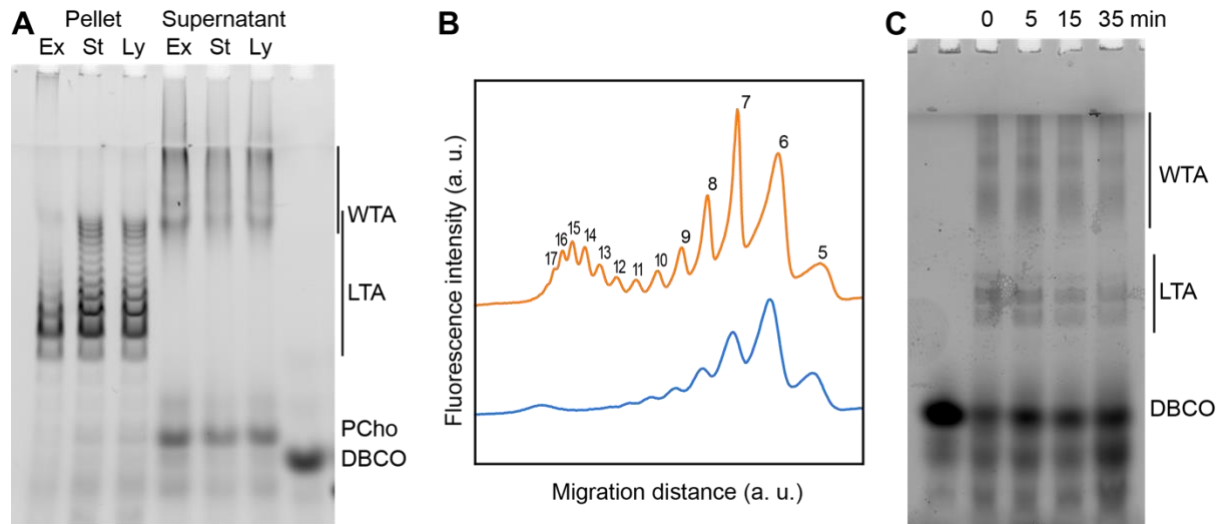
861 **Figure 3.** Super-resolved dSTORM imaging of TA incorporated in growing WT, *ΔtacL* and *ΔlytR* cells
862 after a 5 min pulse of metabolic labeling with 1.5 mM aCho followed by a chase, and subsequent
863 secondary fluorescent labeling by click chemistry using DBCO-AF647. For comparison, the same
864 pulse-chase procedure was applied to reveal the newly synthesized peptidoglycan (PG) with a metabolic
865 labeling using 2 mM azido-D-Ala-D-Ala. Scale bars are 1 μm. Corresponding conventional and bright
866 field images are shown in SI [Fig. S5A](#).

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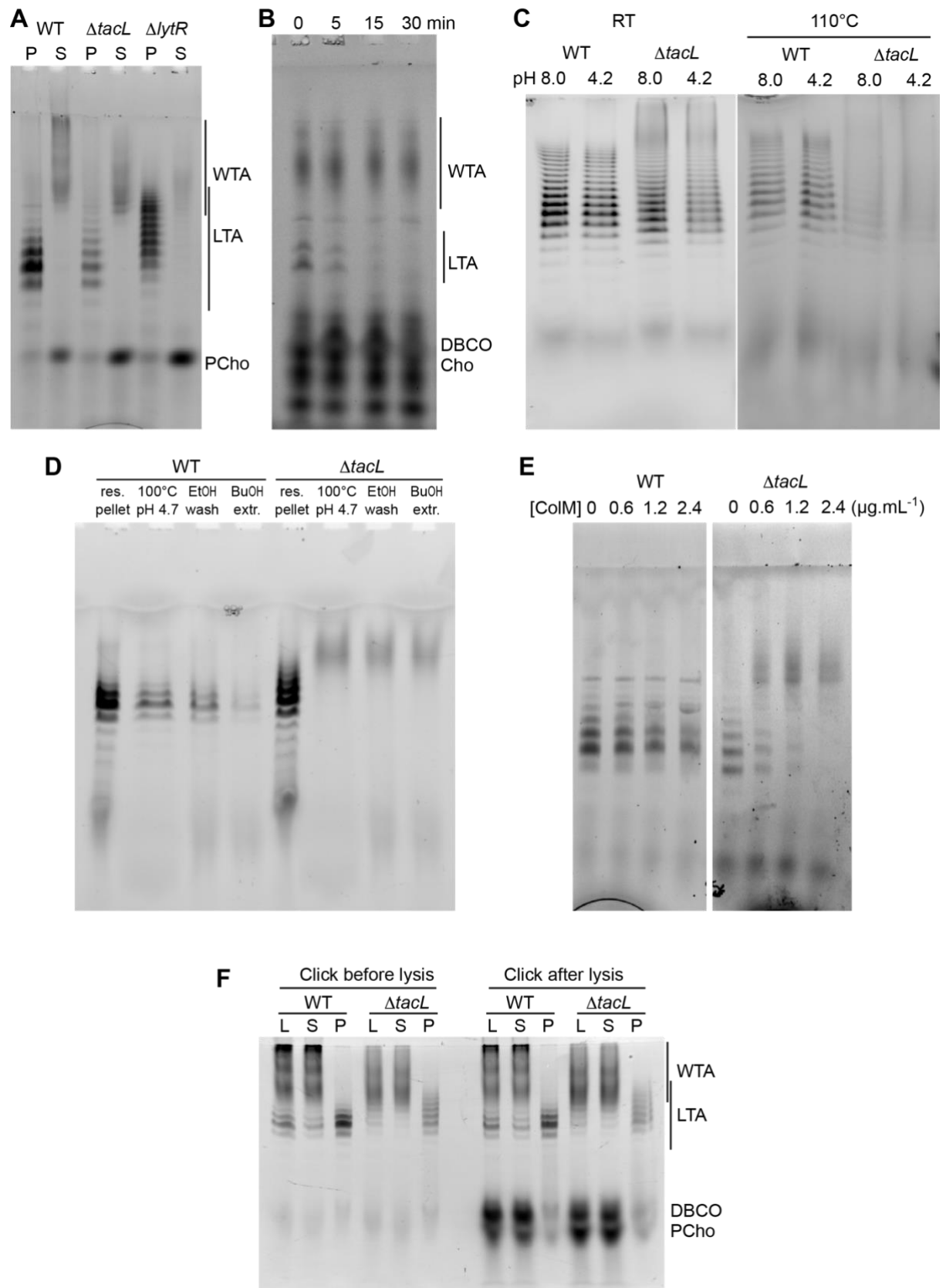
870 **Figure 4. (A)** Polyacrylamide gel electrophoresis of fluorescently labeled TA demonstrating separation
871 of LTA and WTA by centrifugation. Labeled compounds were revealed by UV-transillumination. TA
872 were labeled by growing WT cells in C-medium in the presence of 200 μM aCho. Cells were lysed
873 overnight with lysozyme, mutanolysine and LytA and the azido-groups were modified by reaction with
874 25 μM DBCO-AF488. The lysate was centrifuged for 2 min at 1,250, 5,000 or 20,000 g . The pellets
875 were resuspended in the initial volume. An aliquot of the 20,000 g supernatant was treated with alkaline
876 phosphatase. The buffer with the lysing enzymes, the medium with and without aCho and the culture
877 supernatant were similarly incubated with 25 μM DBCO-AF488. B, lysis buffer with enzymes; M-, C-
878 medium without aCho; M+, C-medium with aCho; M_C, culture supernatant; L, lysate; P, pellet; S,
879 supernatant; AP, alkaline phosphatase-treated. The control samples (B, M-, M+ and M_C were loaded at
880 one-fourth the volume of the cellular samples). Labels on the right side of the gel identify species
881 coupled to the fluorophore AF488. **(B-C)** Titration of cellular TA. WT cells were grown for two
882 generation in the presence of aCho prior to cell lysis. DBCO-AF488 (1.9 μM) was incubated for 24 h
883 with varying amounts of cell lysate corresponding to up to $2.9 \cdot 10^8$ cells mL^{-1} . The remaining DBCO-
884 AF488 was blocked by addition of 100 mM aCho, and the various species were separated by
885 polyacrylamide (17%) gel electrophoresis **(B)**. The bands were quantified and the relative amount of
886 the various species were plotted against the cell concentration **(C)**. Black circles, blocked DBCO-
887 AF488; open circle, phosphocholine; open triangle, TA. A linear regression of the DBCO-AF488 points
888 at low cellular concentration was applied to obtain the titration point as the intercept of the cell
889 concentration axis.
890



891

892 **Figure 5.** Evolution of TA during growth phases. (A) WT cells grown in C-medium containing 200
893 μM aCho were harvested during the exponential growth phase (Ex), at the onset of the
894 stationary phase (St) and during the autolysis (Ly). TA were fluorescently click-labeled with
895 DBCO-AF488 and cells were completely lysed by the addition of PG hydrolases prior to
896 centrifugation. LTA are found in the pellet (P) whereas WTA are observed in the supernatant (S). The
897 amounts of cells at the different culture stages were normalized. The LTA samples are 8-fold
898 concentrated compared to the WTA samples. Fluorescently labeled TA were revealed by UV-
899 transillumination after SDS-polyacrylamide electrophoresis. (B) Densitometric profiles of the
900 fluorescent intensities of exponential (blue) and stationary phase samples (orange) shown in (A). (C)
901 Electrophoretic analysis of TA of WT cells grown in BHI and pulse-labeled for 5 min with 1.5 mM
902 aCho, and chased by further growth in the same medium without added aCho for the indicated duration.

903



904

905

906 **Figure 6.** (A) Electrophoretic analysis of TA from the WT strain, or deleted of the gene encoding the
907 glycosyltransferase TacL, or the phosphotransferase LytR. Cells were grown in C-medium with no
908 choline and 200 μ M aCho, prior to cell lysis with PG hydrolases and secondary labeling with DBCO-
909 AF488. Lysates were fractionated by centrifugation and the pellets were resuspended in one fourth the
910 initial volume: P, pellet; S, supernatant. (B-E) Identification of sedimented TA from lysates of Δ tacL
911 cells as undecaprenyl-pyrophosphate bound precursors. (B) Pulse-chase labeling experiment of TA in
912 strain Δ tacL. Cells grown in BHI were exposed to 1.5 mM aCho for 10 min (pulse), prior to washing
913 and further incubation in the same media without aCho (chase) for various time prior to cell lysis and
914 secondary labeling with DBCO-AF488. Samples were treated with alkaline phosphatase prior to
915 electrophoresis. (C) Pellets containing labeled TA from WT and Δ tacL cells were resuspended in a Tris-
916 or ammonium acetate-buffered solution at pH 8 or pH 4.2 and incubated for 3 h at room temperature or
917 110°C prior to electrophoresis. (D) Labeled TA from WT and Δ tacL cells were submitted to the first
918 steps of the traditional procedure of LTA isolation: boiling in 4% SDS in a citrate-buffered solution at
919 pH 4.7, lyophilization and ethanol wash of the lyophilizate, butanol extraction and retention of the
920 aqueous fraction. (E) Labeled sedimented TA from WT and Δ tacL cells were incubated overnight
921 without or with various concentrations of recombinant *P. aeruginosa* colicin M prior to electrophoretic
922 separation. (F) To probe on which side of the plasma membrane the TA are positioned, secondary click-
923 labeling with DBCO-AF488 was performed prior or after cell lysis of WT and Δ tacL cells grown in C-
924 medium in the presence of 0.2 mM azido-choline. L, whole lysate; S, supernatant of 20,000 g 2 min
925 centrifugation; P, pellet resuspended in one-fourth initial volume.

926

927 **Table 1**

Table 1. Dimensions of cell envelope structures measured on CEMOVIS images

Strain	Mean thickness (nm) ± SD (nb of measurements) ^a			Ratio of thickness PG/PS	GL dist. from membrane ^b (nm)± SD
	PG layer	PS	GL		
WT	15.9 ± 2.5 (35)	10.8 ± 1.4 (35)	3.2 ± 0.8 (35)	1.5	5.0 ± 0.8 (35)
<i>ΔtacL</i>	13.3 ± 3.7 (35) c **/ d **	7.1 ± 2.3 (35) c **/d #	NA	1.87 c **/ d **	NA
<i>ΔlytR</i>	10.2 ± 1.7 (35) c **/ d **	7.7 ± 2.0 (35) c **/d #	NA	1.32 c #/d **	NA

^a Values in nm are presented as mean values ± standard deviations (with numbers of measurements in parentheses). They have been measured using images with a pixel size of 3.7 Å. NA, not applicable.

^b Measured from the outer surface of the cytoplasmic membrane to the inner most side of the GL.

^c Anova test was performed between corresponding structures of WT R800 and mutant cells. #, no statistical difference; *, p= 0.05; **, p= 0.01

^d Anova test was performed between corresponding structures of *ΔlytR* and *ΔtacL* cells. #, no statistical difference; *, p= 0.05; **,p = 0.01.

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932 **Table 2**

Table 2. *S. pneumoniae* strains used in this study

Short name	Construct	Genotype	Source
WT	sspCM514	R800, <i>rpsL1</i> , StrR	Gift from J.-P. Claverys, Toulouse, France
<i>ΔtacL</i>	sspCM246	R800, <i>rpsL1</i> , <i>ΔtacL</i> , StrR	This study
<i>ΔlytR</i>	sspCM493	R800, <i>rpsL1</i> , <i>ΔlytR</i> , StrR	This study

933

934 **SUPPLEMENTARY INFORMATION**

935

936 **Teichoic acids in the periplasm and the cell envelope of**

937 ***Streptococcus pneumoniae***

938

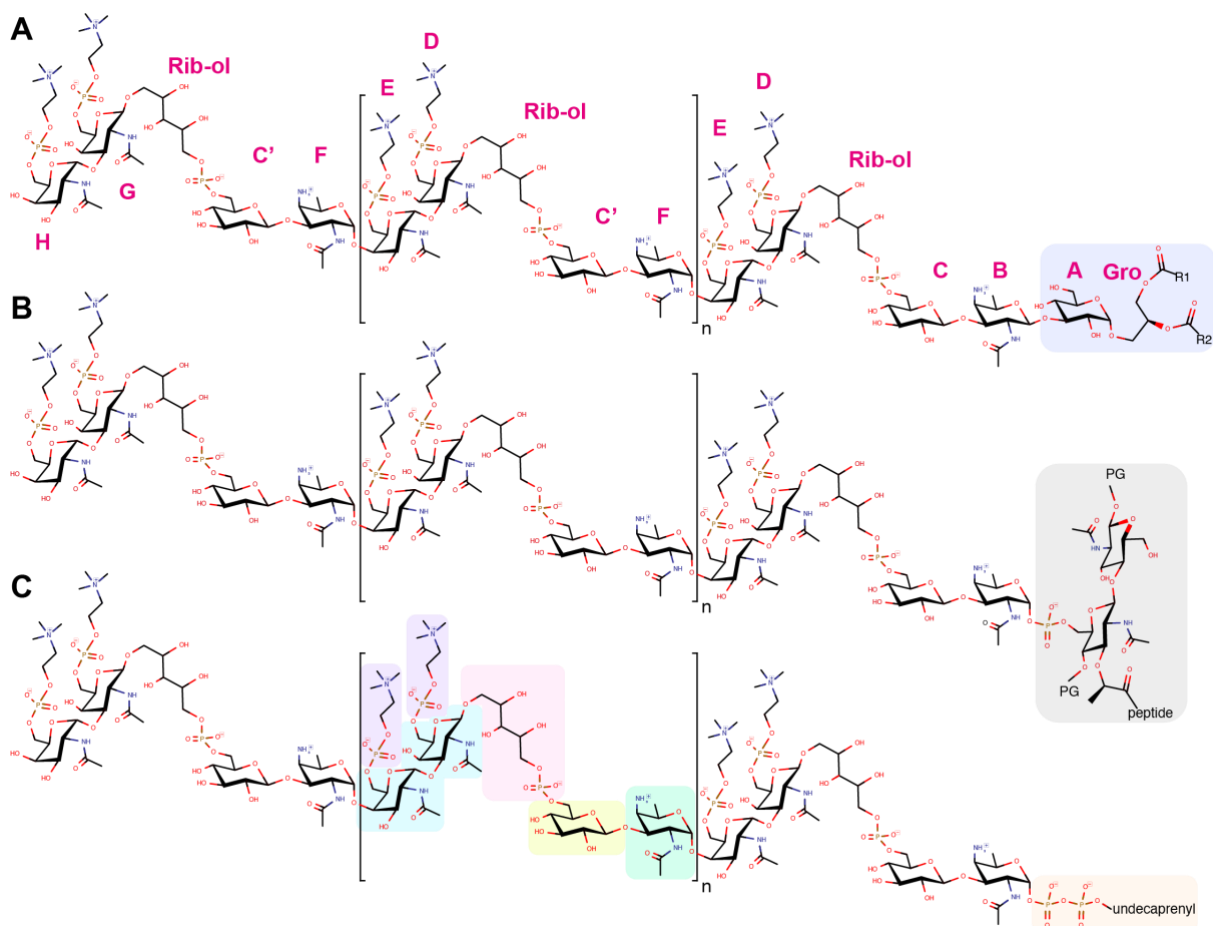
939 Mai Nguyen^a, Elda Bauda^a, Célia Boyat^a, Cédric Laguri^a, Céline Freton^a, Anne Chouquet^a, Benoit
940 Gallet^a, Morgane Baudoin^b, Yung-Sing Wong^b, Christophe Grangeasse^c, Christine Moriscot^c, Claire
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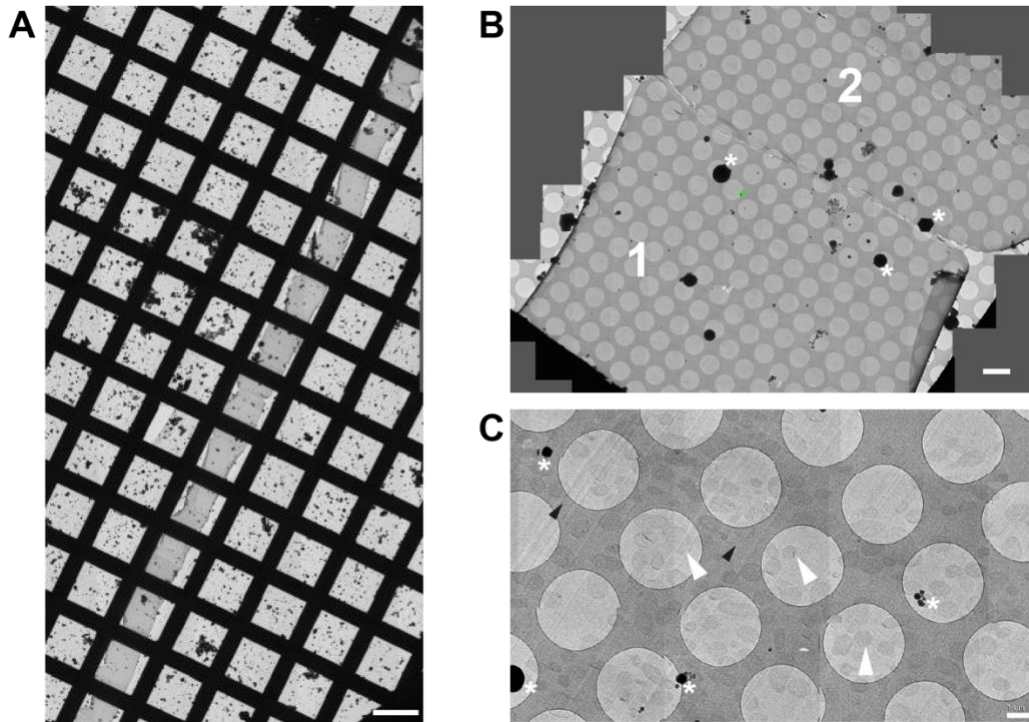
948

949 **Figure S1.** Chemical structure of (A) LTA, (B) WTA, and (C) the membrane-bound precursor. The
 950 constituent of the repeating unit are in green the acetamido-4-amino-6-deoxygalactopyranose
 951 (AATGalpNAc), in yellow the glucopyranose (DGIcp), in pink the ribitol-5-phosphate, in cyan the N-
 952 acetylgalactosamine (DGalpNAc), in purple the phosphocholine. One or both phosphocholine residues
 953 can be absent on the terminal unit. Some units may also lack the phosphocholine on the proximal
 954 DGalpNAc. The most abundant species have 6 repeating units ($n=4$). LTA are β -1-linked to
 955 monoglucosyldiacylglycerol (blue). WTA are α -1-linked via a phosphodiester to position 6 of a N-
 956 acetyl muramic acid of the peptidoglycan (PG, gray). The precursors remain attached to an
 957 undecaprenyl pyrophosphate (orange). The one-letter nomenclature of constituents proposed by Gisch
 958 (1) is given in magenta around the LTA.

959 **Notes on CEMOVIS data quality.**

960 Some crevasses can be observed but the section is devoid of cracks and very low ice
961 contamination is present on the surface (SI [Fig. S2](#)). Knife marks along the cutting direction of the knife
962 cannot be avoided, although compression artifact is rather limited as the cells preserve a round shape.
963 Thus, it can be said reasonably that the sections are exploitable and that artifacts induced by cryo-
964 sectioning do not impair image interpretation and ultrastructure measurement.

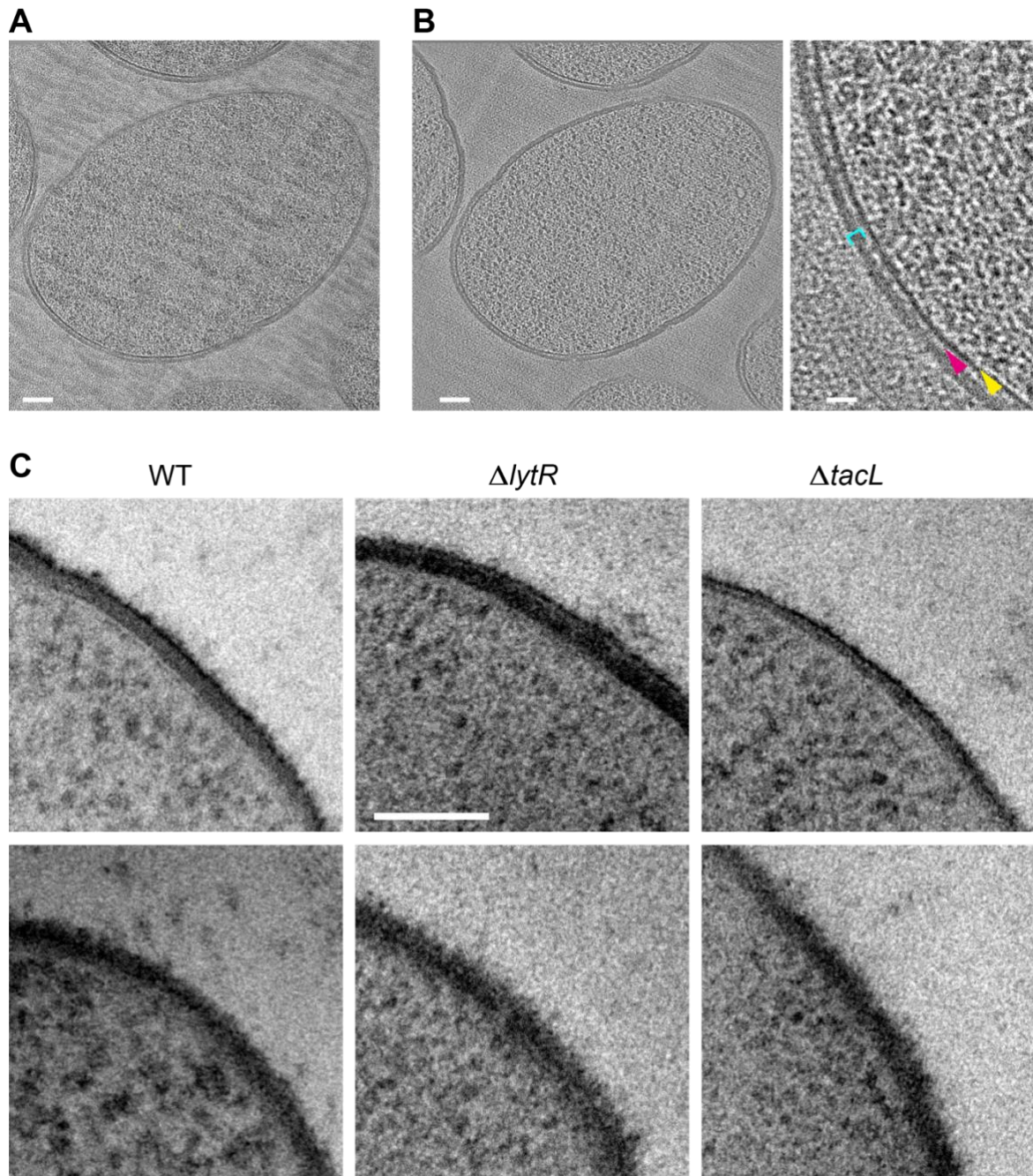
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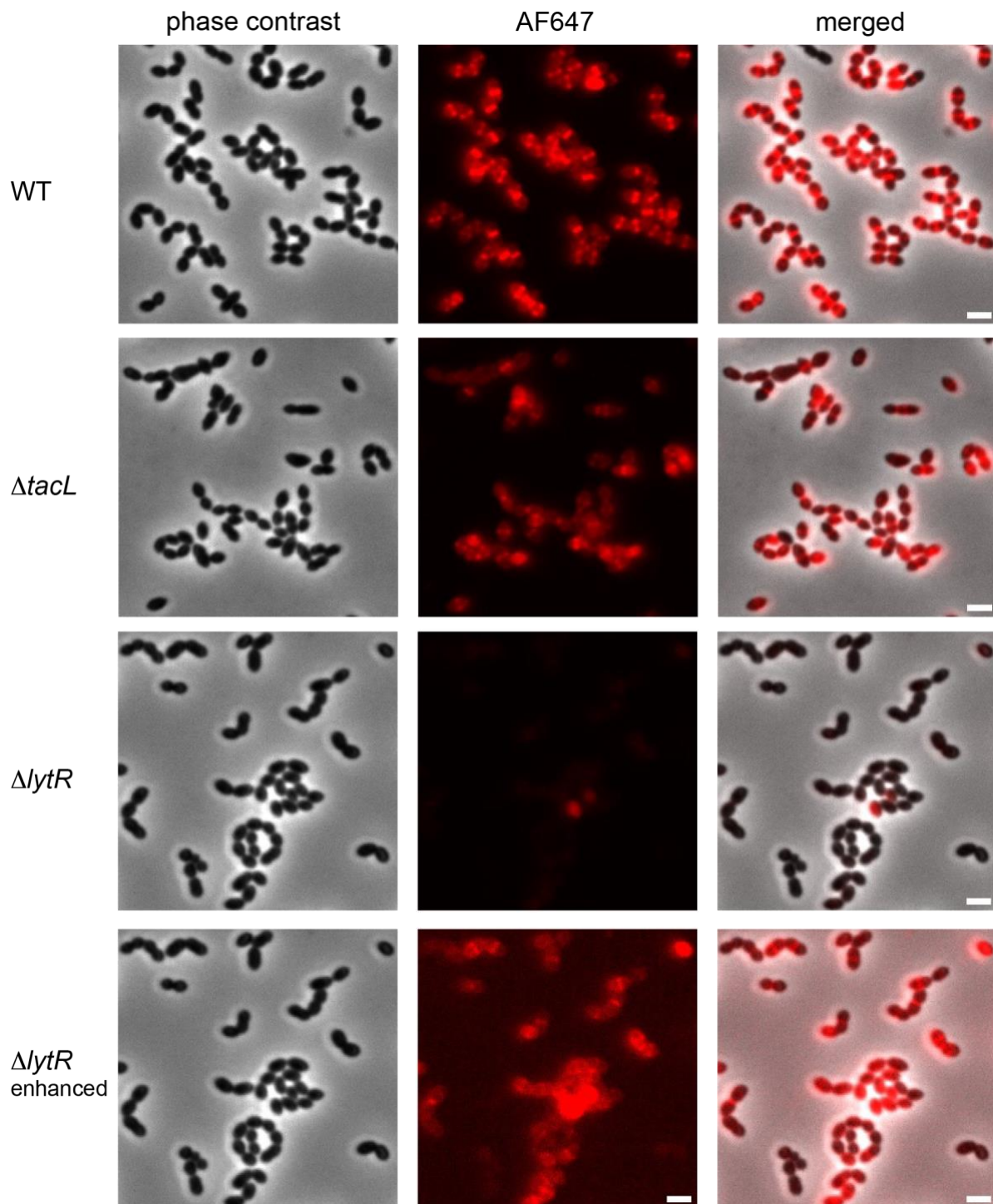
968 **Figure S2.** Low-magnification CEMOVIS images of *S. pneumoniae* $\Delta tacL$. (A) The ribbon spans the
969 entire EM grid. Scale bar, 100 μm . (B) Stitching of several acquisitions areas showing one cryo-section
970 (1) and half of a second one (2) inside the ribbon. Scale bar, 5 μm . (C). Cells are uniformly distributed
971 in the section. Representative acquisition areas, where cells are located at the center of a hole in the
972 carbon, are pointed with a white arrowhead. Scale bar, 1 μm . Black arrowheads: knife marks; asterisks:
973 ice contamination.



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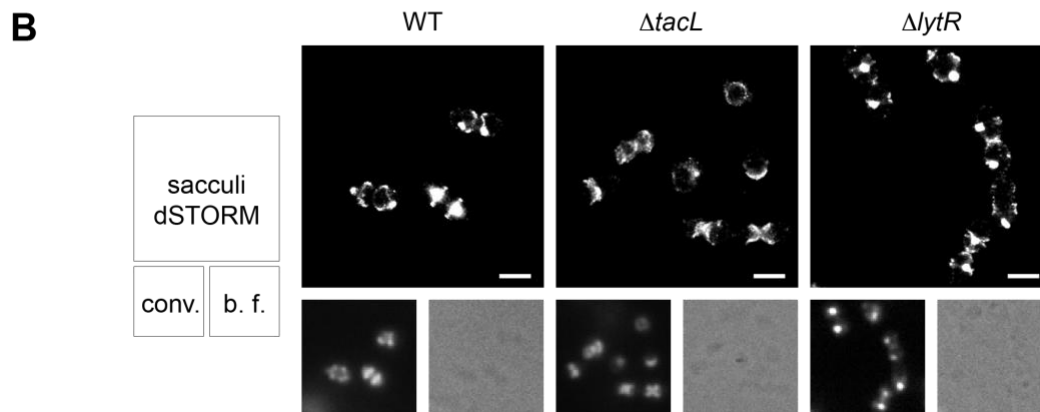
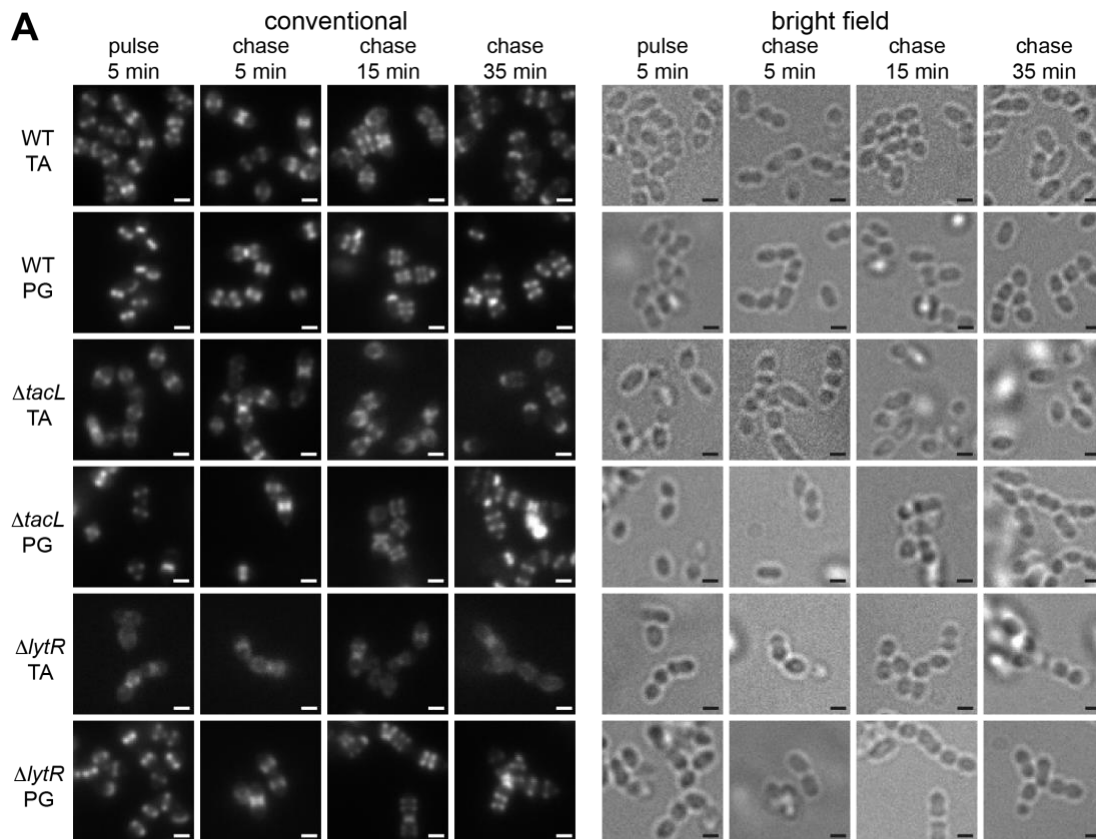
976 **Figure S3. (A-B)** CETOVIS of a $\Delta tacL$ cell section. **(A)** Raw image of a tilt series. **(B)** Full view (scale
977 bar, 100 nm) and zoom (scale bar, 20 nm) of a slice through the cryo-electron tomogram. Yellow arrow,
978 membrane; magenta arrow; periplasmic space; cyan bracket, peptidoglycan. The reduction of the
979 periplasmic space and peptidoglycan layers thickness is observed in the whole volume. No granular
980 layer is detected in the volume. **(C)** TEM micrographs of stained freeze-substituted thin sections of the
981 cell envelope of WT, $\Delta lytR$ and $\Delta tacL$ cells. The appearance varies depending on the angle between the
982 cell envelope and the section plane. Two examples are shown for each strain. No consistent difference
983 could be discerned between the strains. Scale bar 100 nm.



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986 **Figure S4.** Fluorescence microscopy of R800 WT, $\Delta tacL$ and $\Delta lytR$ cells grown in BHI and pulse-
987 labeled with 1.5 mM azido-choline for 5 min prior to fixation and secondary click-labeling with DBCO-
988 AF647. The three top rows are with the same imaging settings. The fluorescent signal of the bottom
989 row was enhanced. Scale bars are 2 μ m.



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991 **Figure S5. (A)** Corresponding conventional and bright field microscopy images of the pulse-chase

992 experiments shown by dSTORM imaging in [Fig. 3](#). New TA were revealed in growing R800 WT, $\Delta tacL$

993 and $\Delta lytR$ cells by a 5 min pulse of metabolic labeling with 1.5 mM azido-choline followed by a chase,

994 and subsequent secondary fluorescent labeling with clickable DBCO-AF647. For comparison, the same

995 pulse-chase procedure was applied to reveal the new peptidoglycan with a metabolic labeling using 1.5

996 mM azido-D-Ala-D-Ala. **(B)** dSTORM imaging (with corresponding conventional and bright field

997 images at half scale) of sacculi prepared from cells pulse-labeled during 5 min and chased for 15 min.

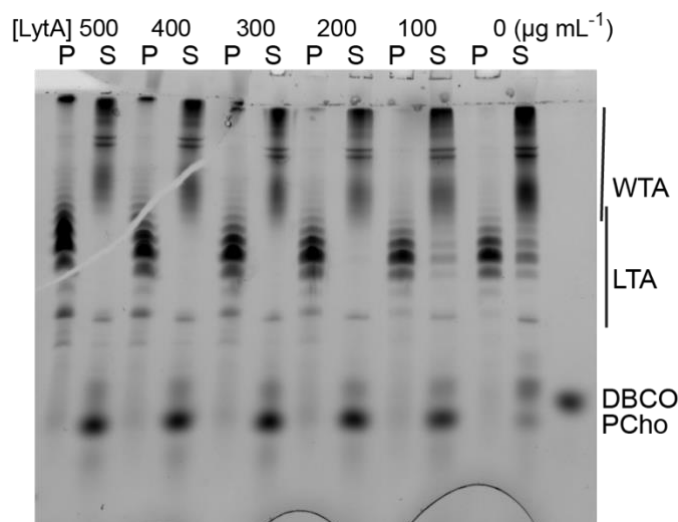
998 Labeled TA in sacculi were revealed by secondary fluorescent labeling with clickable DBCO-AF647.

999 Scale bars are 1 μ m.

1000 **Notes on the fractionation of LTA and WTA**

1001 Although sedimentation of LTA was observed even at relative centrifugal force as low as 2,000
1002 g, the pelleting was not always complete, even after prolonged centrifugation at 20,000 g. Incomplete
1003 cell lysis cannot account for the incomplete pelleting of LTA, since intact cells or large fragments would
1004 be expected to add to the pellet material. We found out that the quality of the separation of LTA from
1005 solubilized WTA by sedimentation depended on the amount of recombinant LytA used during the lysis
1006 (SI [Fig. S6](#)). This parameter was difficult to adjust since the exactly adequate amount appeared to
1007 depend on the particulars of the experiment (strain, cell density). We think that the multivalent choline-
1008 binding domain of LytA crosslinks LTA bounds to membrane vesicles, leading to the formation of large
1009 aggregates that sediment more easily. In support of this explanation, the presence recombinant LytA
1010 also impacts the migration of the solubilized TA, causing species with lower electrophoretic mobilities
1011 to be more abundant at the top of the gels.

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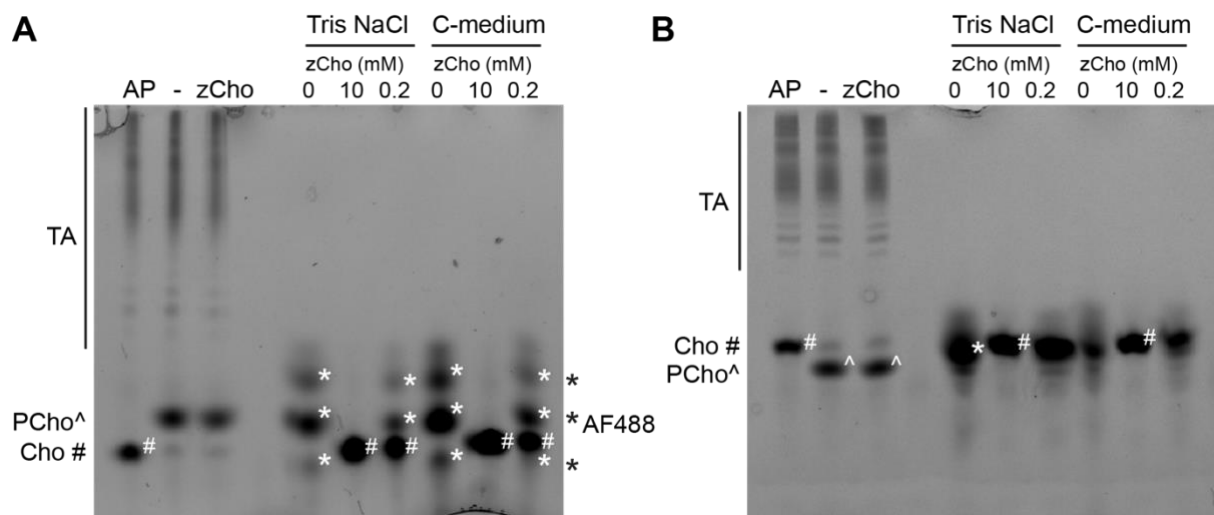
1015 **Figure S6.** Effect of the concentration of added recombinant LytA on the sedimentation of LTA. R800
1016 WT cells grown in C-medium with 200 μM azido-choline were click-labeled with DBCO-AF488 and
1017 lysed overnight with mutanolysine, lysozyme and various concentration of recombinant LytA. Lysates
1018 were centrifuged at 20,000 g for 2 min. The pellets were resuspended in one-fourth the initial volume
1019 and samples were analyzed by gel electrophoresis. Fluorescently labeled compounds were revealed by
1020 UV trans-illumination.

1021 **Notes on the electrophoresis**

1022 Several points should be made regarding the electrophoretic system. We used gels with
1023 acrylamide concentrations of either 12% or 17%. Although the acrylamide concentration had little
1024 consequence on the separation of WTA and LTA, we observed that the migration of small soluble
1025 compounds was greatly affected (SI [Figs S7A-B](#)). Thus, the phospho-choline-triazole-AF488 migrates
1026 a lesser distance than the choline-triazole-AF488 or the DBCO-AF488 in 12% acrylamide gels, whereas
1027 the opposite occurs in 17% gels. The separation of the choline-triazole-AF488 and the DBCO-AF488
1028 is not sufficient in 17% gels.

1029 Also, we found that thiol reducing agents such as DTT routinely included when loading the gels
1030 do react with DBCO-AF488 to yield a mixture of species, denoted with * in SI [Figs S7A-B](#). The extent
1031 of this reaction appeared to depend on the length and conditions of the storage of the samples prior to
1032 analysis and therefore was not controlled.

1033 In some cases, such as in [Figs 6A, 6B or 6E](#), one or multiple additional weak but sharp bands of
1034 fluorescently labeled material appear on the gels above the LTA bands. These bands can be correlated
1035 to Coomassie-stained protein bands (SI [Figs S8D-F](#)) and they are likely products of the reaction of
1036 DBCO-AF488 with protein thiols or primary amines. As stated above for the modification of DBCO-
1037 AF488, this side reaction was not controlled and not always present. A weak non-specific fluorescent
1038 labeling of cellular proteins by DBCO-AF488, which was not a problem when TA were labeled during
1039 the whole culture, contributed to a significant smear blurring the TA bands when labeling was performed
1040 only during a short pulse time such as in [Fig. 5C](#).

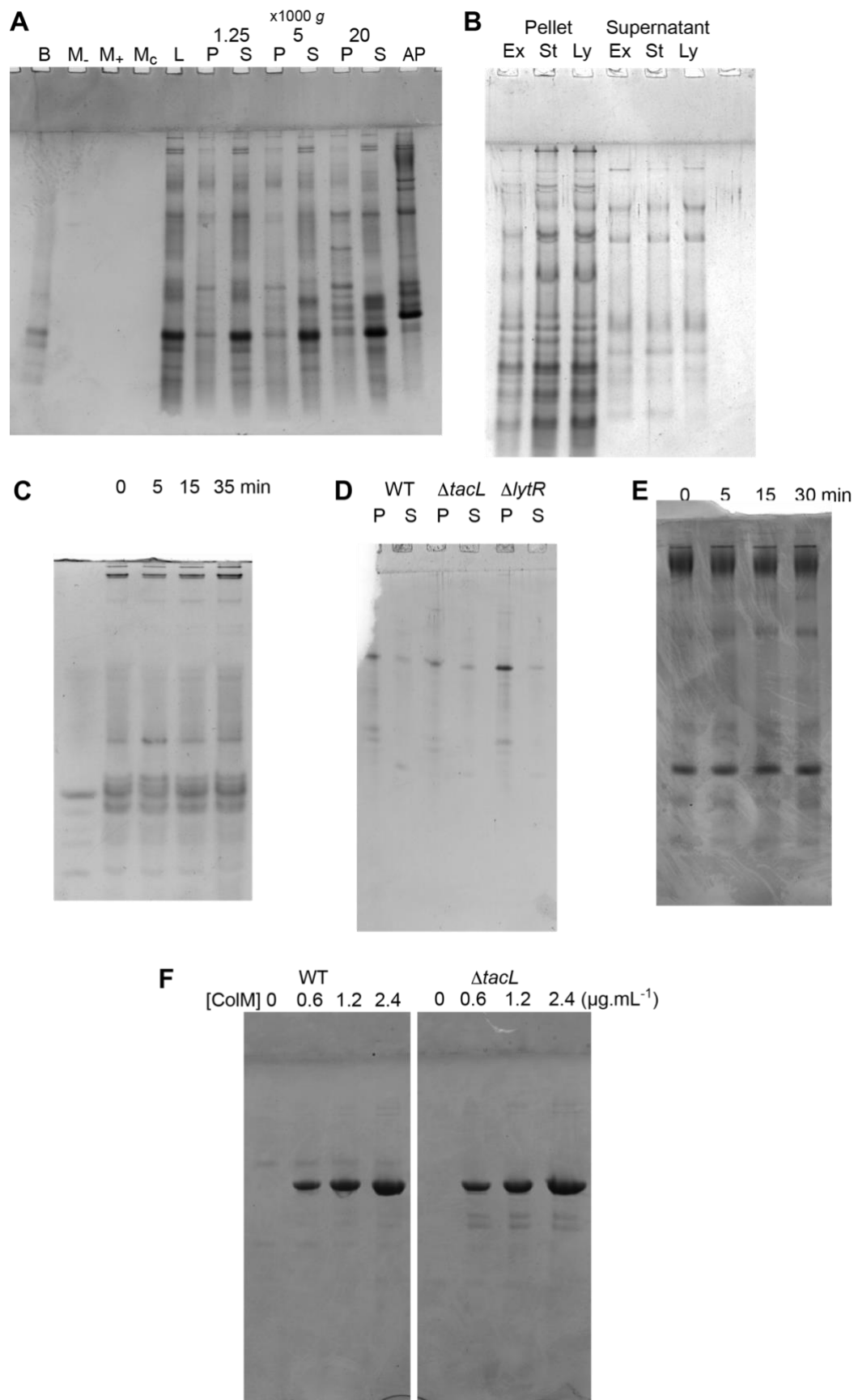


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1043 **Figure S7.** Polyacrylamide gel electrophoresis of click-labeled TA, azido-choline (zCho) and the
 1044 clickable fluorophore DBCO-AF488 with (A) 12% (w/v) and (B) 17% acrylamide. Three lanes on the
 1045 left of the gels: R800 cells were grown in C-medium in the presence of 0.2 μM azido-choline prior to
 1046 lysis and secondary labeling with 10 μM DBCO-AF488 overnight at room temperature. Samples were
 1047 further incubated 4 h with either 1000 $\text{U}\cdot\text{mL}^{-1}$ alkaline phosphatase (AP), nothing (-), or 10 mM
 1048 additional azido-choline. Six lanes on the right of gels. In either 50 mM Tris pH 8, 150 mM NaCl or C-
 1049 medium, 20 μM DBCO-AF488 were incubated at room temperature for 4 h with either 0, 10 mM or
 1050 200 μM azido-choline. Prior to gel loading, samples were supplemented with 8% glycerol, 0.8% SDS,
 1051 8 mM DTT and trace of bromophenol blue. The migration position of the fluorophore-triazole-linked
 1052 species are indicated on the left or on the gels by symbols (PCho[^], phospho-choline; Cho^{*}, choline).
 1053 The position of the DBCO-AF488 reagent is indicated on the right or on the gel with the symbol #.

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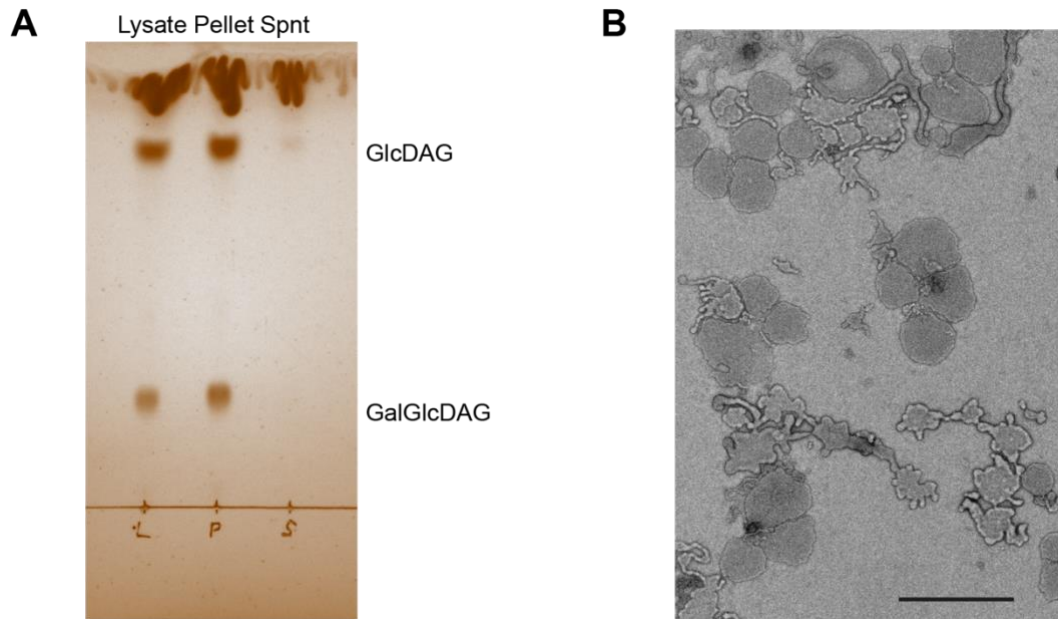


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1057 **Figure S8.** Protein Coomassie staining of TA analysis gels presented in [Figs 4A](#), [5A](#), [5B](#), [6A](#), [6B](#), [6E](#).

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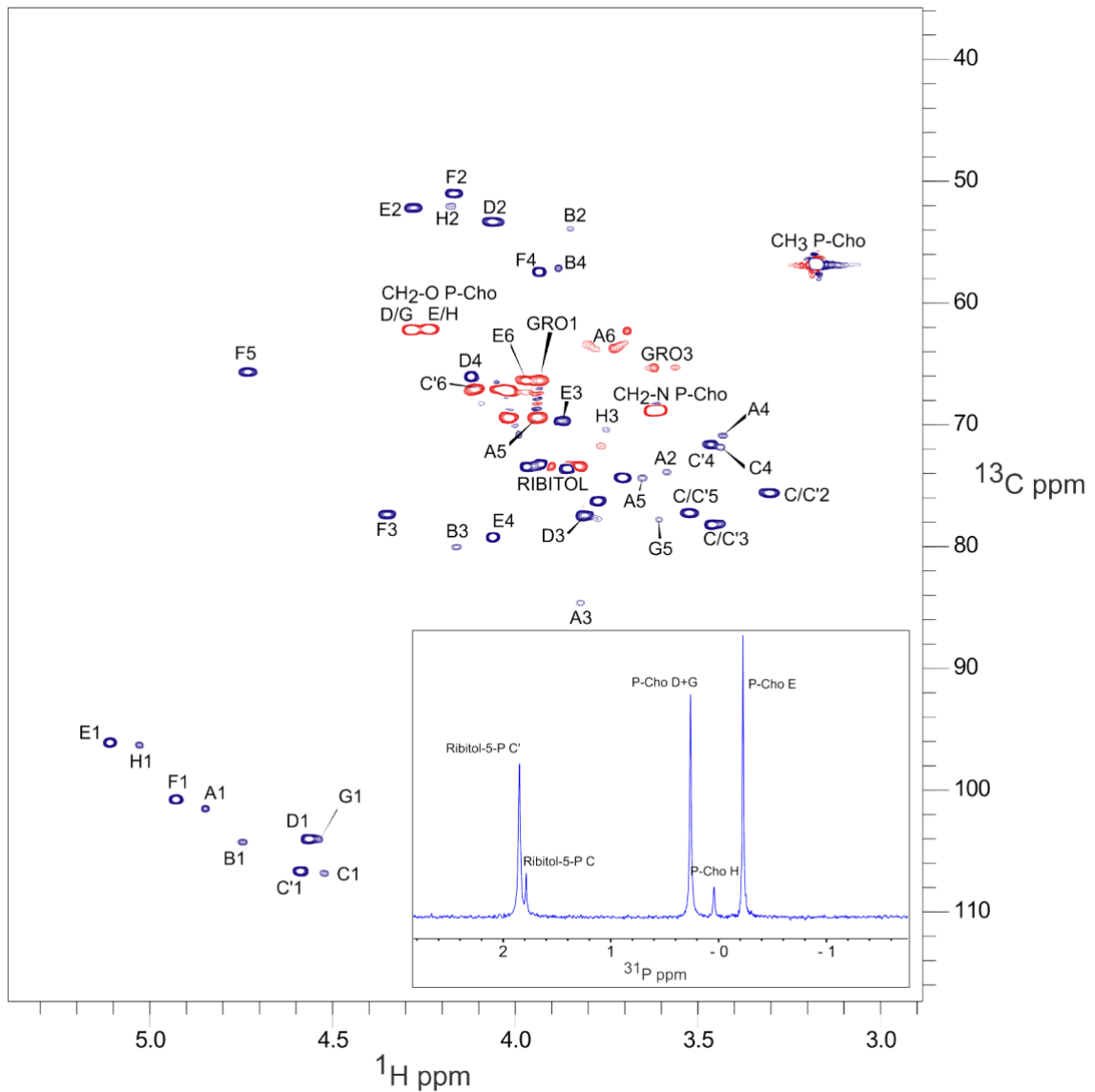
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Figure S9. Characterization of the cell lysate fractionation by sedimentation. **(A)** Iodine vapor-stained thin layer chromatography of lipids extracted from R800 cell lysate, the resuspended low-speed centrifugation pellet and the supernatant (Spnt) of the lysate. Lipids were tentatively identified according (2). GlcDAG, mono-glucosyl-diacyl-glycerol (DGlc p -DAG); GalGlcDAG, galactosyl-glucosyl-diacyl-glycerol (DGalp-DGlc p -DAG). **(B)** Negative stain electron micrograph of the resuspended low-speed centrifugation pellet. Magnification was 4800x. Scale bar is 1 μ m.



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1070 **Figure S10.** NMR spectra of *S. pneumoniae* LTA obtained by low speed centrifugation of cell lysate.

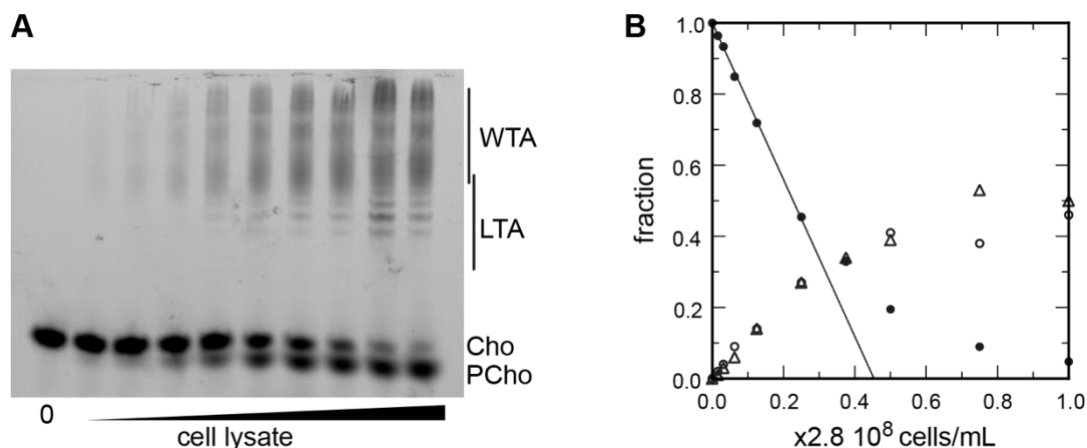
1071 Main is ^{13}C - ^1H HSQC (constant-time) spectrum; ^{13}C nuclei that are coupled to either zero or two other

1072 carbons (red) and those coupled to one or three other carbons (dark blue), exhibit opposite signs. Inset

1073 is ^{31}P 1D spectrum. The peak nomenclature is that proposed by Gisch et al. (1). Peaks A1 to A6, GRO1

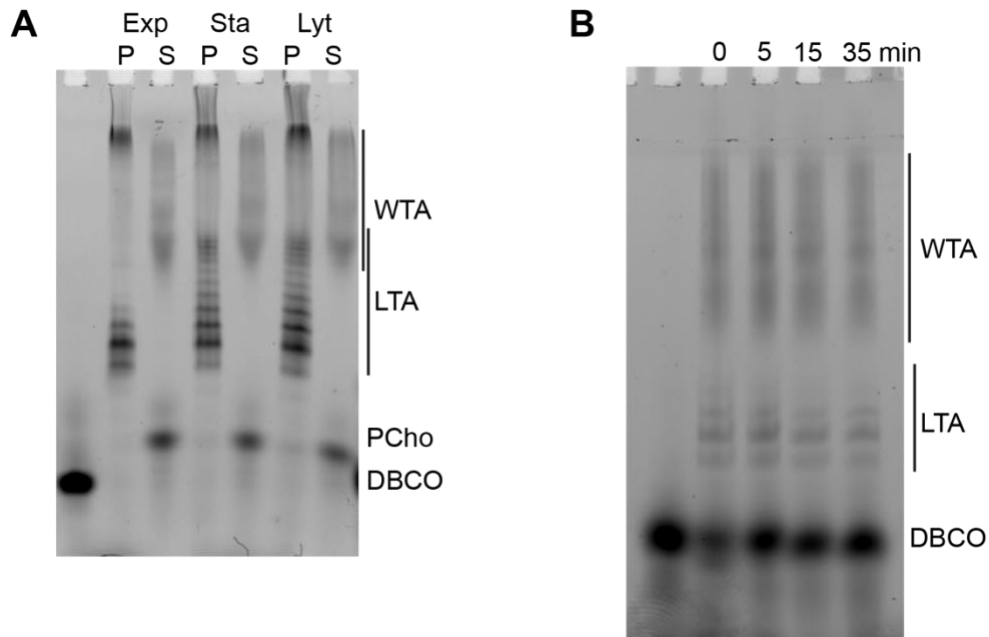
1074 GRO3 are originating from the lipid anchor DGLcp-DAG of LTA.

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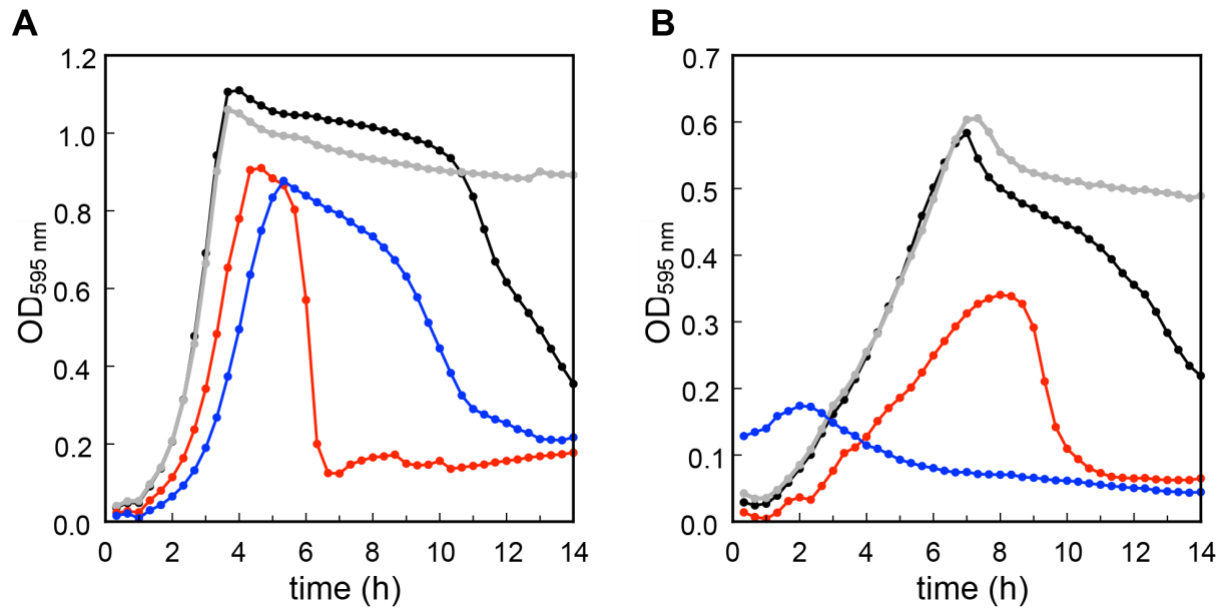
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1078 **Figure S11. (A-B)** Titration of cellular TA. R800 cells were grown for two generation in the presence
1079 of azido-choline. DBCO-AF488 (3.7 μM) was incubated for 24 h with varying amount of cell lysate
1080 corresponding to up to 2.9×10^8 cells mL^{-1} . The remaining DBCO-AF488 was blocked by addition of
1081 100 mM azido-choline, and the various species were separated by polyacrylamide gel electrophoresis.
1082 (A) The gel was imaged by trans-illumination with UV light. (B) The bands were quantified and the
1083 relative amount of the various species were plotted against the cell concentration. Black circles, blocked
1084 DBCO-AF488; open circle, phospho-choline; open triangle, TA. A linear regression of the DBCO-
1085 AF488 points at low cellular concentration was applied to obtain the titration point as the intercept of
1086 the cell concentration axis.



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Figure S12. Evolution of TA during growth. **(A)** $\Delta lytA$ cells grown in C-medium containing 200 μ M azido-choline were harvested during the exponential growth phase (Ex), at the onset of the stationary phase (St) and during the autolysis (Ly). TA were fluorescently click-labeled with DBCO-AF488 and cells were completely lysed by the addition of peptidoglycan hydrolases prior to centrifugation. LTA are found in the pellet (P) whereas WTA are observed in the supernatant (S). The amounts of cells at the different culture stages were normalized. The LTA samples are 8-fold concentrated compared to the WTA samples. Fluorescently labeled TA were revealed by UV-transillumination after SDS-polyacrylamide electrophoresis. **(B)** Electrophoretic analysis of TA of WT cells grown in C-medium supplemented with 0.1% yeast extract and pulse-labeled for 5 min with the addition of 0.2 mM azido-choline, and chased by further growth in the same medium without added azido-choline for the indicated duration.



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Figure S13. Growth of the *S. pneumoniae* WT (black), $\Delta lytA$ (gray), $\Delta tacL$ (red) and $\Delta lytR$ (blue) strains

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in BHI (A) and C-medium 200 μM choline (B).

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1106 **Discussion of the sedimentation of *S. pneumoniae* LTA**

1107 Finally, a technical point requires discussion. The use of fluorescent labeling allowed to track the
1108 TA during cell fractionation and revealed that membranes from *S. pneumoniae* sediment at low relative
1109 centrifugal force ([Fig. 4A](#)). This was a surprise to us, since we were used, as many other laboratories,
1110 to pellet membranes from *E. coli* for the preparation of recombinant proteins at 100,000 *g* after a
1111 clarification step at 40,000 *g*. However, considering the membrane composition could have predicted
1112 this outcome. The sedimentation coefficient s is proportional to $1 - \rho\bar{v}$ where ρ is the density of the
1113 solvent (close to 1 for aqueous biochemical solutions) and \bar{v} is the partial-specific volume of the particle
1114 (the reciprocal of the density). For a complex particle such a membrane, \bar{v} is the weighted sum of its
1115 components $\bar{v} = \sum_i^n w_i \bar{v}_i$. In *E. coli*, glycolipids are absent of the plasma membrane, which consists
1116 mostly of phosphatidylethanolamine (~78%), phosphatidylglycerol (~12%) and cardiolipin (~6%) (3).
1117 The partial-specific volume \bar{v}_i of the various phospholipids is comprised between 0.93 and 1 (4), so
1118 that the partial-specific volume \bar{v} of the *E. coli* membrane is also close to 1 and its sedimentation
1119 coefficient s is very small. In *S. pneumoniae* in contrast, the glycolipids mono-glucosyl-diacylglycerol
1120 (~35%) and galactosyl-glucosyl-diacylglycerol (~45%) are the major constituent of the membrane, with
1121 minor contributions of phosphatidylglycerol (~13%) and cardiolipin (5). Since the partial-specific
1122 volume \bar{v}_i of glucose and galactose is 0.622 (4) and 80% of the pneumococcal lipids carry one or two
1123 hexoses, the resulting partial-specific volume \bar{v} of the membrane should be around 0.8, and
1124 consequently the membranes of *S. pneumoniae* have a larger sedimentation coefficient s than those of
1125 *E. coli*. Note that the LTA themselves further increase the sedimentation coefficient by contributing
1126 additional saccharidic units to the membrane composition.

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1128 **References**

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