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2 *Clostridium difficile*

3

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26 running title: The PASTA Ser/Thr kinase of *Clostridium difficile*

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28

29 Keywords: Hanks kinase, cephalosporin sensitivity, cell morphology, envelope homeostasis,
30 CAMPs, biofilms, peptidoglycan

31 Abstract:

32 *Clostridium difficile* is the leading cause of antibiotic-associated diarrhea in adults. During
33 infection, *C. difficile* must detect the host environment and induce an appropriate survival
34 strategy. Signal transduction networks involving serine/threonine kinases (STKs) play key
35 roles in adaptation, as they regulate numerous physiological processes. PrkC of *C. difficile* is
36 a STK with two PASTA domains. We showed that PrkC is membrane associated and is
37 found at the septum. We observed that deletion of *prkC* affects cell morphology with an in-
38 crease in mean size, cell length heterogeneity, and presence of abnormal septa. When com-
39 pared with the wild-type strain, a $\Delta prkC$ mutant was able to sporulate and germinate but was
40 less motile and formed more biofilm. Moreover, a $\Delta prkC$ mutant was more sensitive to an-
41 timicrobial compounds that target the cell envelope such as the secondary bile salt deoxy-
42 cholate, cephalosporins, cationic antimicrobial peptides, and lysozyme. This increased sus-
43 ceptibility was not associated with differences in peptidoglycan or polysaccharide II compo-
44 sition. However, the $\Delta prkC$ mutant had less peptidoglycan and released more polysaccharide
45 II into the supernatant. A proteomic analysis showed that the majority of *C. difficile* proteins
46 associated with the cell wall were less abundant in the $\Delta prkC$ mutant compared to the wild-
47 type strain. Finally, in a hamster model of infection the $\Delta prkC$ mutant had a colonization
48 delay that did not significantly affect overall virulence.

49

50

51 **Introduction**

52 *Clostridium difficile* is the leading cause of antibiotic-associated infections in adults.
53 Severity of *C. difficile* infection (CDI) symptoms range from diarrhea to life-threatening
54 pseudo-membranous colitis. Major risks associated with CDI include antibiotic exposure
55 leading to dysbiosis of gut microbiota, as well as, advanced aged and hospitalization (1).
56 The impact of CDI is significant in terms of mortality, morbidity, disease management and
57 financial burden. With the emergence of new isolates, the incidence and severity of CDI
58 have increased both in North America and Europe (2). *C. difficile* is acquired from the envi-
59 ronment through the ingestion of spores. This resistant form of the bacterium is thought to be
60 responsible for transmission, environmental persistence and dissemination. When the normal
61 intestinal microbiota is disrupted, the pools of metabolites present in the gut change leading
62 to an increased concentration of cholate conjugates, which in turn triggers spore germination
63 (3, 4). The vegetative cells can then multiply and colonize the dysbiotic gastrointestinal tract.
64 Toxigenic strains of *C. difficile* produce two toxins, TcdA and TcdB, which are its main vir-
65 ulence factors. These toxins cause alteration of the actin cytoskeleton of epithelial cells and
66 neutrophil recruitment causing local inflammation (3). Following the administration of a
67 targeted antibiotic therapy such as metronidazole or vancomycin, CDI relapse can occur,
68 involving spore and possibly biofilm formation (5, 6). Additional factors including those
69 involved in stress adaptation and surface associated proteins, such as adhesins, the fibron-
70 ectin-binding protein (FbpA), the surface layer protein (SlpA) and the flagellum also partici-
71 pate in the colonization process, allowing *C. difficile* to establish its intestinal niche (7).

72 Gut dysbiosis is an essential step that allows *C. difficile* to colonize the colon and cause
73 an infection. Certain antibiotics such as cephalosporins, clindamycin and fluoroquinolones
74 are known to increase the risk of developing a CDI. Most of *C. difficile* strains are resistant
75 to these antibiotics, which is of major concern (1). Fluoroquinolone resistance in *C. difficile*
76 strains is associated with amino acid substitution in the gyrases GyrA or GyrB (1). The
77 mechanisms responsible for cephalosporin resistance by *C. difficile* remain poorly character-
78 ized (1). However, some progress has been made, as a class D β -lactamase was identified in
79 the genomes of most *C. difficile* strains (8). In addition to antibiotics, vegetative cells also
80 encounter secondary bile salts (4), host secreted antimicrobial peptides and also reactive ox-
81 xygen and nitrogen species produced during inflammation (3, 9). To survive in the host, *C.*
82 *difficile* must detect the host environment and induce an appropriate survival strategy, which
83 may include activating the general stress response, sporulation, biofilm formation, and pro-

84 ducing virulence factors (5, 6, 9).

85 Protein phosphorylation is a reversible post-translational modification used to transduce
86 signal and regulate cellular processes. Bacterial serine/threonine kinases of the Hanks family
87 (STKs) share structural similarities with eukaryotic serine/threonine kinases and undergo
88 substrate phosphorylation on serine or threonine residues (10). STKs and their associated
89 phosphatases (STP) regulate numerous physiological processes including translation, carbon
90 and cell-wall metabolism, antibiotic tolerance, cell division, developmental processes, and
91 virulence (10, 11). Phosphoproteomic studies revealed that STKs might phosphorylate a
92 broad spectrum of substrates (12) including enzymes, components of the cellular machinery
93 involved in translation, division or repair and also several transcriptional regulators that
94 mainly form part of two-component systems (10, 13). Therefore, STKs are key integration
95 nodes in signaling networks that control bacterial physiology, growth and stationary phase.
96 In *B. subtilis*, PrkC is a trans-membrane kinase with an extracellular signal receptor domain
97 containing penicillin-binding and STK associated (PASTA) repeats. PrkC interacts with pep-
98 tidoglycan fragments and β -lactam *in vitro* (14, 15), while a PASTA-STK (Stk1) homologue
99 in *Staphylococcus aureus* interacts with lipid II (16). In *B. subtilis*, PrkC is a key-signaling
100 enzyme that controls stationary phase survival, biofilm formation, sporulation, germination
101 (13) and peptidoglycan (PG) metabolism (17). Inactivation of genes encoding PASTA do-
102 main-containing STKs are associated with a range of phenotypes among Gram-positive bac-
103 teria, with defects in cell division and changes in cell-wall metabolism commonly observed
104 (10, 17, 18). In *S. aureus*, Stk1 plays a role in virulence, antibiotic resistance and cell-wall
105 remodeling (17) whereas in *Streptococcus pneumoniae*, StkP controls cell division, compe-
106 tence, virulence, adherence to eukaryotic cells, stress response and cell wall metabolism (17,
107 19).

108 The role of STKs has not been studied in anaerobic firmicutes. *C. difficile* has two genes
109 encoding STKs, *CD2148* and *prkC* (*CD2578*) and one gene encoding a STP (*CD2579*). In
110 this study, we characterized the role of the *C. difficile* PASTA-STK, PrkC, in the regulation
111 of the physiological processes important in CDI. Inactivation of the *prkC* gene resulted in
112 changes in the morphology and the properties of the cell envelope as well as increased sensi-
113 tivity to antimicrobial compounds targeting the cell wall.

114

115 **Materials and methods**

116 **Bacterial strains and growth conditions**

117 *C. difficile* strains and plasmids used in this study are listed in Table S1. *C. difficile* strains
118 were grown anaerobically (5% H₂, 5% CO₂, 90% N₂) in TY broth (Bacto tryptone 30 g.l⁻¹,
119 yeast extract 20 g.l⁻¹, pH 7.4), in Brain Heart Infusion broth (BHI) or in a peptone-containing
120 medium (Pep-M) (20). Sporulation medium (SM) (21) was used for sporulation assays and
121 spores were produced in SMC medium (22). Solid media were obtained by adding agar to a
122 final concentration of 17 g.l⁻¹. When necessary, cefoxitin (Cfx, 25 µg.ml⁻¹) or thiamphenicol
123 (Tm, 15 µg.ml⁻¹) was added. *E. coli* strains were grown in LB broth. When indicated, ampi-
124 cillin (100 µg.ml⁻¹) or chloramphenicol (15 µg.ml⁻¹) was added. Anhydrotetracycline (ATc)
125 was used to induce expression of the *prkC* gene from the P_{tet} promoter of pDIA6103 (23).

126 **Construction of plasmids and strains**

127 The allelic exchange cassette used to delete of the *CD2578* gene (*prkC*) was constructed in a
128 derivative of pMTLSC7315 (24) lacking the multi-cloning site. DNA sequences flanking the
129 *prkC* gene (1038 and 1117-bp) were amplified by PCR using primers IMV854 and IMV855
130 or IMV856 and IMV857 (Table S2 and Fig S1). A DNA fragment obtained by overlapping
131 PCR was digested by XhoI and BamHI and then ligated into pMTLSC7315ΔMCS, giving
132 pDIA6401. *E. coli* HB101 (RP4) containing pDIA6401 was mated with *C. difficile*
133 630Δ*erm*. Transconjugants were selected on BHI-Cfx-Tm plates. Colonies were restreaked
134 onto the same medium to identify faster growing single-crossover integrants that were then
135 streaked onto CDMM supplemented with 50 µg/ml fluorocytosine (FC) to select for second
136 cross-over events. FC^R Tm^S clones that have lost pDIA6401 were analyzed by PCR using
137 IMV867 and IMV868 to distinguish between wild-type and Δ*prkC* clones (Fig S1). For
138 complementation, the *prkC* gene (positions -22 to +2205 from the translational start site) was
139 amplified using primers IMV879 and IMV880. The PCR fragment was cloned between the
140 StuI and BamHI sites of pDIA6103 (25) to produce pDIA6413. To construct a plasmid ex-
141 pressing PrkC-HA (pDIA6713), an reverse PCR was performed using pDIA6413 as template
142 and primers EC01 and EC02. The same strategy was used to introduce a point mutation into
143 the *prkC* gene [lysine at position 39 replaced by an alanine (K39→A)]. The resulting plas-
144 mid, pDIA6714, was then used to construct pDIA6716 expressing HA-tagged
145 *prkC*(K39→A). These plasmids were transferred into *C. difficile* strains by conjugation (Ta-
146 ble S1).

147 To construct a translational SNAP-PrkC fusion, we amplified the SNAP coding sequence
148 fused to a linker in 3' (GGATCCGCAGCTGCT) using pFT58 as a template and primers
149 EC238 and EC239. pDIA6413 (pDIA6103-*prkC*) was amplified by inverse PCR using the

150 primers EC236 and EC237. Using these two fragments, pDIA6855 was obtained by Gibson
151 Assembly. pDIA6855 was conjugated in the 630 Δ *erm* strain to obtain CDIP1357 (Table S1).
152 To determine if *prkC* is co-transcribed with upstream genes (*CD2579* and *rlmN*), we carried
153 out an RT-PCR experiment using RNA extracted from exponentially growing cells. After
154 cDNA synthesis using IMV843, a primer targeting the *prkC* gene, PCR amplification was
155 tested with pairs of primers located in adjacent genes (*prkC/CD2579* or *CD2579/rlmN*).
156 Non-treated RNAs were used as a negative control.

157 **Antimicrobial sensitivity tests**

158 Cultures of *C. difficile* strains (OD_{600nm} of 0.3) were plated on BHI agar plates. 6-mm paper
159 disks containing antibiotics were placed onto the agar surface. Lysozyme (800 μ g) was also
160 placed on a 6-mm paper disk using Pep-M agar plates. The growth inhibition diameter was
161 measured after 24 h of incubation at 37°C. Minimal Inhibitory Concentrations (MICs) were
162 determined on BHI plates by E-test (bioMérieux) after 24 h incubation at 37°C. To test sus-
163 ceptibility for antimicrobial peptides, protocols were modified from previous published work
164 (26, 27). Each well of a 24-well microplate was inoculated with 1 ml of a BHI bacterial in-
165 oculum (OD_{600nm} of 0.01). After addition of bacitracin (Sigma Aldrich), polymixin B (Sigma
166 Aldrich) or nisin (Sigma Aldrich), microplates were incubated at 37°C for 20 h. The MIC
167 was defined as the lowest concentration of antimicrobial peptide preventing growth.

168 5 μ l of an overnight culture of *C. difficile* strains was streaked on BHI plates without or with
169 SDS (0.005% to 0.009%). The plates were incubated at 37°C for 48 h. Deoxycholate (DOC)
170 resistance tests were performed in 24-well microplates. Each well was inoculated with 1 ml
171 of TY with or without DOC (0.03%). The OD_{600nm} of each culture was monitored. For autol-
172 ysis, cells (OD_{600nm} of 1) were resuspended in 50 mM potassium phosphate buffer (pH 7.0)
173 containing 0.01 % of Triton X-100 and incubated at 37°C. The OD_{600nm} was determined eve-
174 ry 5 min.

175 **Sporulation, germination, motility and biofilm formation assays**

176 Sporulation assays were performed as described previously (28). To purify spores, 100 μ l of
177 culture plated on solid SMC were grown at 37°C for 7 days. Spores were scraped off with
178 water and then incubated 7 days at 4°C to allow the release of spores from the cells. Spores
179 were purified by centrifugation using a HistoDenz (Sigma-Aldrich) gradient as described
180 previously (22). For the germination efficiency tests, we monitored OD_{600nm} of purified
181 spores incubated under anaerobiosis in BHIS supplemented with 0.5 % taurocholate, with
182 bryostatin or mucopeptides.

183 To measure motility, plates containing 25 ml of BHI agar (0.3% w/v), Tm and ATc were
184 inoculated with 5 μ l of an exponential-phase culture. Plates were incubated for 48h at room
185 temperature and the zone of motility was then measured. For the biofilm assay, 1 ml of BHI-
186 S medium containing 0.1 M glucose, 0.1% cysteine and polymixin B (20 μ g.ml⁻¹) or DOC
187 (0.01%) was inoculated and deposited in a well of a 24-well microplate. Microplates were
188 incubated at 37°C. The biofilm was washed with PBS (Phosphate buffered saline), stained
189 with 1 ml of crystal violet (0.2%) followed by two washes with PBS. The OD_{570nm} was
190 measured after resuspension of the cells in methanol/acetone (80%/20%) using non-
191 inoculated medium as a negative control. For dispersion of preformed biofilms, 24 h and 48
192 h biofilms were treated with DNase I (100 μ g/ml in 150 mM NaCl, 1 mM CaCl₂) under an-
193 aerobic conditions at 37°C for 1 h (29). Control wells were treated with buffer without
194 DNase. Biofilms were then washed, stained, and quantified as described above.

195 **Phase contrast and transmission electron microscopy**

196 *C. difficile* strains were grown 5 h in TY medium. For phase contrast microscopy, cells were
197 analyzed using an axioskop microscope (Zeiss). For transmission electron microscopy
198 (TEM), cultures were mixed with 1 volume of a fixative solution containing 4% paraformal-
199 dehyde (PFA) and 4% glutaraldehyde (GA) in PHEM buffer 1x pH 7.3 (60 mM Pipes,
200 25mM Hepes, 10 mM EGTA, 2mM MgCl₂) and then incubated for 30 min at room tempera-
201 ture. After centrifugation, pellets were resuspended in a second fixative solution (2% PFA
202 and 2% GA in PHEM buffer pH= 7.3) and incubated for 1 h. Samples were then washed
203 twice in PBS prior to being subjected to a high pressure (>2000 bar) freezing in 1-
204 hexadecane using a BAL-TEC HPM 010 (LEICA). Freeze substitution was done with 2%
205 OsO₄ in acetone followed by several steps: -90°C for 42 h, warmed up to -30°C (5°C/h),
206 incubated for 12 h, warmed up to 0°C (10°C/h) and incubated for 1h. Samples were then
207 washed with acetone on ice and incubated in EPON/acetone at different volume ratio (1/3
208 for 3 h, 1/1 for 3 h; 2/1 ON; 3/1 for 4 h), followed by an incubation in pure EPON (2 h,
209 overnight; 6 h). Samples were then incubated overnight in EPON and the hardener BDMI
210 prior to polymerization at 60°C for 48 h. Sections (60-70 nM) were obtained on a FC6/UC6
211 ultramicrotome (Leica), transferred on 200 Mesh Square Copper grids coated with formvar
212 and carbon (CF-200-Cu50, Delta Microscopy). Samples were stained with 4% uranyl acetate
213 and counterstained with lead citrate. Images were recorded with TECNAI SPIRIT 120 kv
214 (with a bottom-mounted EAGLE 4Kx4K Camera).

215 **SNAP labelling, fluorescence microscopy and image analysis**

216 For membrane and chromosome staining, 500 μ l of exponential-phase cells were centrifuged
217 and resuspended in 100 μ l of PBS containing the fluorescent membrane dye FM 4-64 (1
218 μ g/ml, Molecular Probes, Invitrogen) and the DNA stain 4,6-Diamidino-2-phenylindole
219 (DAPI) (2 μ g/ml, Sigma). Samples were incubated for 2 min in the dark and mounted on
220 1.2% agarose pad. Strain CDIP1357 was grown 2 h in TY and expression of the SNAP^{Cd}-
221 *prkC* fusion was induced with 50 ng/ml of ATc for 2 h. For SNAP labelling, the TMR-Star
222 substrate (New England Biolabs) was added at 250 nM, and the mixture was incubated for
223 30 min in the dark under anaerobiosis. Cells were then collected by centrifugation, washed
224 and resuspended in PBS. Cell suspension (3 μ l) was mounted on 1.7% agarose-coated glass
225 slides. The images were taken with 300 ms exposure times for the autofluorescence and 900
226 ms exposure times for the SNAP using a Nikon Eclipse TI-E microscope 100x Objective and
227 captured with a CoolSNAP HQ2 Camera. The images were analysed using ImageJ (30).

228 **Detection of phospho-threonine by western blot on soluble and insoluble fractions.**

229 Cells were grown for 6 h in TY and then harvested by centrifugation. The pellets were re-
230 suspended in PBS containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich)
231 and 0.12 μ g/ml of DNase. Cells were lysed for 45 min at 37°C and then centrifuged. The
232 soluble fraction was diluted twice with 2X Sample buffer (150 mM Tris-HCl pH6.8, 30%
233 glycerol, 1.5 % SDS, 15% β -mercaptoethanol, 2 μ g/ml of bromophenol blue). After 2 wash-
234 es with PBS, the insoluble fraction was resuspended in 2X PBS containing 1% SDS, and
235 mixed with 2X Sample buffer. Western blots were performed using an anti-P-Thr primary
236 antibody (Cell signaling) followed by a goat anti-rabbit horseradish-peroxidase-conjugated
237 secondary antibody (Sigma-Aldrich) and developed using the SuperSignalWest Femto
238 chemiluminescent Kit (Thermo scientific).

239 **Peptidoglycan and polysaccharide II analysis**

240 Peptidoglycan (PG) samples were prepared from 1.5 l of *C. difficile* grown in BHI (OD_{600nm}
241 of 1) as previously described (31). Purified PG was then digested with mutanolysin (Sigma
242 Aldrich) and the soluble muropeptides were separated by reverse phase high-performance
243 liquid chromatography (RP-HPLC) (31). Polysaccharides II (PSII) were extracted from *C.*
244 *difficile* grown in BHI (OD_{600nm} of 1) as previously described (32). PSII was lyophilized and
245 its structure was checked by [¹H] and [¹³C] NMR. Spectra were acquired on a 400 MHz
246 Bruker spectrometer equipped with a Prodigy probe. PSII was lyophilized and its structure
247 was determined by [¹H] NMR. For the quantification of PG and PSII, 500 ml of culture
248 (OD_{600nm} of 1) was used to purify PSII covalently linked to PG (PG-PSII). PG-PSII was pu-
249 rified as previously described (33) without the acetone treatment. Linkage between PG and

250 PSII was disrupted after an incubation for 48 h at 4°C in hydrofluoric acid (48%). The pellet
251 containing PG was washed three times in H₂O and the hydrofluoric acid supernatant that
252 contains PSII subunits was evaporated and resuspended in H₂O. Both, PG and PSII were
253 lyophilized and then weighted.

254 For the PSII dot blot, exponential phase cultures were harvested by centrifugation. Superna-
255 tant and total crude cell extracts were kept as separate fractions. The supernatant fraction
256 was recovered and precipitated with 10% TCA for 30 min. The supernatant and the total
257 crude cell fractions were treated with 100 µg/ml of proteinase K (Sigma) for 1 h at 37°C.
258 Samples were then serially diluted and 5 µl of each dilution were spotted onto an activated
259 polyvinylidene difluoride membrane. The membrane was washed in H₂O, blocked for 15
260 min in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween20, pH7.5) containing 10%
261 milk, and then washed in 5% milk in TBST for 2 min. After overnight incubation in PSII-
262 LTB rabbit antiserum (1:8,000) (34), the membrane was washed once in TBST with 5%
263 milk, twice in TBST for 5 min, and once in TBST with 5% milk for 10 min. Following incu-
264 bation with goat anti-rabbit horseradish-peroxidase-conjugated secondary antibody at
265 1:10,000 dilution for 1 h, the membrane was washed 5 times in TBST for 5 min, and re-
266 vealed using the SuperSignalWest Femto chemiluminescent substrate.

267 **Isolation of cell-wall proteins and proteomic analysis**

268 *C. difficile* strains were grown for 6 h in TY at 37°C and 20 mL of each culture was then
269 centrifuged. The cell pellets were washed with PBS, resuspended (OD_{600nm} of 100) in 75
270 mM Tris-HCl pH6.8, 15% glycerol, 7.5% β-mercaptoethanol, 0.75% SDS and boiled for 10
271 min at 100°C. Proteins were precipitated with 10% TCA, washed with 90% cold acetone,
272 dried, resuspended in urea 8 M/NH₄HCO₃ 100 mM and sonicated. Total protein extracts (50
273 µg) were reduced with TCEP (10 mM) (Sigma) for 30 min and alkylated with iodoacetamide
274 20 mM (Sigma) for 1 h. Proteins were digested with rLys-C 1 µg (Promega) for 4 h at 37°C
275 and with Trypsin 1µg (Promega) overnight at 37°C. The digestion was stopped with Formic
276 acid (FA) 4%. Peptides were desalted on C18 Sep-Pak Cartridge (WAT054955, Waters) and
277 eluted with Acetonitrile (ACN) 50%/FA 0.1% and then ACN 80%/FA 0.1% before being
278 dried in vacuum centrifuge. Peptides were resuspended with ACN 2%/FA 0.1%. A na-
279 nochromatographic system (Proxeon EASY-nLC 1000, Thermo Fisher Scientific) was cou-
280 pled on-line to a Q ExactiveTM HF Mass Spectrometer (Thermo Fisher Scientific). Peptides
281 (1 µg) were injected onto a 47 cm C18 column (1.9 µm particles, 100 Å pore size, ReproSil-
282 Pur Basic C18, Dr. Maisch GmbH) and separated with a gradient from 2 to 45 % ACN at a
283 flow rate of 250 nl/min over 132 min. Column temperature was set to 60°C. Data were ac-

284 quired as previously described (35). Raw data were analyzed using MaxQuant software ver-
285 sion 1.5.1.2 (36) using the Andromeda search engine (37). The MS/MS spectra were
286 searched as previously described (35) against an internal *C. difficile* database containing
287 3,957 proteins and the contaminant file included in MaxQuant. The statistical analysis was
288 performed with Perseus 1.5.2.6 (38) as previously described (35). Missing values for LFQ
289 intensities were imputed and replaced by random LFQ intensities that are drawn from a
290 normal distribution at the low detection level. Statistical significance was assessed with a
291 two-sided *t*-test of the Log2 transformed LFQ intensities with a permutation-based FDR cal-
292 culation at 5% and $S_0=2$ (39). Differentially regulated proteins are visualized on a Volcano-
293 Plot. The mass spectrometry proteomics data have been deposited to the ProteomeXchange
294 Consortium via the PRIDE (40) partner repository with the dataset identifier PXDX012241.

295 **Golden Syrian hamster infections**

296 Golden Syrian hamsters were first treated with a single oral dose of 50 mg/kg of
297 clindamycin. Five days after the antibiotics treatment, the hamsters were infected by gavage
298 with 5000 spores of either the 630 Δ erm strain or the Δ *prkC* mutant. Spore inocula were
299 standardized before challenge. Eight animals per strain per experiment were used for the
300 infection and we performed two independent experiments. Colonization was followed by
301 enumeration of *C. difficile* cells in feces samples and was started 2-day post infection and
302 each day until the death of the animal. Briefly, feces were resuspended in PBS at 10 mg.ml⁻¹
303 and serially diluted with PBS before plating on BHI supplemented with 3% of defibrinated
304 horse blood and the *C. difficile* selective supplement containing cycloserine (25 μ g/ml) plus
305 cefoxitine (8 μ g/ml). All animal experiments were conducted according to the European
306 Union guidelines for the handling of laboratory animals and Procedures for infection, eutha-
307 nasia, and specimen collection were approved by the Central Animal Care Facilities and Use
308 committee of University Paris-Sud (agreement 92-019-01; protocol number 2012-107).

309

310

310 **Results**

311 **CD2578 is the PASTA-STK of *C. difficile***

312 CD2578 (PrkC) from *C. difficile* contains a cytosolic N-terminal kinase domain and an
313 extracellular motif containing repeats of a PASTA that are separated by a trans-membrane
314 segment (Fig 1A). Based on its predicted amino acid sequence, the kinase domain of PrkC
315 has a high level of sequence identity with that of *B. subtilis* (PrkC), *S. aureus* (Stk1), *S.*
316 *pneumoniae* (StkP), *Enterococcus faecalis* (IreK) and *Mycobacterium tuberculosis* (PknB)
317 (43 to 50%). The conserved lysine within the ATP-binding P loop of the kinase domain is

318 present and corresponds to position 39 in PrkC (Fig 1A). This residue is essential for the
319 phosphotransfer in other STKs (41). As observed for the PASTA-STKs in other firmicutes, a
320 trans-membrane segment is predicted from amino acid 375 to 397 (42, 43). The extracellular
321 sequence of PrkC contains 2 PASTA motifs (Fig 1A) while up to 7 PASTA motifs are typi-
322 cally present in STKs of other firmicutes (17). Interestingly, *C. difficile* contains a very atyp-
323 ical Ser-Gly-Asn (SGN) rich region of 100 amino acids at the C-terminal part of the protein
324 (Fig 1A). This SGN-rich region is absent from PASTA-STKs of other firmicutes. Interest-
325 ingly, SGN-rich regions are found in some cell wall anchored proteins of bacilli and in
326 CwpV, a surface associated protein in *C. difficile* but their function have yet to be character-
327 ized.

328

329

330 **The *prkC* locus of *C. difficile***

331 The *prkC* gene likely belongs to a large cluster of genes, which ranges from *dapF* (*CD2590*)
332 to *CD2578-prkC* (Fig 2A). As usually observed in other firmicutes (44, 45), a gene
333 (*CD2579-stp*) encoding a PP2C-type phosphatase (STP), which is probably involved in the
334 dephosphorylation of the PrkC substrates, is adjacent to *prkC*. Upstream of *stp*, we found
335 genes encoding proteins involved in translation (*rlmN*, *rsmB*, *def* and *fmt*), transcription
336 (*rpoZ*), DNA replication (*priA*) and metabolism (*gmk* and *coaBC*). This cluster is conserved
337 in *B. subtilis* and *B. cereus* with the exception of 3 genes that are only present in *C. difficile*;
338 *CD2582* and *CD2583* encoding membrane proteins and *dapF* encoding a diaminopimalate
339 epimerase (Fig 2A). To determine if *prkC* is co-transcribed with *stp* as observed in other
340 firmicutes, and the other genes located upstream of *stp* such as the *rlmN* gene, RT-PCR ex-
341 periments were performed. PCR products were detected using primer pairs located in adja-
342 cent genes (*prkC/stp* or *stp/rlmN*) with RNAs processed by a reverse transcriptase but not
343 with untreated RNA used as negative control (Fig 2B). This result indicated that the *prkC*
344 gene is in operon with *stp* but also *rlmN*. Using a genome-wide transcriptional start site
345 (TSS) mapping (25), we identified a unique TSS in the *prkC* locus located 23 bp upstream of
346 *dapF* suggesting that this gene might be the first gene of a large operon. We found a TG-N-
347 TATAAT extended -10 box specific for the consensus of σ^A -dependent promoters upstream
348 of the TSS (Fig 2A). However, we cannot exclude the existence of additional promoters be-
349 tween *dapF* and *prkC* that were not identified in the genome-wide TSS mapping (25).

350

351 **Localization of the PrkC protein**

352 To determine the cellular location of PrkC, we expressed the *prkC* gene fused to a HA tag
353 under the control of the inducible promoter P_{tet} (pDIA6103-P_{tet}-*prkC*-HA). A western blot
354 analysis of the membrane or cytoplasmic fraction with an anti-HA antibody revealed two
355 bands corresponding to proteins of about 100 kDa and 50 kDa (Fig S2A). These two proteins
356 were only detected in the membrane fraction (Fig 1B). The expected molecular weight of the
357 PrkC-HA tagged protein is around 75 kDa and the band detected around 100 kDa likely cor-
358 responds to PrkC-HA. Indeed, the PrkC and StkP proteins of *B. subtilis* and *S. pneumoniae*
359 are detected with an apparent molecular weight higher than their expected size (42, 43) and
360 the phosphorylation of the kinase results in a reduced electrophoretic mobility (46). The
361 lower band at 50 kDa could be a form of degradation product that might correspond to the
362 extracellular domain fused to HA. It is worth noting that PrkC of *B. subtilis* is sensitive to
363 protease cleavage (42).

364 To determine the localization of PrkC, we used a 630 Δ *erm* strain containing a plasmid en-
365 coding a SNAP^{Cd}-PrkC protein fusion produced under the control of the P_{tet} promoter. The
366 production of the SNAP^{Cd}-PrkC protein is stable as shown by western blot using an anti-
367 SNAP antibody (Fig S2B). After 2 h of induction, we detected the SNAP^{Cd}-PrkC fusion pro-
368 tein fusion at the septum of dividing cells (Fig 1C). Thus, the PrkC protein is membrane as-
369 sociated and localizes at the septum during cell growth.

370

371 **Deletion of the *prkC* gene in *C. difficile* and detection of PrkC activity**

372 To study the role of PrkC in the physiology of *C. difficile*, we inactivated the *prkC* gene by
373 allelic exchange in the strain 630 Δ *erm* (24). Deletion of the *prkC* gene from codon two to the
374 stop codon was confirmed by PCR (Fig S1). The *prkC* mutant was complemented with the
375 wild-type *prkC* gene (pDIA6103-P_{tet}-*prkC*) or a modified copy of *prkC* (pDIA6103-P_{tet}-
376 *prkC*-K39 \rightarrow A) with the lysine residue required for phosphotransfer replaced by an alanine.

377 The kinase activity of PrkC was demonstrated by comparing the profiles of phosphorylation
378 of the wild-type (WT) strain or the Δ *prkC* mutant carrying either pDIA6103, pDIA6103-P_{tet}-
379 *prkC* or pDIA6103-P_{tet}-*prkC*-K39 \rightarrow A. We assumed that PrkC and PrkC-K39 \rightarrow A were pro-
380 duced to the same level and were located in membrane because we detected similar levels in
381 the membrane fraction of these proteins with an HA tag fusion (PrkC-HA or PrkC-K39 \rightarrow A-
382 HA) (Fig S2A). We detected proteins phosphorylated on threonine in the WT strain both in
383 soluble and insoluble fractions by western blot analysis with an antibody against phosphory-
384 lated threonine residues (α -PThr (Fig 1D). Some bands that were detected in the WT strain
385 and with a lower intensity in the complemented strain disappeared in the Δ *prkC* mutant and

386 in the strain producing a modified PrkC-K39→A protein. This result strongly suggested that
387 PrkC has a kinase activity *in vivo* and that the lysine at position 39 (Fig 1A) is essential for
388 the phosphorylation of PrkC targets as observed in the PASTA-STKs of other firmicutes (17,
389 41).

390

391 **Impact of *prkC* deletion on growth, sporulation and germination**

392 The 630 Δ *erm* strain and the Δ *prkC* mutant exhibited nearly identical doubling time and
393 growth yield in TY (Fig S3A). No difference in CFU was also observed between the two
394 strains (Fig S4A and data not shown). We also performed co-culture of the WT and Δ *prkC*
395 mutant strains in TY (Fig S3C). We observed that the Δ *prkC* mutant was less abundant than
396 the wild-type strain by two-fold after 8 h or 24 h of growth. This suggests that there is no
397 drastic difference in fitness associated with the inactivation of *prkC*. The growth of the
398 Δ *prkC* containing pDIA6103-P_{tet}-*prkC* was similar to that of the WT strain in the presence of
399 15 ng/ml of ATc but was affected when we added 50 ng/ml of ATc (Fig S3B). This growth
400 defect was also observed in the Δ *prkC* mutant expressing *prkC*-K39→A (Fig S3B). In con-
401 trast, the addition of increasing concentrations of ATc had no effect on the growth of the WT
402 strain harboring an empty plasmid (Fig S3B). A qRT-PCR analysis also showed that expres-
403 sion of *prkC* was similar in the WT and complemented strains in the presence of 15 ng/ml of
404 ATc (Fig S3D). In the presence of 50 ng/ml of ATc, the expression of *prkC* probably in-
405 creased in the Δ *prkC* strain containing pDIA6103-P_{tet}-*prkC* because we detected a drastic
406 overproduction of the SNAP-PrkC fusion in the presence of 50 ng/ml when compared to 20
407 ng/ml (Fig S2). Over-production of PrkC is likely responsible for the growth defect observed
408 in the presence of 50 ng/ml of ATc suggesting that a proper level of *prkC* expression is im-
409 portant for the fitness of *C. difficile*. Since the kinase activity of PrkC is apparently not re-
410 sponsible for the growth defect when the protein is overexpressed, the trans-membrane seg-
411 ment or the extracellular domain are probably involved in this toxicity.

412 To determine if PrkC is involved in sporulation, we measure sporulation levels of the WT
413 and the Δ *prkC* mutant strains after 24 h or 72 h of growth in SM. No significant differences
414 were observed between both strains for the quantity of spores produced (Fig S4A). When we
415 tested the ability of purified spores to germinate, we observed that the drop in the OD_{600nm} of
416 spore suspensions after the addition of taurocholate was similar for the WT and the Δ *prkC*
417 mutant (Fig S4 B). In *B. subtilis*, PrkC is involved in the germination of spores in the pres-
418 ence of *B. subtilis* muropeptides (47). However, purified muropeptides from *C. difficile* (0.1
419 to 0.01 mg/ml) failed to induce spore germination of the WT or the Δ *prkC* mutant (data not

420 shown). Bryostatins are molecules involved in STK activation that induces germination of *B.*
421 *subtilis* spores (47). This compound at 1 μ M or 10 μ M had no effect on *C. difficile* spores
422 (data not shown). All these results indicated that PrkC is not involved in the control of
423 sporulation and spore germination in *C. difficile*.

424

425 ***prkC* deletion affects cell morphology**

426 To determine if deletion of *prkC* had an effect on the cell morphology, we analyzed by phase
427 contrast microscopy cells of the 630 Δ *erm* strain, the Δ *prkC* mutant, and the complemented
428 strain during exponential growth phase (Fig S5A). The Δ *prkC* mutant cells were more elon-
429 gated than those of the WT strain and the complemented strain (Fig S5A). We then meas-
430 ured the cell size using cells labeled with DAPI and FM4-64 (Fig 3A and 3B). The Δ *prkC*
431 mutant cells had an average cell size of 9.2 μ m whereas the WT and the complemented
432 strains had an average cell size of 4.5 μ m and 6.4 μ m, respectively (Fig 3B). We detected
433 elongated cells (Fig 3A, blue arrows) and cells with several septa and undivided (Fig 3A,
434 green arrows). Interestingly, we observed septation defects for the Δ *prkC* mutant (Fig 3A,
435 white square, Fig 3C and Fig S5B). Our analysis revealed that 4% of the cells had abnormal
436 septation (Fig 3C and S5B, yellow arrows) and that 1% of the cells had adjacent septa which
437 created mini-cell lacking DAPI-stained DNA (Fig 3C and Fig S5B, white arrows). We then
438 analyzed by TEM the cell morphology and the structure of the septa for the WT, the Δ *prkC*
439 mutant and the complemented strains. Cells with normal septation were observed for the
440 WT strain, complemented strain and Δ *prkC* mutant (Fig 4A) while some cells of the Δ *prkC*
441 mutant displayed aberrant septation with the presence of multiple and adjacent septa (Fig
442 4B). Mini-cells attached to each other were also observed (Fig 4B left) and these probably
443 arise from the formation of several septa in close proximity (Fig 4B, right, red arrows).
444 These results indicated that deletion of the *prkC* gene in *C. difficile* affects bacterial cell
445 morphology and septum formation.

446

447 ***prkC* deletion increases sensitivity to detergents and autolysis**

448 To determine if *prkC* deletion also affects the cell envelope, we tested the sensitivity of the
449 *prkC* mutant to compounds with a detergent activity. The presence of 0.006% SDS affected
450 the growth of the Δ *prkC* mutant but did not affect the WT and the complemented strains (Fig
451 5A). It is worth noting that in the gastrointestinal tract, *C. difficile* is subjected to the bacteri-
452 cidal effects of secondary bile salts that solubilize phospholipids, impair membrane integrity
453 and weaken cell wall (48). We therefore tested the sensitivity of the Δ *prkC* mutant to the

454 secondary bile salt, deoxycholate (DOC) and to the primary bile salt, cholate. While the sen-
455 sitivity to cholate (0.03% or 0.04 % in TY) was similar for all the strains (data not shown),
456 we observed that the growth of the $\Delta prkC$ mutant was reduced in the presence of 0.03%
457 DOC (Fig 5B), a physiological concentration encountered in the gut (49, 50). In addition, the
458 autolysis in the presence of 0.01% of the non-ionic detergent Triton X-100 is more rapid for
459 the $\Delta prkC$ mutant than for the WT and the complemented strains (Fig 5C) while no differ-
460 ence in autolysis was observed in the absence of Triton (Fig S6A). This result suggested that
461 the $\Delta prkC$ mutant is more sensitive to the PG hydrolysis.

462

463 **PrkC controls sensitivity to antimicrobial compounds targeting the cell envelop**

464 Given that the deletion of *prkC* affected cell morphogenesis, we tested the sensitivity of the
465 *prkC* mutant to antibiotics targeting cell wall biosynthesis (46, 51). Using disk assays, we
466 showed that the size of the zone of inhibition increased for the $\Delta prkC$ mutant compared to
467 the WT and the complemented strains for all β -lactams tested (Fig 6A). Thus, the *prkC* mu-
468 tant is more susceptible to ticarcillin, amoxicillin, imipenem and several cephalosporins
469 (cefoxitin, ceftazidime and cefepime). In contrast, we did not observe any differences for
470 antibiotics that target transcription or translation (Fig 6A and Fig S6B). We also observed an
471 important reduction in the MIC of the $\Delta prkC$ mutant compared to the WT strain for the gly-
472 copeptide, teicoplanin (>12-fold), but not for vancomycin (2-fold) (Table 1). In addition, we
473 observed a slight increase in the susceptibility of the mutant to amoxicillin and imipenem (4-
474 fold) and a more substantial increase for the cephalosporins tested (Table 1). The MIC for
475 the second-generation cephalosporin, cefoxitin, was reduced by more than 6-fold for the
476 $\Delta prkC$ mutant compared to the WT strain whereas the MIC for the third generation cephalo-
477 sporins, ceftazidime, cefepime and cefotaxime, was reduced for the $\Delta prkC$ mutant by 20-
478 17.5- and 8-fold, respectively. The same increase in sensitivity to cefoxitin, ceftazidime and
479 cefepime was seen for the $\Delta prkC$ mutant carrying pDIA6103- P_{tet} -*prkC*-K39 \rightarrow A (Fig 6A),
480 suggesting that the kinase activity of PrkC is required for *C. difficile* to express an intrinsic
481 high-level of resistance to cephalosporins (1).

482 Cationic antimicrobial peptides (CAMPs) that are produced by both bacteria and the host
483 also target the cell envelop and/or membrane of bacteria (52). Thus, we tested the resistance
484 of the WT and mutant strains for several CAMPS of bacterial origin. The MIC of the $\Delta prkC$
485 mutant for polymixin B, bacitracin and nisin was 6-, 22- and 4.5-fold lower, respectively
486 than the MIC for the WT strain (Table 1). In the gastrointestinal tract, host-produced antimi-
487 crobial proteins such as lysozyme inhibit colonization of many Gram-positive pathogens by

488 hydrolyzing the PG and breaking the integrity of the cell wall. However, *C. difficile* is
489 known for its intrinsic resistance to lysozyme that might be associated with high level of
490 deacetylation of its PG (26). Interestingly, using an antimicrobial disk assay realized on Pep-
491 M plates, we observed a greater size of the zone of inhibition for the $\Delta prkC$ mutant (17 mm)
492 when compared to the WT strain (7.25 mm) in presence of 800 μg of lysozyme (Fig 6B).
493 These results indicated that *prkC* deletion is involved in resistance to CAMPs and lysozyme.

494

495 ***prkC* deletion affect motility, sedimentation and biofilm formation**

496 To identify other phenotypes associated with cell envelop properties, we first tested motility
497 on semi-solid BHI plates and observed a reduced motility for the $\Delta prkC$ mutant compared to
498 the WT and complemented strains (Fig 7A). After 48 h, the motility of the WT strain is 1.35
499 mm +/- 0.2 mm while the motility of the $\Delta prkC$ mutant is 0.8 mm +/- 0.1 mm. We also ob-
500 served that the $\Delta prkC$ mutant formed more aggregates at the bottom of the tube than the WT
501 and complemented strains (Fig S7A). We also tested the ability of these strains to form bio-
502 film after an exposure to compounds triggering an envelope stress such as DOC or polymix-
503 in B. In the presence of sub-inhibitory concentration of polymixin B (20 $\mu\text{g}/\text{ml}$) or DOC
504 (0.01%), the $\Delta prkC$ mutant formed, respectively, 6- and 10-fold more biofilm than the WT
505 and complemented strains after 24 h, respectively (Fig 7B and C). However, no difference in
506 biofilm formation was observed without these compounds (data not shown). When we quan-
507 tified the biofilm formed in presence of DOC at 24 h, 48 h and 72 h (Fig S7B), we found that
508 the $\Delta prkC$ mutant formed biofilm earlier than the WT strain (at 24 h) while more biofilm
509 was produced by the WT strain than the $\Delta prkC$ mutant at 48 h. A previous work show that
510 extracellular DNA (eDNA) is a major component of the matrix in DOC-induced biofilms
511 (29). In both the $\Delta prkC$ mutant and the WT strain, DOC-induced biofilms were rapidly dis-
512 persed when treated with DNase (Fig S7C). Moreover, we detected eDNA in the extracellu-
513 lar matrix for the $\Delta prkC$ mutant at 24 h and 48 h but only at 48 h for the WT strain (Fig 7D).
514 Since eDNA is required for biofilm stability (29), the early release of eDNA detected in the
515 $\Delta prkC$ mutant might be responsible for the premature biofilm formation. Thus, deletion of
516 *prkC* seems to affect the motility, the ability of *C. difficile* to sediment and to form biofilm
517 under conditions that induce cell envelop stress.

518

519 **Comparison of the PG structure and composition between the WT strain and the** 520 ***prkC* mutant**

521 Based on the changes in cell-shape and increased susceptibility to β -lactams and lysozyme of
522 the $\Delta prkC$ mutant, we hypothesized that these phenotypes could be due to modification of
523 the PG structure or composition. However, we showed that the muropeptide profiles of the
524 WT strain (black) and the $\Delta prkC$ mutant (red) are almost identical (Fig S8). Indeed, we
525 found no difference in the abundance of dimers containing a 3-3 cross-link (peaks 9, 10, 11,
526 13, 14, 15 and 17) generated by L,D-transpeptidases that might be associated with the high
527 level of intrinsic resistance of *C. difficile* to some β -lactams (31) or containing a 4-3 cross-
528 link (peaks 18, 19, 21) catalyzed by D,D-transpeptidases, a target of β -lactam (Fig S8).
529 Moreover, the ratio of 4-3 to 3-3 cross-linking was not affected in our conditions. In addi-
530 tion, the amount of deacetylated muropeptides (peaks 4, 7, 9, 10, 11, 13, 14, 15, 17, 18, 19,
531 21) (31), was similar in the WT strain and the $\Delta prkC$ mutant (Fig S8). As muropeptides
532 deacetylation contribute to a high level of resistance to lysozyme in *C. difficile* (26), the in-
533 creased sensitivity of the $\Delta prkC$ mutant to lysozyme cannot be explained by changes in the
534 level of PG deacetylation (Fig S8). Our results suggested that susceptibility of the *prkC* mu-
535 tant to some β -lactams or lysozyme is not related to changes of its PG structure or composi-
536 tion.

537

538 **Impact of the *prkC* deletion on the PSII**

539 In firmicutes, the wall teichoic acids (WTA) are involved in cell division, maintenance of
540 cell-shape and susceptibility to CAMPs or β -lactam (53). The 630 Δerm strain of *C. difficile*
541 has an atypical wall teichoic acid known as polysaccharide II (PSII) composed of hexaglyco-
542 sylphosphate repeats (34, 54). To know if the phenotypes observed for the $\Delta prkC$ mutant
543 were associated with changes in the abundance, localization and/or structure of the PSII, we
544 first purified the PSII of the WT and $\Delta prkC$ mutant strains. Their structure was analyzed by
545 NMR and showed that the ^1H NMR and ^{13}C HSQC spectra from the 630 Δerm PSII were in
546 good agreement with previous data (32, 54) and that no difference in the PSII structure was
547 detected between the *prkC* mutant and the WT strain (Fig S9).

548 We also investigated a possible role for PrkC in controlling PSII localization. Using an im-
549 munoblot analysis with an antibody raised against PSII (34), we observed that more PSII
550 was shed in the supernatant during growth by the $\Delta prkC$ mutant when compared to the pa-
551 rental and complemented strains during growth (Fig 8A). By contrast, we detected similar
552 quantity of PSII in membrane fraction for all strains (Fig S10). Thus, this result suggests that
553 the deletion of *prkC* leads to an enhanced release of PSII into the supernatant.

554 To precisely quantify the amount of PSII and PG in the cell wall of the WT and the $\Delta prkC$
555 mutant strains, we fractionated cells to recover the cell wall containing the PSII covalently
556 linked to PG. We then separated these two compounds and quantified the amount of each
557 product. Interestingly, we observed that the amount of PG purified from the $\Delta prkC$ mutant
558 was lower compared to the WT strain, which represented 55 % of the quantity purified from
559 the WT strain (Fig 8B). Similarly, but to a lesser extent, a reduction of 33 % in the quantity
560 of PSII was observed for the $\Delta prkC$ mutant (Fig 8C). It is possible that the reduction in PG
561 for the $\Delta prkC$ mutant could interfere with the localization of PSII and could explain the in-
562 crease shedding of PSII in the supernatant (Fig 8A).

563

564 ***prkC* deletion affects cell wall associated proteins**

565 Several properties of the bacterial cell envelop can be partly attributed to proteins localized
566 at the cell surface. We extracted proteins non-covalently anchored to the cell wall of the
567 $\Delta prkC$ mutant and the WT strains (55) and compared their relative abundance by mass spec-
568 trometry (Table 2 and Fig S11). A first family of proteins is the CWPs that contain a CWB2
569 motif responsible for their anchorage to the cell wall by interacting with PSII (56, 57). In-
570 deed, we found that 27 CWP proteins with CWB2 (out of 29 in strain 630) including the S-
571 layer protein, SlpA, were more abundant in the cell wall of the WT strain than in the $\Delta prkC$
572 mutant while a unique CWP protein, Cwp7, was more abundant in the $\Delta prkC$ mutant. Other
573 non-covalently anchored proteins present of the cell surface (with SH3_3 or PG4 motifs)
574 were also found in different amount in the WT strain and the $\Delta prkC$ mutant. Interestingly,
575 some of them are involved in cell wall metabolism including L,D-transpeptidases, carboxy-
576 peptidases and putative cell wall hydrolases.

577

578 **Virulence and colonization of the $\Delta prkC$ mutant in the hamster model**

579 The different phenotypes of the *prkC* mutant suggest that PrkC can play a role during critical
580 steps of the infectious cycle of *C. difficile* (4). To determine a possible role of PrkC in CDI,
581 we compared the virulence of the WT strain and the $\Delta prkC$ mutant in the acute Golden Syri-
582 an hamster model of infection. Despite a trend towards a delay in the death of the hamsters
583 infected with the *prkC* mutant, no significant difference was observed for the average time of
584 post challenge survival between hamsters infected with the WT and the $\Delta prkC$ mutant
585 strains (Fig 9A). When we monitored the daily level of gut colonization of the WT and the
586 $\Delta prkC$ mutant, we found no significant difference in the average level of gut colonization the
587 day the hamster died as determine by the average CFU per g of feces for each hamster (Fig

588 9B). However, a significant change in gut colonization was observed between the two strains
589 40 h post infection (Fig 9C). Despite the fact that the $\Delta prkC$ mutant has a delay in gut colo-
590 nization, this delay has no significant effect on the virulence in this infection model.

591

592 **Discussion**

593 In this work, we showed that the *prkC* mutant of *C. difficile* has pleiotropic phenotypes
594 such as an increased sensitivity to various antimicrobial compounds (CAMPs, lysozyme,
595 DOC, β -lactams), modifications in motility, cell aggregation and biofilm formation, and also
596 changes in cell morphology and septum formation or localization. The increased sensitivity
597 to several antimicrobial compounds detected for the *C. difficile* $\Delta prkC$ mutant has been ob-
598 served in other firmicutes. The *prkA* mutant of *L. monocytogenes* is more sensitive to lyso-
599 zyme and several CAMPs (58) while the *ireK* mutant of *E. faecalis* has an increased sensi-
600 bility to bile and cholate (45) but not to DOC as observed for the *C. difficile* $\Delta prkC$ mutant.
601 The inactivation of the PASTA-STKs in *S. pneumoniae*, *S. pyogenes*, *E. faecalis*, *L. mono-*
602 *cytogenes* and *S. aureus* results in an increased susceptibility toward β -lactams but the extent
603 and pattern of the effects vary among species and strains (18, 45, 58, 59). These specific
604 patterns of sensitivity observed for the PASTA-STK mutant for each firmicute might be as-
605 sociated with differences in the targets phosphorylated by the PASTA-STK, in the penicil-
606 lin-binding proteins (PBPs) and in their affinity for the different β -lactams. As observed in
607 *B. subtilis* and *S. aureus* (18, 42), the PrkC kinase of *C. difficile* controls biofilm formation
608 under cell-membrane stresses conditions. Several factors probably contribute to the in-
609 creased biofilm formation in the $\Delta prkC$ mutant in the presence of DOC or polymixin B. This
610 may include a decreased motility, an increased sedimentation and changes in the amount of
611 CWPs that modify adhesive properties of the cell surface. For example, the Cwp84 protein,
612 which is less abundant in the $\Delta prkC$ mutant is known to negatively control biofilm formation
613 (60). In addition, premature autolysis by the $\Delta prkC$ mutant probably releases eDNA earlier
614 in presence of DOC resulting in the increased biofilm formation observed at 24 h for the
615 mutant (29). By controlling cell lysis, PrkC seems able to affect biofilm formation in re-
616 sponse to cell membrane stresses.

617 Changes in the homeostasis and the integrity of the cell envelop might explain most of the
618 phenotypes of the *C. difficile* $\Delta prkC$ mutant including modification of cell shape that is de-
619 termined by the orderly processing and assembly of each cell wall component (53, 61, 62).
620 The absence of PrkC probably modifies the abundance, composition and/or structure of
621 component(s) of the cell envelop, including the PG, the glycopolymers (PSII and/or LTA-

622 PSIII) and/or cell-wall associated proteins (56). In other Gram-positive bacteria, PASTA-
623 STK phosphorylates enzymes involved in PG and/or teichoic acid synthesis, modification,
624 assembly and/or turnover. This includes enzymes of the Glm pathway, Mur enzymes, MviM,
625 a flippase involved in the transport of the lipid II anchored compounds across the membrane,
626 a penicillin-binding protein, LTA synthetases but also transcriptional regulators such as
627 WalR-WalK and GraR that control cell wall metabolism (17, 18, 63). In *C. difficile*, we did
628 not detect a transcriptional effect of the *prkC* deletion on genes encoding enzymes involved
629 in the synthesis of envelop components (E. Cuenot, unpublished data). These results strongly
630 suggest that PrkC does not act by modifying the activity of a transcriptional regulator but
631 rather by phosphorylating one or several protein(s) directly or indirectly controlling the syn-
632 thesis, assembly or turnover of at least one component of the envelop.

633 Teichoic acids play a role in protecting bacteria from stressful conditions by modifying
634 the properties of the cell surface. Changes in teichoic acids resulted in phenotypes similar to
635 those we observed in the $\Delta prkC$ mutant. This include increased β -lactam susceptibility, au-
636 tolysis, modification of cell morphology and defect in septa positioning and numbers (53,
637 64). In *C. difficile*, the biosynthesis of the PSII, its properties as well as the phenotypes asso-
638 ciated with defect in PSII are poorly studied (32, 34). Furthermore, less information is avail-
639 able on atypical LTA (32). However, we noted that the $\Delta prkC$ mutant shares common phe-
640 notypes with the *lcpB* mutant encoding a protein involved in tethering PSII (34) that includes
641 elongated cell, presence of multiple septa and increased ability to form biofilms (34). While
642 the structure of PSII is not altered in the $\Delta prkC$ mutant, we observed a slightly reduction in
643 the amount of PSII anchored to the cell wall and an increased quantity in PSII released in the
644 supernatant of the $\Delta prkC$ mutant. These changes in PSII localization might be caused by the
645 dysregulation of PSII synthesis/anchorage or the production/assembly of another cell enve-
646 lope component such as PG in the absence of PrkC. Indeed, anchoring of the PSII to PG is
647 probably affected by the reduced amount of PG detected in the $\Delta prkC$ mutant.

648 As observed for *L. monocytogenes* and *E. faecalis*, *C. difficile* is inherently resistant to
649 cephalosporin and PASTA-STK inactivation leads to a robust increase in sensitivity towards
650 these antibiotics that target PG synthesis by inactivating PBPs (45, 65). In *C. difficile*, we
651 showed that the kinase activity of PrkC is required for the high level of resistance to cepha-
652 losporin. The sensitivity of the $\Delta prkC$ mutant to teicoplanin, an antibiotic that affects trans-
653 glycosylation by PBPs and to bacitracin that interferes with PG synthesis through its role on
654 lipid II recycling also suggests a possible role for this kinase in controlling PG metabolism
655 (66). Other phenotypes such as lysozyme resistance or autolysis are also related to PG struc-

656 ture or metabolism. However, we failed to detect any modification in the composition of PG,
657 in reticulation of PG, 3-3 to 4-3 cross-link ratio or PG deacetylation under our experimental
658 conditions. Changes to these would have offer, to a certain extent, an explanation for the
659 increased sensitivity to cephalosporin and lysozyme. Nevertheless, it is possible that local-
660 ized and/or subtle changes in PG composition occur at the septum, which is where PrkC is
661 localized during growth. These changes would be difficult to detect. The main difference
662 between the $\Delta prkC$ and the WT is a decrease in the total amount of PG. This might be linked
663 to a reduced size of the glycan chains formed. It is interesting to note that proteins potential-
664 ly involved in PG metabolism are also detected in different amount in the cell wall of the
665 WT and $\Delta prkC$ mutant strains (Table 2). All these results suggest that PrkC has an effect
666 directly or indirectly on PG synthesis or turnover in *C. difficile*. In *L. monocytogenes*, it has
667 been suggested that the PASTA kinase might specifically regulate PBPs resulting in cepha-
668 losporin resistance(58). In *Enterococcus faecium*, mutants in class A PBPs are sensitive to
669 cephalosporins and suppressor mutations that restore cephalosporin resistance are found in
670 *ireK* and *ireP* encoding a STK and a STP, respectively (67). In *E. faecalis*, IreB negatively
671 controls cephalosporin resistance and IreB is a small protein of unknown function and the
672 only substrate of IreK kinase identified to date (68). However, the molecular mechanisms
673 linking IreB, IreK and maybe PBPs that could explain their role in the control of cephalo-
674 sporin resistance remain unknown. Interestingly, CD1283, the IreB-like protein of *C. dif-*
675 *ficile*, contains one threonine (T7) and this residue is phosphorylated in IreB of *E. faecalis*.
676 CD1283 might contribute to the regulatory pathway downstream of PrkC. In addition, the
677 PrkC-mediated phosphorylation of proteins controlling cell division and the synthesis of cell
678 envelop components is another interesting hypothesis. Indeed, PrkC is localized at the sep-
679 tum and the deletion of the *prkC* gene affects *C. difficile* cell morphology with elongated
680 cells, abnormal septum localization and defect in cell separation. In *S. pneumoniae* and other
681 streptococci, $\Delta stkP$ mutants have longer cells than the WT and a modified shape and exhibits
682 cell division and separation defects (69). Several proteins involved in cell division such as
683 DivIVA, MapZ, GspB or FtsZ are phosphorylated by the PASTA-STKs in *B. subtilis*, *S.*
684 *pneumoniae* or *S. aureus* (17, 18, 69). In *S. pneumoniae*, phosphorylation of DivIVA con-
685 trols cell shape and the localization of PG synthesis machinery required for cell elongation
686 and cell constriction (69). The phosphorylation by PrkC of an orthologous of one of these
687 proteins, especially DivIVA, could explain several cell morphology associated phenotypes
688 observed for the *prkC* mutant. Altogether, our results indicate that the PASTA-STK of *C.*

689 *difficile*, PrkC, is probably involved in the control of the envelop biogenesis and/or cell divi-
690 sion.

691 Finally, the virulence of the $\Delta prkC$ mutant is similar to that of the WT strain while a col-
692 onization delay of the hamster gut is observed for the mutant. This can be explained by a
693 global increased in sensitivity of the $\Delta prkC$ mutant to antimicrobial compounds and the pos-
694 sible changes in its cell envelop properties. Our results highlight the involvement of PrkC in
695 controlling several processes corresponding to critical steps of CDI including resistance to
696 DOC, known to inhibit growth of vegetative cells in the gastrointestinal tract (4), to lyso-
697 zyme, a critical component of the innate immune system and to CAMPs produced by the
698 microbiota and/or by the host (3) that are compounds present in the hamster model. Fur-
699 thermore, dissecting the role of PrkC in controlling the resistance to antibiotics promoting
700 CDI such as cephalosporins (1) could pave the way to new strategies for the prevention of
701 these infections.

702

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711

712 **Table 1. MIC of the strain 630 Δerm and of the $\Delta prkC$ mutant for antibiotics targeting**
713 **cell wall and CAMPS**

| Compound tested | MIC ($\mu\text{g/ml}$) | | Median-fold sensitization |
|---------------------------------|--------------------------|---------------|---------------------------|
| | 630 Δerm | $\Delta prkC$ | |
| Antibiotics targeting cell wall | | | |
| Vancomycin | 1.5 | 0.75 | 2 |
| Teicoplanin | 0.19 | <0.016 | >12 |
| Amoxicillin | 4 | 1 | 4 |
| Imipenem | 6 | 1.3 | 4 |
| Cefoxitine | >256 | 44 | >6 |
| Ceftazidime | 60 | 3 | 20 |
| Cefepime | 70 | 4 | 17.5 |
| Cefotaxime | 128 | 16 | 8 |
| Antimicrobial peptides | | | |
| Polymixin B | 340 | 55 | 6 |
| Bacitracin | 550 | 25 | 22 |
| Nisin | 140 | 30 | 4.5 |

714

MICs for antibiotics were determined using E-test with the exception of cefotaxime.

715

The MICs for cefotaxime and antimicrobial peptides were determined by the method of dilution.

716

717
718**Table 2. Change in cell wall associated proteins in *C. difficile* between the 630 Δ erm strain and the Δ prkC mutant.**

| Gen ID | name | function | Fold-change 630 Δ erm/ Δ prkC* | -Log (P-value) | Detected in surface proteome |
|---|---------------------------------|---|--|-------------------|------------------------------------|
| Cell-wall associated proteins CWB2 motif | | | | | |
| Down in Δ prkC | | | | | |
| CD1233 | <i>cwp26</i> | Cell wall binding protein of skin element | 31.3 | 2.7 | |
| CD1469 | <i>cwp20</i> | Cell-wall penicillin-binding protein | 6.7 | 6.5 | |
| CD2518 | <i>cwp29</i> | Cell-wall binding protein | 6.4 | 4.6 | |
| CD2795 | <i>cwp11</i> | Cell-wall binding protein | 6.1 | 6.5 | |
| CD0440 | <i>cwp27</i> | Cell-wall binding protein | 5.1 | 4.3 | |
| CD2735 # | <i>cwp14</i> | Cell-wall binding protein (also SH3) | 4.8 | 5.2 | |
| CD2798 | <i>cwp9</i> | Cell-wall binding protein | 4.7 | 4.5 | |
| CD2767 | <i>cwp19</i> | Cell-wall binding protein, autolysin | 4.6 | 5.9 | |
| CD1803 | <i>cwp23</i> | Cell-wall binding protein | 4.4 | 4.9 | |
| CD2786 | <i>cwp5</i> | Cell-wall binding protein | 4.4 | 5.4 | |
| CD1751 | <i>cwp13</i> | Cell-wall binding protein, protease | 4.2 | 5.4 | |
| CD2787 | <i>cwp84</i> | Cell-wall binding protein, protease | 4.0 | 5.4 | + |
| CD2193 | <i>cwp24</i> | Cell-wall binding protein, glucosaminidase domain | 3.9 | 4.4 | + |
| CD0844 | <i>cwp25</i> | Cell-wall binding protein | 3.9 | 5.9 | + |
| CD3192 | <i>cwp21</i> | Cell-wall binding protein | 3.9 | 6.8 | |
| CD2789 | <i>cwp66</i> | Cell-wall binding protein | 3.8 | 4.7 | |
| CD2791 | <i>cwp2</i> | Cell-wall binding protein | 3.7 | 6.6 | + |
| CD2793 | <i>slpA</i> | Precursor of the S-layer proteins | 3.6 | 4.90 | + |
| CD2713 # | <i>ldtcd2</i> , <i>cwp22</i> | Cell-wall binding protein, L,D-transpeptidases | 3.6 | 5.1 | |
| CD2794 | <i>cwp12</i> | Cell-wall binding protein | 3.6 | 5.6 | |
| CD0514 | <i>cwpV</i> | Hemagglutinin/adhesin | 3.4 | 4.8 | + |
| CD1987 | <i>cwp28</i> | Cell-wall binding protein | 3.1 | 1.5 | |
| CD1035 | <i>cwp16</i> | Cell-wall binding protein, amidase domain | 3.1 | 5.7 | |
| CD1036 | <i>cwp17</i> | Cell-wall binding protein, amidase domain | 2.9 | 3.3 | |
| CD2796 | <i>cwp10</i> | Cell-wall binding protein | 2.8 | 4.8 | |
| CD2784 | <i>cwp6</i> | Cell-wall binding protein, amidase domain | 2.8 | 3.6 | + |
| CD1047 | <i>cwp18</i> | Cell-wall binding protein | 2.4 | 3.5 | |
| CD2799 | <i>cwp8</i> | Cell-wall binding protein | 2.1 | 4.0 | |
| Up in Δ prkC | | | | | |
| CD2782 | <i>cwp7</i> | Cell-wall binding protein | 0.2 | 2.1 | |
| SH3 3 motif | | | | | |
| Down in Δ prkC | | | | | |
| CD0183 # | | Putative cell-wall hydrolase | 10.2 | 1 | |
| CD2768 # | | Putative cell-wall hydrolase | 3.8 | 2.9 | |
| CD1135 # | | Putative endopeptidase | 3 | 4.8 | |
| CD2402 # | | Putative cell wall hydrolase phosphatase-associated protein | 2.3 | 2.6 | |
| PG4 motif | | | | | |

| Up in $\Delta prkC$ | | | | | |
|---------------------|---------------|--|-----|-----|--|
| CD1436 | | Putative hydrolase [^] | 0.2 | 2.4 | |
| CD2963# | <i>ldtcd1</i> | L,D-transpeptidases [^] | 0.3 | 2.5 | |
| CD2149 | | Putative vancomycin resistance protein, VanW family [^] | 0.3 | 1.1 | |

719 # cell wall metabolism
720 *FDR<0.05
721 [^]protein containing one trans-membrane domain
722 SH3 domain (PF08239), CWB 2 motif (PD04122) and PG4 motif (PF12229)
723

724 Figure Legend

726 Figure 1. Organization, localization and kinase activity of the PrkC protein.

727 A. Organization of the domains of *C. difficile* CD2578-PrkC. PrkC contains a cytoplasmic
728 kinase domain in N-terminal part (brown), a trans-membrane (TM) segment, two PASTA
729 domains (green) and an atypical SGN (Ser, Gly, Asn) rich domain in C-terminal part (pink).
730 The conserved lysine residue (K39) within the ATP-binding P loop of the kinase that is re-
731 quired for phosphotransfer is indicated.

732 B. Localization of the PrkC-HA tagged protein. Cells expressing *prkC* fused to HA were
733 grown in the presence of 15 ng/ml of ATc, and harvested during exponential growth. Sam-
734 ples were fractionated into membrane (Mb) and cytoplasm (Cy) fractions. Protein fractions
735 were analyzed by western blot using an antibody raised against HA.

736 C. Localization of the SNAP-PrkC fusion during growth. The SNAP-PrkC protein was pro-
737 duced during exponential growth phase in the presence of 50 ng/ml ATc. After labeling with
738 the TMR-star substrate, PrkC-SNAP localization was analyzed by fluorescence microscopy.
739 AF (autofluorescence). The scale bar represents 5 μ m.

740 D. Western blot performed after fractionation in a soluble fraction (1) and an insoluble frac-
741 tion (2) obtained from exponential phase cultures of the WT, the $\Delta prkC$ mutant, the com-
742 plemented strain and the $\Delta prkC$ mutant carrying pDIA6103-*prkC*-K39 \rightarrow A (K \rightarrow A). An α -P-
743 Thr antibody was used to detect phosphorylated threonine. We indicated by red arrows the
744 bands detected in the WT strain that disappeared in the $\Delta prkC$ mutant.

746 Figure 2. Genetic organization of the *prkC* locus

747 A. Schematic representation of the gene cluster present upstream of *prkC*. The locus between
748 *CD2590-dapF* and *prkC* includes genes encoding proteins involved in translation, transcrip-
749 tion, DNA replication, metabolism and membrane proteins. The genome-wide TSS mapping
750 (25) indicated the presence of a promoter upstream of *dapF*. The extended -10 box and the
751 TSS are indicated in bold.

752 B. PCR realized with primers annealing in *prkC* and *stp* (IMV936 and IMV908, line 1 and 2)
753 or *stp* and *rlmN* (IMV935 and IMV907, line 3 and 4) either on RNA extracted from
754 630 Δerm (line 1 and 3) or on cDNA synthesized by reverse transcription from the same
755 RNA using primer IMV843 (line 2 and 4). Smart Ladder (200-10,000 bp).

757 Figure 3. Morphology of the $\Delta prkC$ mutant

758 A. Fluorescence microscopy was carried out on the WT, the $\Delta prkC$ mutant and the comple-
759 mented (Comp) strains. Membranes and DNA were visualized with FM4-64 (red) and DAPI
760 (blue), respectively. A blue arrow showed an elongated cell and green arrows non-separated
761 cells. The white square indicates the presence of a septum with an aberrant structure. Scale
762 bars represent 5 μ m.

763 B. Cell size distribution for the WT, the $\Delta prkC$ mutant and the complemented (Comp)
764 strains. The measurement was done on cells labeled with FM4-64 and DAPI. Means and
765 errors of the means were calculated after the measurement of at least 600 cells for each
766 strain. The analysis was performed with the software Image J.
767 C. Presence of aberrant septation and anucleated minicells in $\Delta prkC$ mutant cells. Fluores-
768 cence microscopy of cells staining with FM4-64 and DAPI revealed the presence of aberrant
769 septum (yellow arrows) and anucleate minicells (white arrows) in the $\Delta prkC$ mutant. Scale
770 bars represent 5 μm .

771

772 **Figure 4. Presence of abnormal septa in the $\Delta prkC$ mutant cells**

773 A. TEM pictures showing normal septal structure in the 630 Δerm (WT), the $\Delta prkC$ mutant
774 and the complemented (Comp) strains. Scale bars represent 100 nm.

775 B. TEM pictures showing aberrant septal structure in the $\Delta prkC$ mutant cells. In the right
776 panel, we can distinguish the beginning of the synthesis of two septa (white arrows) has be-
777 gun near an apparent normal septum. Scale bars represent 200 nm.

778

779 **Figure 5. *prkC* deletion increases sensitivity to detergents and autolysis**

780 A. Resistance to SDS stress of the $\Delta prkC$ mutant, the 630 Δerm (WT) and the complement
781 strains (Comp) was tested on BHI plates containing 0.006 % of SDS. This experiment was
782 performed in triplicate and this plate is representative of the results obtained.

783 B. Growth of the WT strain (black circle), the $\Delta prkC$ mutant (white circle) and the comple-
784 mented strain (white square) in 24-well microplates containing TY medium in the presence
785 of 0.03% DOC. A growth curve without DOC is presented in Fig S3. 4 independent cultures
786 were done.

787 C. Autolysis of the WT (black circle), $\Delta prkC$ (white circle) and complemented (black
788 square) strains in the presence of 0.01% Triton X-100. The OD_{600nm} of the samples incubated
789 at 37°C was determined every 5 min until complete cell lysis was reached. 4 independent
790 experiments were done.

791

792 **Figure 6. Sensitivity of the $\Delta prkC$ mutant to antibiotics targeting cell-wall and to lyso-** 793 **zyme.**

794 A. Sensitivity to antibiotics targeting the cell wall. Histograms representing the diameters of
795 growth inhibition area after 24 h of incubation on BHI plates for the 630 Δerm strain
796 pDIA6103 (WT, black), the $\Delta prkC$ mutant pDIA6103 (medium grey), the complemented
797 strain (Comp, dark grey) and the $\Delta prkC$ mutant expressing PrkC-K39A (pale grey). We used
798 antibiogram disks containing Ticarcillin 75 μg , Amoxicillin 25 μg , Imipenem 10 μg ,
799 Ceftazidime 30 μg , Cefepime 30 μg or Erythromycin 15 μg . Cefoxitin was tested at 100 μg .
800 The results presented correspond to 7 experiments (Ticarcillin, Amoxicillin, Imipenem) or 4
801 experiments for cephalosporins. Data were analyzed by t test. * indicates P<0.05.

802 B. Sensitivity to lysozyme was determined on Pep-M plates. 800 μg of lysozyme was added
803 to a 6-mm disk. Histograms representing the diameter of growth inhibition measured for the
804 630 Δerm pDIA6103 (WT, black), the $\Delta prkC$ mutant pDIA6103 (medium grey), and the
805 complemented strain (Comp, dark grey). The experiment was performed in quadruplicate.
806 Data were analyzed by t test. * indicates P<0.05.

807

808 **Figure 7. Sedimentation, biofilm formation and motility of the $\Delta prkC$ mutant**

809 A. Motility test on 0.3% agar BHI plates. The plates shown are representatives of 3 in-
810 dependent tests.

811 B and C Mean values of the OD_{570nm} measured after crystal violet staining of the mass of
812 biofilm obtained after 24 h in presence of 20 μM polymixin B (B) or 0.01% DOC (C). Error

813 bars show standard deviation of three independent experiments performed in triplicate. Data
814 were analyzed by t test. P values were 0.031 and 0.0045 for polymixin B (B) and DOC (C),
815 respectively. On the picture, crystal violet staining revealing the amount of biofilm formed
816 after 24 h corresponding to the values represented above.

817 D. Detection of e-DNA in the matrix of 24 h and 48 h DOC-induced biofilms formed by the
818 WT strain and the $\Delta prkC$ mutant. The experience was done in triplicate. We presented a gel
819 representative of the results obtained.

820

821 **Figure 8. Impact of $prkC$ deletion on PSII localization and PG and PSII production**

822 A. Immunoblot detection of PSII using a serum antibody raised against this glycopolymer
823 (34) in supernatants of the WT, $\Delta prkC$ and complemented strains. For each sample, we nor-
824 malized by using the OD_{600nm} of the corresponding culture. ND, not diluted. All immunob-
825 lots are representative of at least four replicates.

826 B and C. Quantification of the PG (B) and PSII (C) present at the surface of the WT and the
827 $\Delta prkC$ mutant strains. The values were normalized to the WT levels considered as 1. Error
828 bars represents standard deviation of at least 4 independent experiments.

829

830 **Figure 9. $prkC$ deletion affect gut establishment and not virulence**

831 A. Average survival time (h) post-infection for hamsters challenged with 630 Δerm or $\Delta prkC$
832 spores.

833 B and C. Average quantity of CFU per g of feces determined the day of animal death (B) or
834 40 h post infection (C) for hamsters challenged with 630 Δerm or $\Delta prkC$ spores. The average
835 values (B) were calculated with 16 and 12 hamsters for the WT strain and the $\Delta prkC$ mutant,
836 respectively due to the absence of feces at this time point for four hamsters.

837 The average values (C) were calculated with the totality of hamsters for both groups. Data
838 were analyzed by a Mann-Whitney test. * indicates P<0.05. The sensitivity threshold of the
839 method is 10³ g/feces.

840

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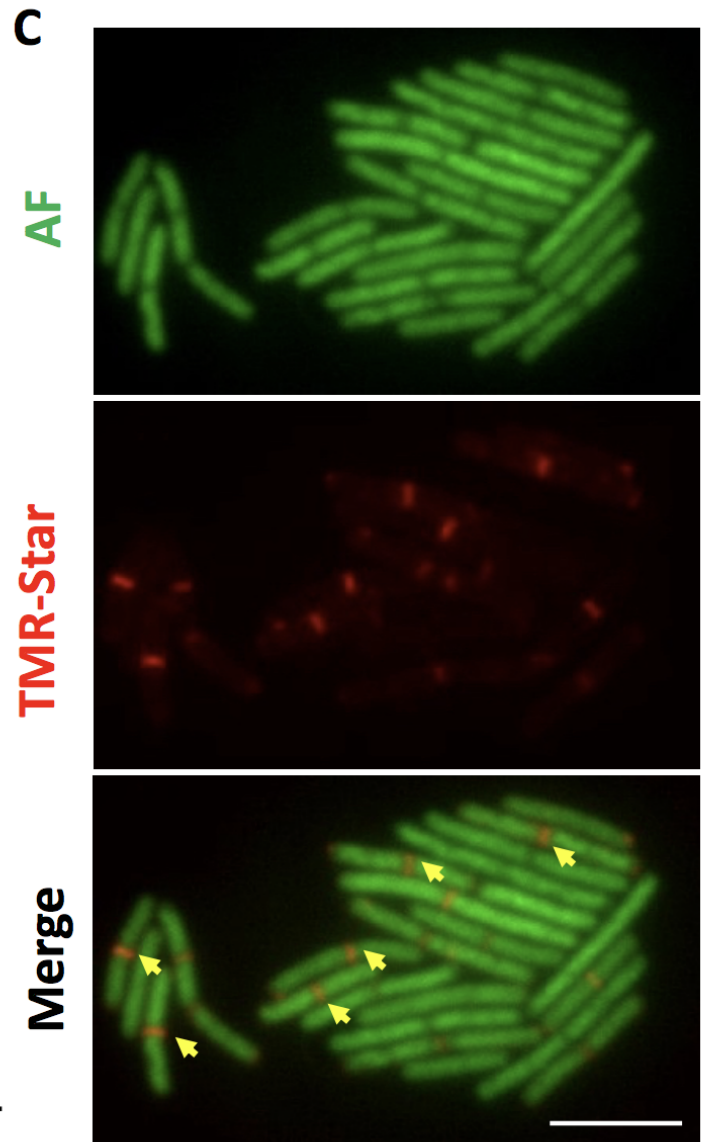
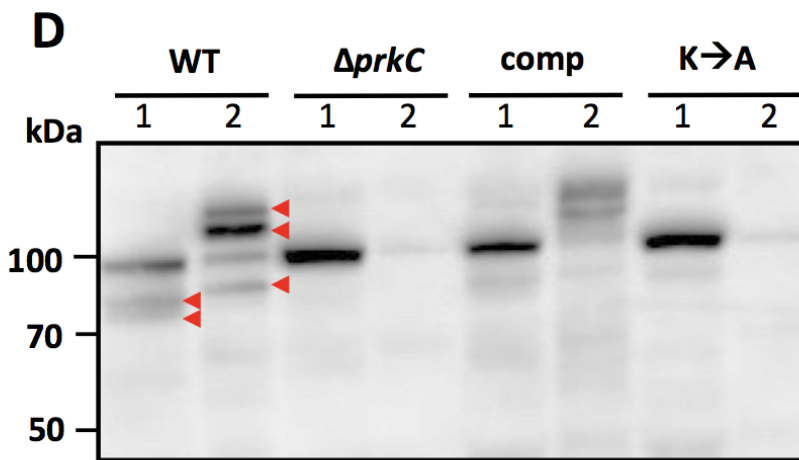
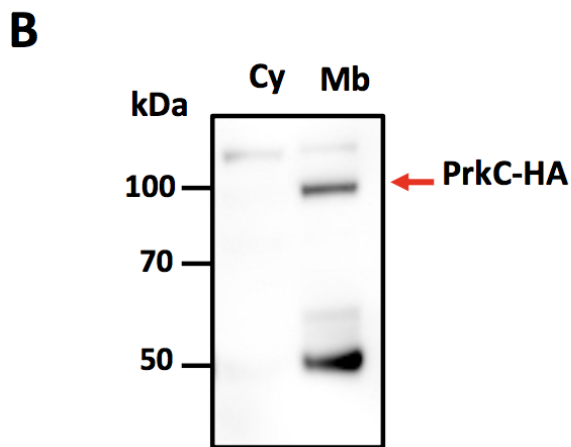
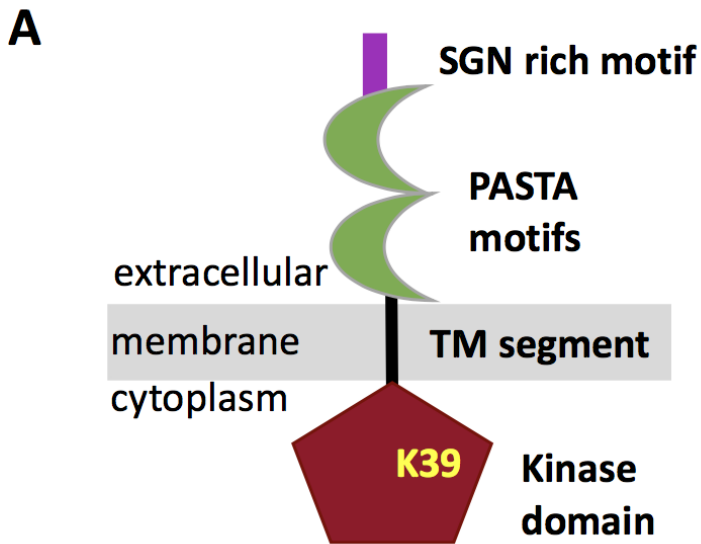
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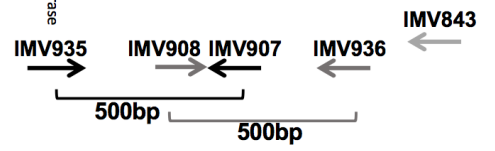
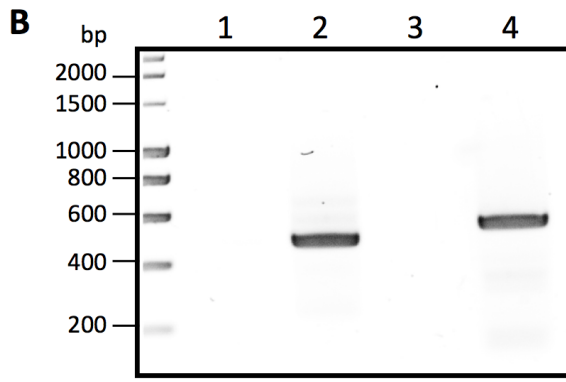
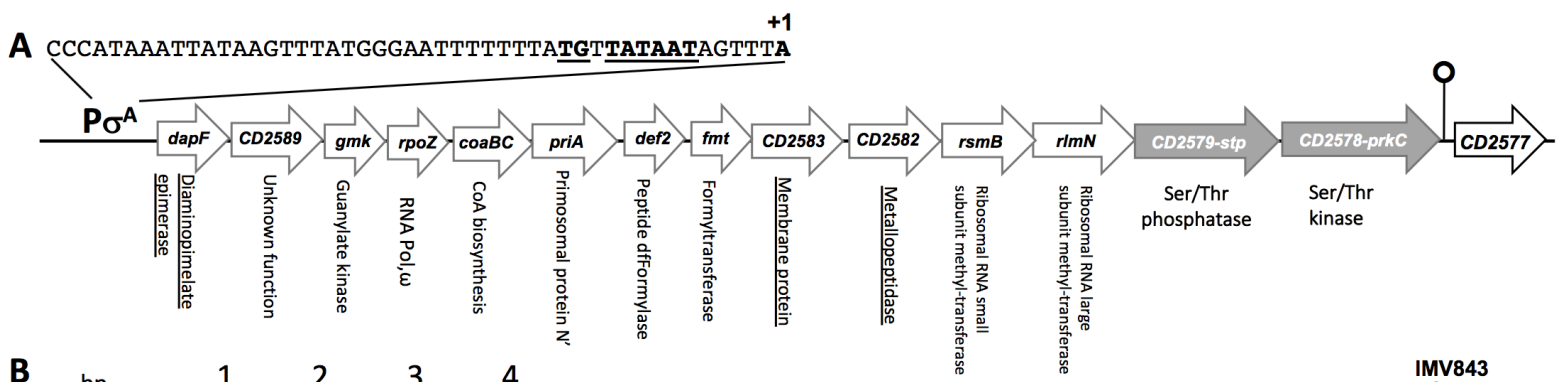
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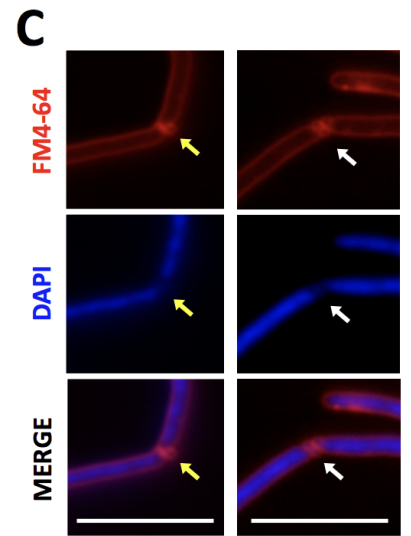
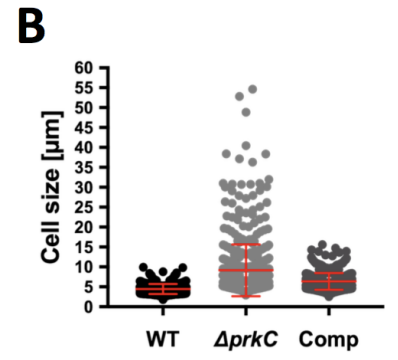
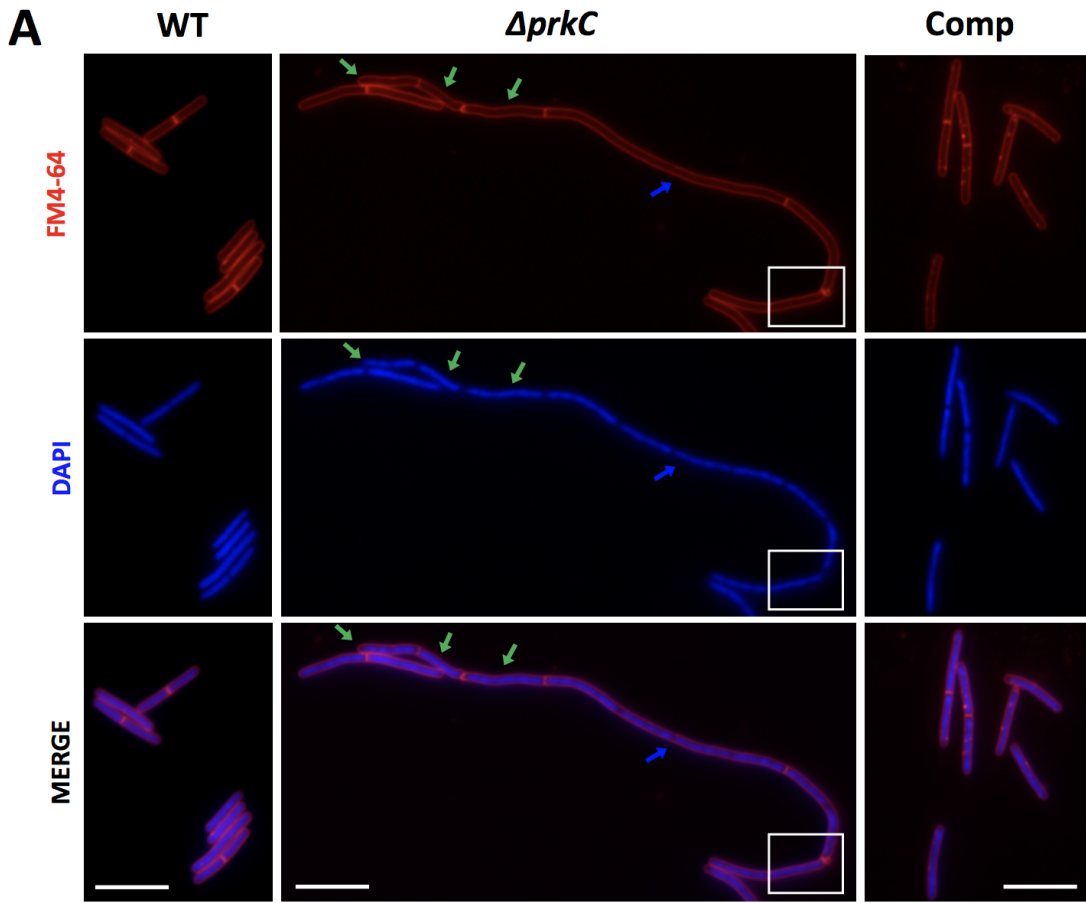
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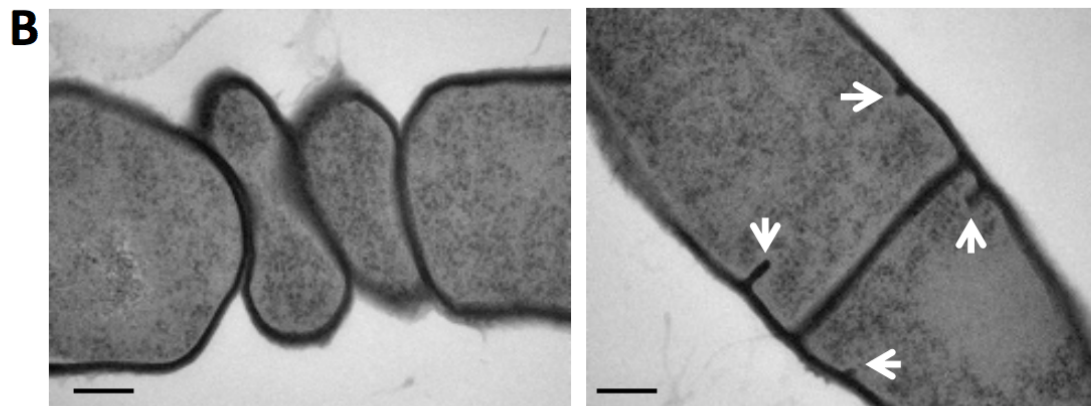
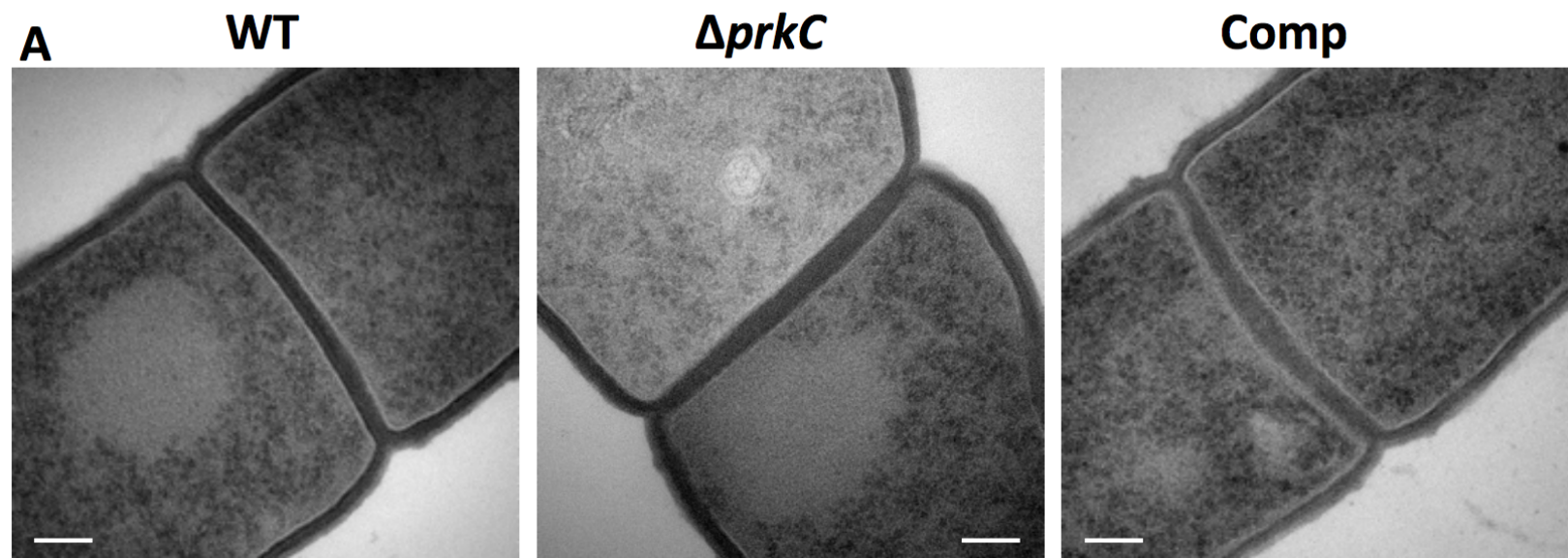
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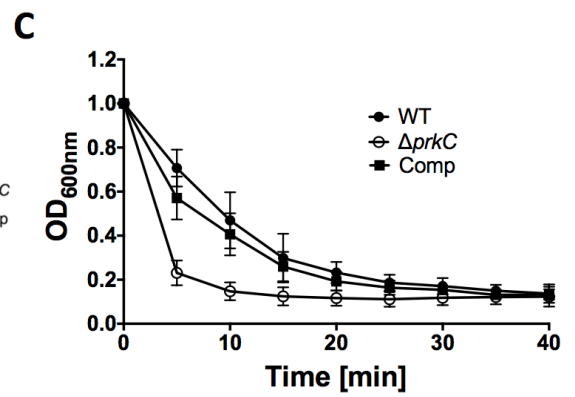
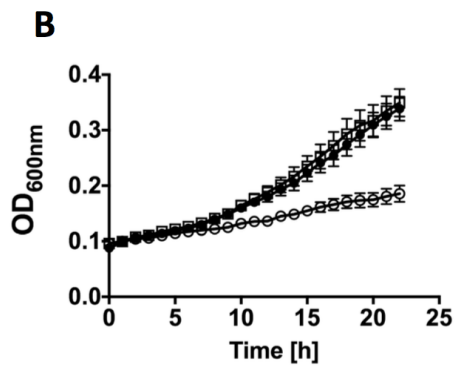
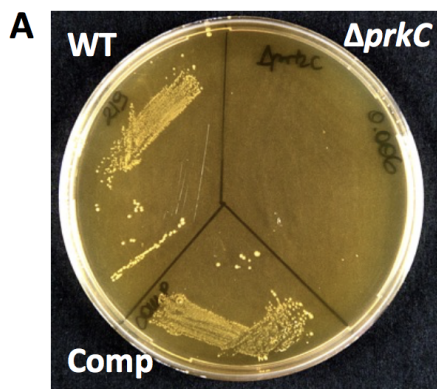
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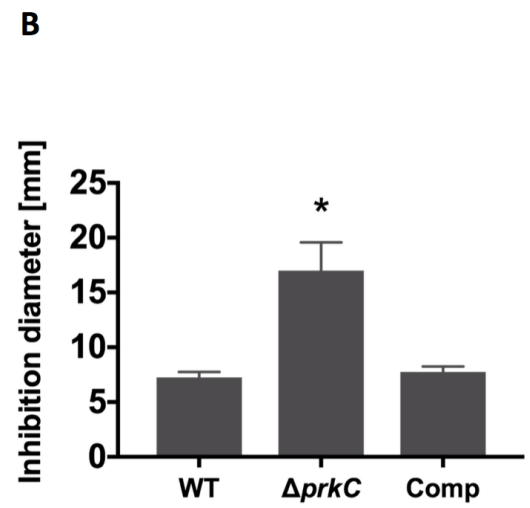
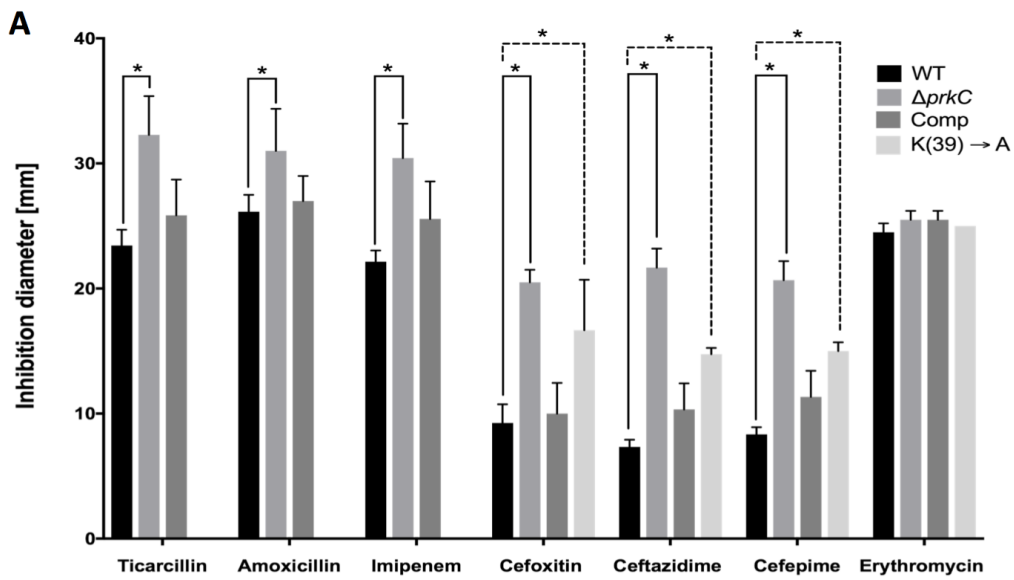


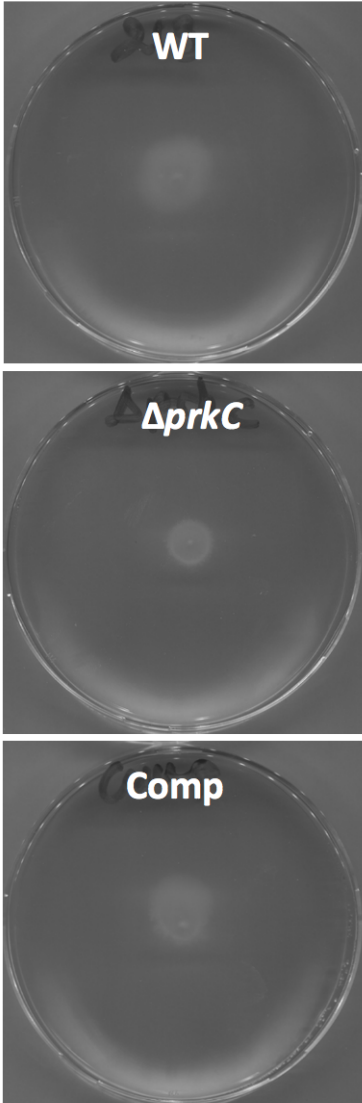
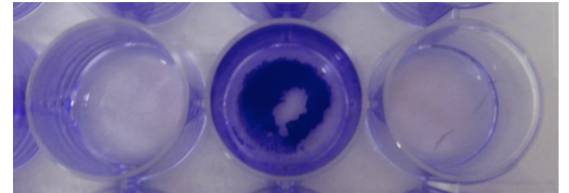
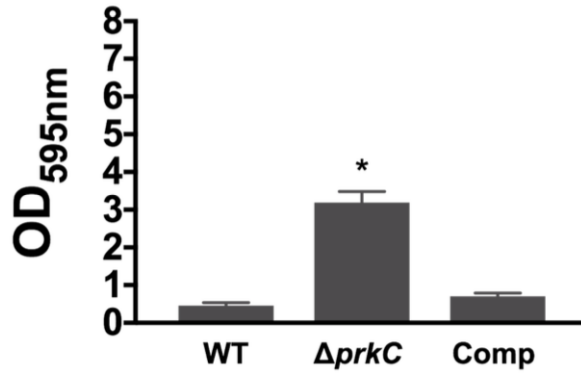




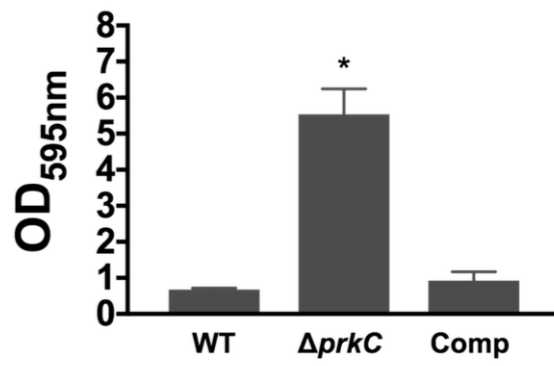






A**B**

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C**D**