Membrane binding properties of the cytoskeletal protein bactofilin

Ying Liu[1,#], Rajani Karmakar[2], Maria Billini[1], Wieland Steinchen[3,4], Saumyak Mukherjee[2,$], Rogelio Hernández-Tamayo[5,6], Thomas Heimerl[4], Gert Bange[3,4,7], Lars V. Schäfer[2], Martin Thanbichler[1,4,7*]

[1] Department of Biology, Marburg University, 35032 Marburg, Germany
[2] Theoretical Chemistry, Ruhr University Bochum, 44780 Bochum, Germany
[3] Department of Chemistry, Marburg University, 35032 Marburg, Germany
[4] Center for Synthetic Microbiology (SYNMIKRO), 35032 Marburg, Germany
[5] Microcosm Earth Center, 35032 Marburg, Germany
[6] Max Planck Fellow Group Bacterial Cell Biology, Max Planck Institute for Terrestrial Microbiology, 35043 Marburg, Germany
[7] Max Planck Fellow Group Molecular Physiology of Microbes, Max Planck Institute for Terrestrial Microbiology, 35043 Marburg, Germany

[#] Present address: Universitätsklinikum Schleswig-Holstein, 24105 Kiel, Germany
[$] Present address: Department of Theoretical Biophysics, Max Planck Institute of Biophysics, 60438 Frankfurt am Main, Germany

*Address correspondence to
Martin Thanbichler (thanbichler@uni-marburg.de)

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Abstract

Bactofilins are a widespread family of cytoskeletal proteins with important roles in bacterial morphogenesis, chromosome organization and motility. They polymerize in a nucleotide-independent manner, forming non-polar filaments that are typically associated with the cytoplasmic membrane. Membrane binding was suggested to be mediated by a short N-terminal peptide, but the underlying mechanism and the conservation of this interaction determinant among bacteria remain unclear. Here, we use the bactofilin homolog BacA of the stalked bacterium *Caulobacter crescentus* as a model to analyze the membrane-binding behavior of bactofilins. Based on site-directed mutagenesis of the N-terminal region, we identify the full membrane-targeting sequence of BacA (MFSKQAKS) and pinpoint amino acid residues that are critical for its function *in vivo* and *in vitro*. Molecular dynamics simulations then provide detailed insight into the molecular mechanism underlying the membrane affinity of this peptide. Collectively, these analyses reveal a delicate interplay between the water exclusion of hydrophobic N-terminal residues, the arrangement of the peptide within the membrane and the electrostatic attraction between positively charged groups in the peptide and negative charges in the phospholipid molecules. A comprehensive bioinformatic analysis shows that the composition and properties of the membrane-targeting sequence of BacA are conserved in numerous bactofilin homologs from diverse bacterial phyla. Importantly, our findings reveal cooperative effects between the membrane-binding and polymerization activities of BacA. Moreover, they demonstrate that both of these activities critically contribute to the recruitment of the BacA client protein PbpC, a membrane-bound cell wall synthase that uses a conserved peptide in its N-terminal cytoplasmic tail to interact with BacA assemblies. Finally, we show that PbpC can functionally replace the endogenous membrane-targeting sequence of BacA when provided at elevated levels *in trans*, indicating that client proteins can make a significant contribution to the membrane association of bactofilin polymers. Together, these results unravel the mechanistic underpinnings of membrane binding by bactofilin homologs, thereby illuminating a previously obscure but important aspect in the biology of this cytoskeletal protein family.

Introduction

Cytoskeletal proteins mediate a variety of fundamental cellular processes in bacteria, including cell growth, cell division and DNA segregation (Cabeen and Jacobs-Wagner, 2010; Wagstaff and Löwe, 2018). Many important components of the bacterial cytoskeleton have eukaryotic homologs. The conserved cell division protein FtsZ, for instance, is a member of the tubulin superfamily (Löwe and Amos, 1998; Mukherjee et al., 1993), forming dynamic polymers at the cell division site that control the assembly and function of the cell division apparatus (McQuillen and Xiao, 2020). By contrast, the morphogenetic protein MreB, which governs cell growth and morphology in most rod-shaped bacteria (Jones et al., 2001; van den Ent et al., 2001), is homologous to actin (Rohs and Bernhardt, 2021). Some bacteria also possess intermediate-filament-like proteins, such as the coiled-coil-rich protein crescentin, which promotes cell curvature in the crescent-shaped alphaproteobacterium *Caulobacter crescentus* (Ausmees et al., 2003; Liu et al., 2024). Apart from these universally conserved cytoskeletal protein families, bacterial cells can contain various polymer-forming proteins that are typically absent from eukaryotes (Wagstaff and Löwe, 2018). A prominent member of this group of proteins is bactofilin (Kühn et al., 2010).

Bactofilin homologs are widespread among bacteria and involved in diverse cellular functions. In many species, they have important roles in cell shape determination, including the establishment or adjustment of cell curvature in *Helicobacter pylori* (Sycuro et al., 2010), *Campylobacter jejuni* (Frirdich et al., 2023), *Leptospira biplexa* (Jackson et al., 2018) and *Rhodospirillum rubrum* (Pöhl et al., 2024), the modulation of cell size in *Chlamydia trachomatis* (Brockett et al., 2021), the formation of buds in *Hyphomonas neptunium* (Pöhl et al., 2024), the stabilization of rod shape in *Myxococcus xanthus* (Koch et al., 2011) and *Proteus mirabilis* (Hay et al., 1999) and the synthesis of stalk-like cellular extensions in *C. crescentus* (Kühn et al., 2010), *Asticcacaulis biprosthecum* (Caccamo et al., 2020) and *Rhodomicrobium vannielii* (Richter et al., 2023). Other functions include DNA organization in *M. xanthus* (Anand et al., 2020; Lin et al., 2017) as well as cell motility in *Bacillus subtilis* (El Andari et al., 2015). Bactofilins are relatively small proteins characterized by a central Bactofilin A/B domain (InterPro ID: IPR007607) that is typically flanked by unstructured terminal regions of varying lengths. The central domain is ~110 amino acids long and folds into a compact right-handed β-helix with a triangular geometry, measuring roughly 3 nm along its longitudinal axis (Kassem et al., 2016; Shi et al., 2015; Vasa et al., 2015; Zuckerman et al., 2015). This basic unit polymerizes into extended non-polar filaments through alternating head-to-head and tail-to-tail interactions between individual subunits (Deng et al., 2019). The resulting protofilaments can further assemble into higher-order structures such as bundles, lattices and 2D crystalline sheets *in vitro* (Holtrup et al., 2019; Kühn et

55 al., 2010; Pöhl et al., 2024; Sichel et al., 2022; Vasa et al., 2015; Zuckerman et al., 2015) and, potentially,
56 also *in vivo* (Kühn et al., 2010). Previous studies have indicated that bactofilins are typically associated
57 with the cytoplasmic membrane (Deng et al., 2019; Hay et al., 1999; Koch et al., 2011; Kühn et al., 2010;
58 Lee et al., 2023). However, only a small subset of them, including certain gammaproteobacterial represen-
59 tatives (Hay et al., 1999), contain predicted transmembrane helices, suggesting that canonical bactofilins
60 use an unconventional membrane-targeting mechanism that may have specifically evolved in this cyto-
61 skeletal protein family. Consistent with this notion, the membrane-binding activity of bactofilin homologs
62 from *Thermus thermophilus* (Deng et al., 2019) and *C. trachomatis* (Lee et al., 2023) has recently been
63 shown to depend on a short peptide in the N-terminal unstructured region of the proteins. However, the
64 conservation of this peptide, the precise mechanism underlying its affinity for the lipid bilayer, and the
65 interplay between membrane attachment and bactofilin polymerization still remain to be investigated.

66 In this study, we comprehensively analyze the membrane-binding behavior of bactofilins, using BacA from
67 *C. crescentus* as a model protein. Previous work has shown that BacA forms membrane-associated poly-
68 meric sheets at the old pole of the *C. crescentus* cell, which recruit the cell wall synthase PbpC to stimulate
69 stalk formation at this subcellular location (Billini et al., 2019; Hughes et al., 2013; Kühn et al., 2010). By
70 systematically exchanging residues in the N-terminal region of BacA, we identify a short N-terminal pep-
71 tide (MFSKQAKS) that acts as the membrane-targeting sequence (MTS) of BacA and pinpoint residues
72 critical for its membrane-binding activity *in vivo* and *in vitro*. We then clarify the molecular mechanism
73 underlying the membrane affinity of this sequence using molecular dynamics simulations. A compre-
74 hensive bioinformatic analysis suggests that the mode of action identified for the MTS of BacA may be
75 broadly conserved among bactofilins from various different phyla. Importantly, our findings indicate that
76 membrane association and polymerization are cooperative processes that are both required for robust
77 BacA assembly, with polymerization in turn promoting the interaction of BacA with its client protein PbpC,
78 and vice versa. Together, these findings reveal the mechanistic basis of membrane binding by bactofilin
79 homologs and highlight the implications of this process for bactofilin function, thereby shedding light on
80 a thus-far poorly understood aspect in the biology of this widespread cytoskeletal protein.

Results

81 **The membrane-binding activity of BacA depends on an N-terminal amphipathic peptide**

82 Previous work has proposed a small N-terminal peptide as the membrane-targeting sequence (MTS) of
83 canonical bactofilin homologs (Deng et al., 2019). In the case of *C. crescentus* BacA, the suggested region
84 comprises the sequence MFSKQAKS at the N-terminal end of the protein (**Figure 1A**). To verify the impor-
85 tance of this peptide for BacA function, we compared the localization pattern of wild-type BacA with that
86 of a mutant derivative lacking the predicted membrane-targeting sequence (MTS). For this purpose, the
87 two proteins were fused to the monomeric yellow fluorescent protein mVenus and produced in a strain
88 carrying an in-frame deletion in the endogenous *bacA* and *bacB* genes (**Figure 1B**). As expected, the wild-
89 type protein was fully functional and formed distinct fluorescent foci at the stalked pole of the cells, which
90 have been previously shown to reflect the formation of small patches of membrane-associated BacA poly-
91 mers (Kühn et al., 2010). The mutant protein (Δ2-8), by contrast, was evenly distributed in the cytoplasm,
92 supporting the idea that the N-terminal region of BacA is involved in membrane binding. The diffuse local-
93 ization also suggests a defect in polymerization, which however may be a secondary effect resulting from
94 the loss of membrane association, since previous work has shown that the unstructured terminal regions
95 are not essential for bactofilin assembly (Sichel et al., 2022; Vasa et al., 2015). In line with this finding,
96 transmission electron microscopy (TEM) analysis confirmed that the Δ2-8 variant formed polymers similar
97 to those of wild-type BacA when overproduced in *Escherichia coli* (**Figure 1–figure supplement 1**). Thus,
98 although not essential, membrane binding may facilitate BacA polymerization at native expression levels
99 by promoting its enrichment at the membrane surface and thereby stimulating inter-subunit interactions.
100 To further evaluate the role of polymerization in BacA localization, we generated a mutant variant of BacA
101 impaired in self-assembly by replacing a conserved phenylalanine residue in the C-terminal polymerization
102 interface of its Bactofilin A/B domain (F130 in *C. crescentus* BacA) with a charged arginine residue. Consis-
103 tent with previous work in other species (Deng et al., 2019; Jacq et al., 2024; Zuckerman et al., 2015), TEM
104 analysis (**Figure 1–figure supplement 1**) and size-exclusion chromatography studies (**Figure 1–figure**
105 **supplement 2**) verified that the mutant protein (F130R) failed to form polymeric structures *in vitro*. Impor-
106 tantly, the corresponding mVenus fusion protein no longer formed membrane-associated polar foci but
107 was dispersed within the cytoplasm (**Figure 1B**), suggesting that polymerization and membrane binding
108 could stimulate each other.

109 Apart from transmembrane segments, proteins commonly employ amphiphilic helices to associate with
110 the inner face of the cytoplasmic membrane, as exemplified by the bacterial actin homologs FtsA (Pichoff

111 and Lutkenhaus, 2005) and MreB (Salje et al., 2011). As a first step to clarify the mechanism underlying
112 the membrane-binding activity of the MTS, we therefore determined whether it was able to adopt an
113 amphiphilic helical structure. However, neither machine-learning-based pattern recognition, as employed
114 by AmphipaSeeK (Sapay et al., 2006) (**Figure 1A**), nor helical wheel analysis (**Figure 1C**) provided any sup-
115 port for this possibility, suggesting a different mode of action.

116 To obtain more insight into the function of the MTS, we systematically exchanged single or multiple amino
117 acid residues in the N-terminal eight amino acids of BacA-mVenus (**Figure 1–figure supplement 3**) and
118 analyzed the localization patterns of the mutant proteins. Among the single exchanges, substitution of
119 the hydrophobic phenylalanine residue at position 2 (F2Y and F2E) led to a disperse or, occasionally,
120 patchy distribution of the fusion protein (**Figure 1B**). Other single substitutions, by contrast, had relatively
121 mild effects, including the formation of multiple and/or mispositioned bactofilin complexes (**Figure 1–**
122 **figure supplement 4**). Notably, however, a double exchange replacing the two positively charged lysine
123 residues in the MTS with polar serine residues (K4S-K7S) strongly increased the fraction of delocalized
124 protein (**Figure 1B**), although distinct fluorescent foci or patches were still detectable, suggesting residual
125 membrane-binding activity. An even stronger effect, as reflected by a complete dispersion of the fluores-
126 cence signal, was observed when negatively charged glutamate residues were introduced at these posi-
127 tions (K4E-K7E). Collectively, these results point to a central role of F2 and K4/K7 in the association of BacA
128 with the cytoplasmic membrane.

129 To further characterize the mutant BacA-mVenus variants, we analyzed their mobility within the cell using
130 single-particle tracking (**Figure 2–video 1** and **Figure 2–figure supplement 1**). As expected, given its ability
131 to interact with the membrane and polymerize, the wild-type protein exhibited a very low diffusional
132 mobility. Mutant variants that were partially delocalized (F2Y and K4S-K7S) were significantly more mobile,
133 and variants with a largely diffuse localization (Δ2-8, F2E, K4E-K7E and F2E-K4E-K7E) displayed the highest
134 diffusion rates, approaching the mobility of free mVenus (**Figure 2A** and **Supplementary file 1**). These
135 results support the notion that the MTS is required for the formation of stable membrane-associated BacA
136 assemblies.

137 As a direct means to analyze the membrane-binding activity of the fusion proteins, we next performed
138 cell fractionation studies. To this end, cells producing different BacA-mVenus variants were lysed and
139 membranes were separated from soluble components by ultracentrifugation. Western blot analysis re-
140 vealed that a large part of the wild-type protein was detected in the membrane fraction, whereas the
141 variant lacking the MTS (Δ2-8) was completely soluble (**Figure 2B**). A severe reduction in membrane-

142 binding activity was also observed for all fusion proteins lacking residues F2 and K4/K7 (**Figure 2C**), con-
143 firming the importance of these residues in the function of the MTS.

144 After having identified a role of the MTS in the association of BacA with membranes *in vivo*, we aimed to
145 verify the membrane-targeting activity of this peptide in a defined system *in vitro*. For this purpose, we
146 purified wild-type BacA and various mutant derivatives, using a cleavable N-terminal His_6-SUMO affinity
147 tag (Marblestone et al., 2006), which allowed us to obtain proteins without any non-native extensions
148 that could potentially interfere with the analysis. This approach also made it possible to obtain two addi-
149 tional BacA variants, one lacking the N-terminal methionine of BacA (ΔM) and another one lacking the N-
150 terminal methionine and containing a glutamate instead of the phenylalanine normally located at the
151 second position of the MTS (xE). We then incubated the different proteins with small unilamellar vesicles
152 (liposomes) made of phosphatidylglycerol, which has been identified as the most abundant membrane
153 lipid in *C. crescentus* under standard growth conditions (Contreras et al., 1978; Stankeviciute et al., 2019).
154 Subsequently, the liposomes were collected by ultracentrifugation and analyzed for the amount of bound
155 protein (**Figure 3**). Consistent with the *in vivo* data, a large proportion (~80%) of the wild-type protein was
156 associated with the liposome pellet, whereas only a small fraction sedimented in control reactions lacking
157 liposomes, likely reflecting large, poorly soluble polymer bundles. Variants lacking the entire MTS (Δ2-8)
158 or carrying the F2E or K4E/K7E exchanges, by contrast, showed severe defects in membrane binding, with
159 sedimentation efficiencies similar to those obtained in the liposome-free control reactions. Residual bind-
160 ing was still observed for the F2Y and K4S/K7S variants, consistent with the milder defects observed for
161 these proteins in the localization and single-particle tracking studies (compare **Figures 1B** and **2A**). Notably,
162 while the behavior of the xE variant closely resembled that of the F2E variant, the absence of residue M1
163 (ΔM) alone only partially abolished the membrane association of BacA, indicating that the N-terminal
164 methionine plays an important but not decisive role in the function of the MTS (**Figure 3**). Together, these
165 findings verify the existence of an N-terminal MTS in BacA. Moreover, they identify the hydrophobic
166 residues M1 and F2 and the two positively charged lysine residues K4 and K7 as key determinants of its
167 membrane-binding affinity.

168 **BacA polymerization and membrane binding are mutually stimulating processes**

169 Our results showed that the disruption of the polymerization interface (F130R) resulted in a diffuse, cyto-
170 plasmic localization of BacA, suggesting that polymerization might be a prerequisite for efficient mem-
171 brane binding (**Figure 1B**). To investigate this possibility, we clarified the membrane-binding activity of the
172 polymerization-deficient F130R variant of BacA-mVenus. Cell fractionation experiments showed that the

173 fusion protein was barely detectable in the membrane fraction (**Figure 4A**). In line with this finding, only
174 marginal membrane-binding activity was observed for the untagged BacA-F130 variant in liposome-bind-
175 ing assays (**Figure 4B**). These results suggest that the MTS has a relatively low affinity for the membrane,
176 so that a stable interaction can only be achieved by cooperative binding of multiple MTS-containing N-
177 terminal tails, arrayed on the surface of bactofilin polymers. Further support for this idea came from do-
178 main swapping experiments that showed that the MTS of BacA was able to mediate stable membrane
179 attachment when fused to a derivative of the polymer-forming protein crescentin from *C. crescentus* lack-
180 ing its native membrane-targeting sequence (**Figure 4–figure supplement 1**).

181 Interestingly, the diffuse localization and high diffusional mobility of BacA variants containing a defective
182 MTS (**Figures 1B and 2A**) indicate that there may also be a converse stimulatory effect of membrane bind-
183 ing on BacA polymerization. To further assess this possibility, we analyzed whether an MTS-defective BacA
184 variant could regain the ability to form membrane-associated polymeric complexes if equipped with a
185 heterologous membrane-targeting sequence. To this end, two copies of the N-terminal amphiphilic helix
186 of *E. coli* MreB (Salje et al., 2011) were fused to a BacA-mVenus variant lacking the native MTS-containing
187 peptide (Δ2-8). Microscopic analysis revealed that the fusion protein condensed into distinct foci at the
188 old pole or the cell center (**Figure 4C**), reminiscent of the aberrant localization patterns observed for
189 mutant proteins with exchanges in the MTS that did not completely abolish membrane binding (compare
190 **Figure 1–figure supplement 4**). The restoration of fluorescent foci was accompanied by a strong decrease
191 in diffusional mobility, as determined by single-molecule tracking (**Figure 4D**). In addition, cell fractiona-
192 tion experiments showed that the fusion protein was highly enriched in the membrane pellet (**Figure 4E**).
193 Importantly, when its polymerization interface was disrupted by introduction of the F130R exchange,
194 focus formation was abolished, membrane binding was strongly reduced, and the diffusional mobility of
195 the fusion protein increased to a value similar to that of an F130R variant containing the native MTS of
196 BacA (**Figure 4C-E**). Collectively, these findings demonstrate that the polymerization and membrane-
197 binding activities of BacA strongly stimulate each other.

198 **Molecular dynamics simulations identify M1, K4 and K7 as key residues of the BacA MTS**

199 To investigate the molecular underpinnings of the interaction between the MTS of BacA and the mem-
200 brane, we turned to all-atom molecular dynamics (MD) simulations. For this purpose, peptides comprising
201 the ten N-terminal bactofilin residues were simulated in the presence of a multi-component lipid bilayer
202 that was composed of approximately 50% monoglucosyldiglyceride (GLY), 33% phosphatidylglycerol (PG)
203 and 16% diacylglycerol (DAG) lipids (see **Supplementary file 2** for details), approximating the native com-

204 position of *C. crescentus* cell membranes under standard growth conditions (Chow and Schmidt, 1974;
205 Contreras et al., 1978; Stankeviciute et al., 2019). In addition to the wild-type peptide MFSKQAKSNN, our
206 analysis also included the mutant F2Y and K4S-K7S variants (each in a separate simulation).

207 The starting structures of the peptides were modeled in an extended conformation without any secondary
208 structure. At the beginning, a single peptide (wild-type, F2Y or K4S-K7S) was placed in the aqueous phase
209 at a distance of approximately 3 nm from the lipid headgroups. During the 500-ns simulations, all three
210 peptides rapidly bound to the membrane and established multiple contacts with the lipids at the mem-
211 brane/water interface (illustrated for the wild-type MTS in **Figure 5A**). In doing so, the peptides did not
212 adopt any persistent secondary structures but remained disordered, with a preference for somewhat
213 extended conformations (**Figure 5B,C** and **Supplementary file 3**). Notably, in control simulations in which
214 the peptides were initially modeled as α-helices, the peptides unfolded rapidly, either in the aqueous
215 phase or after binding to the membrane/water interface, eventually yielding the same results (within the
216 statistical uncertainties) as the simulations starting from an extended conformation.

217 Excluding the initial phase of the simulations in which the peptides were not yet bound to the membrane,
218 we then determined the density profiles of the three different peptides along the membrane normal
219 (**Figure 5D** and **Figure 5–figure supplement 1**). The results show that the N-terminal residues 1 and 2
220 insert deeply into the lipid bilayer, assuming positions below the phosphate headgroup region, while the
221 positions of the remaining residues (3 to 10) are gradually shifted towards the aqueous phase. The
222 peptides thus bind to the membrane in a tilted orientation, with their N-terminal region protruding more
223 deeply into the hydrophobic parts of the lipid headgroup region (**Figure 5B**). Interestingly, in case of the
224 F2Y variant, the presence of tyrosine at position 2 pulls residue M1 and Y2 further towards the head-
225 group/water interface (**Figure 5–figure supplement 1B**). This result can be explained by the preferential
226 hydration of tyrosine compared to phenylalanine, as is also reflected in the difference of the solvation
227 free energies (ΔG_{solv}) of the corresponding side-chain analogs *p*-cresol (-25.6 kJ mol^{-1}) and toluene (-3.2 kJ
228 mol^{-1}) (Wolfenden et al., 1981).

229 To characterize the interactions between the peptides and the membrane in more detail, the contacts
230 between the individual peptide residues and the different lipid species in the bilayers were counted. The
231 results confirm the above finding that residues 1 and 2 show the strongest association with lipids and that
232 lipid interactions gradually decrease for residues further down the peptide chain (**Figure 6A**). Notably,
233 among these interactions, contacts with anionic PG lipids dominate, even though the percentage of PG
234 lipids (33%) in the simulated bilayer is smaller than that of GLY lipids (50%) (see above and **Supplementary**

235 **file 2**), which can largely be attributed to the lysine residues at positions 4 and 7. In line with this result,
236 the K4S-K7S variant shows a strong reduction in the number of PG contacts at these positions, which is
237 partially compensated by a higher prevalence of GLY contacts, particularly for residues 1 to 5 (**Figure 6A**).
238 The F2Y variant, by contrast, did not show a marked decrease in the number of total lipid contacts for
239 position 2, with fewer PG contacts (of residues 1 and 2) again compensated by more GLY lipid contacts
240 (**Figure 6A**). Taken together, the lipid contact analysis shows that the N-terminal residues M1 and F2
241 establish the closest contacts with the lipid bilayer, and that the lysine residues K4 and K7 strongly interact
242 with PG lipids.

243 The contact analysis described above only provides insight into the spatial proximity of the peptides and
244 the lipids. To additionally determine the strength of the interactions, we analyzed the MD simulations in
245 terms of the interaction energies between the different peptide residues and the lipid molecules (**Figure**
246 **6B**). For both the wild-type peptide and its F2Y variant, the strongest (most favorable) peptide-lipid inter-
247 action energy is seen for residues 1, 4 and 7, which can be attributed to strong electrostatic attraction
248 between the PG lipids and the charged NH_3^+ group at the N-terminus (residue M1) as well as the side
249 chains of residues K4 and K7, respectively. Notably, out of all residues, M1 interacts most strongly with
250 the membrane. Nevertheless, its removal (ΔM) had only a moderate effect on the membrane-binding
251 activity of BacA *in vitro* (**Figure 3**), corroborating the notion that the largest part of the interaction energy
252 is provided by the N-terminal NH_3^+ group of M1 rather than its hydrophobic side chain. However, taken
253 together, the two lysines K4 and K7 make an even larger contribution to the interaction, consistent with
254 the significant reduction in membrane association observed for the K4S-K7S variants of BacA (**Figures 1C,**
255 **2C and 3**). It is interesting to note that in terms of the interaction energy, residue F2 does not seem to
256 play a prominent role in the peptide-membrane association (**Figure 6B**), although the F2Y exchange
257 strongly reduces the membrane-binding affinity of BacA (**Figures 1C, 2C and 3**). The deleterious effect of
258 this exchange may be explained by the preferential hydration of tyrosine compared to phenylalanine,
259 which is not reflected in the peptide-membrane interaction energy but in the difference in hydration free-
260 energy (see above). Furthermore, residues M1 and Y2 in the F2Y peptide are located in closer proximity
261 to the aqueous phase (see **Figure 5–figure supplement 1B**), which leads to a substantial decrease in the
262 favorable interaction energy of the N-terminal NH_3^+-group with PG lipids that is only incompletely com-
263 pensated by slightly more favorable interactions with GLY lipids (**Figure 6B**). Together, these results pro-
264 vide a rational basis for the experimental finding that the F2Y and K4S-K7S variants of BacA have a con-
265 siderably lower membrane-binding affinity than the wild-type protein.

266 **The N-terminal membrane-targeting sequence is conserved among bactofilin homologs**

267 The results of the MD simulations confirmed that the short peptide (MFSKQAKS) at the N-terminus of
268 BacA acts as an MTS. To assess whether this mode of interaction was more widespread among bactofilins,
269 we set up a bioinformatic pipeline to analyze the conservation of the N-terminal region of bactofilin homo-
270 logs from a broad range of bacterial lineages (**Figure 7–figure supplement 1**). To this end, all proteins
271 containing an annotated bactofilin domain were retrieved from the UniProt database (UniProt
272 Consortium, 2023). After the exclusion of entries from non-bacterial origin, we eliminated all bactofilin
273 homologs containing predicted N-terminal transmembrane helices as a membrane anchor. These proteins
274 were relatively rare (~3.2%) but widely distributed across the bacterial phylogeny, with the majority found
275 in the gammaproteobacteria (**Figure 7–figure supplement 1**). To avoid biases arising from the over-repre-
276 sentation of certain bacterial species in UniProt, we next grouped highly similar (>90% identity) bactofilin
277 sequences, which typically represent orthologs from different sequenced strains, and only kept one
278 sequence per group. For each of the remaining proteins, we then determined the N-terminal region pre-
279 ceding the bactofilin domain. Based on the length of the MTS identified for BacA, we eliminated proteins
280 whose predicted N-terminal tails were shorter than eight amino acids, potentially due to misannotation
281 of the translational start sites. The remaining proteins (14337 sequences) were then used to search for
282 conserved N-terminal amino acid motifs.

283 A global analysis of the entire protein set yielded a conserved motif with the consensus sequence
284 MFSKKKK, which resembled the MTS identified for *C. crescentus* BacA and contained both the two hydro-
285 phobic N-terminal residues that insert into the core of the lipid bilayer as well as the two lysin residues
286 that make electrostatic interactions with the headgroups of PG lipids (**Figure 7**). This result suggests that
287 the presence of an N-terminal MTS is a common feature of bacterial bactofilin homologs. However, this
288 global approach might obscure lineage-specific adaptations of the MTS to evolutionary differences in
289 membrane lipid composition. To address this issue, we conducted an additional analysis in which we sort-
290 ed the proteins according to their phylogenetic origin before performing a motif search. In doing so, we
291 focused on the phyla Pseudomonadota, Bacteroidota, Bacillota, Spirochaetota and Thermodesulfobac-
292 teriota, which contribute the majority of the bactofilin homologs in the UniProt database. This refined
293 analysis confirmed the presence of conserved N-terminal motifs in each of the five phyla, although there
294 is variability in the consensus sequences of these motifs both within and between phyla (**Figure 7–figure**
295 **supplement 1**). A comparison of the most prevalent motifs (**Figure 7**) revealed that bactofilin homologs
296 from Pseudomonadota and Bacillota share highly similar N-terminal sequences, which largely correspond
297 to the global motif described above. A similar N-terminal motif is also found in most bactofilin homologs

298 from Bacteroidota, although these proteins often display a negatively rather than a positively charged
299 residue at position 4. The most prevalent motif identified in Spirochaetota and Thermodesulfobacteriota,
300 by contrast, is fundamentally different from the global consensus motif and may thus have a function
301 unrelated to membrane binding (**Figure 7**). However, there is also a subset of sequences from these phyla
302 that do share MTS-like sequences (**Figure 7–figure supplement 2**), suggesting that the ability of bactofilins
303 to interact with membranes is still widespread in these phyla. Together, these results strongly suggest
304 that the majority of bactofilin homologs known to date feature an N-terminal MTS whose composition
305 and mode of action are similar to that of *C. crescentus* BacA.

306 **BacA polymerization promotes client-protein binding**

307 The only client protein of BacA reported to date is the cell wall synthase PbpC, a bitopic membrane protein
308 related to *E. coli* PBP1A (Yakhnina et al., 2013; Strobel et al., 2014). Previous work has shown that its N-
309 terminal cytoplasmic tail was sufficient for its bactofilin-dependent recruitment to the stalked cell pole of
310 *C. crescentus*, suggesting that this region of the protein contains all critical interaction determinants
311 (Hughes et al., 2013; Kühn et al., 2010). However, its precise mode of interaction with the polar bactofilin
312 cluster and the role of this interaction in bactofilin assembly has remained unclear.

313 To clarify which of the two bactofilin paralogs of *C. crescentus* interacts with PbpC to mediate its polar
314 localization, we analyzed the subcellular distribution of an mVenus-tagged PbpC variant in different bacto-
315 filin mutants. The fusion protein retained its wild-type localization pattern in the Δ*bacB* background but
316 failed to condense into polar foci in Δ*bacA* cells, identifying BacA as its main interactor (**Figure 8** and **Figure**
317 **8–figure supplement 1A**). Next, we aimed to pinpoint the regions in the cytoplasmic tail of PbpC that
318 mediate its recruitment to BacA. For this purpose, we retrieved the amino acid sequences of PbpC homo-
319 logs and aligned their predicted cytoplasmic tails (**Figure 8–figure supplement 2**). This analysis identified
320 four distinct segments, including (i) a highly conserved peptide at the N-terminal end of PbpC (region C1),
321 (ii) a proline-rich medial region, (iii) a second highly conserved peptide (Region C2) and (iv) a region rich
322 in positively charged amino acids immediately adjacent to the transmembrane helix. To assess the impor-
323 tance of these segments, we generated mVenus-PbpC derivatives whose cytoplasmic tail either lacked
324 region C1 (Δ2-13) or only comprised region C1, with the remaining parts being replaced by an unstructured
325 region taken from the periplasmic protein DipM of *C. crescentus* (Izquierdo-Martinez et al., 2023) (**Figure**
326 **8–figure supplement 3**). Microscopic analysis showed that the removal of region C1 resulted in the loss
327 of polar localization, whereas the chimeric variant showed the same localization pattern as the wild-type
328 fusion protein (**Figure 8A**). Similar results were obtained for cells that were cultivated under phosphate-

329 limiting condition to stimulate stalk growth (Schmidt, 1968). Interestingly, the delocalization of mVenus-
330 PbpC upon deletion of region C1 did not reduce its ability to promote stalk elongation (**Figure 8–figure**
331 **supplement 4**). Region C1 thus appears to be necessary and sufficient for the recruitment of PbpC to the
332 cell pole-associated BacA assembly, but dispensable for its role in stalk biogenesis.

333 To verify the ability of region C1 to directly associate with BacA, we generated a synthetic peptide com-
334 prising the N-terminal 13 residues of PbpC and analyzed its interaction with purified BacA polymers using
335 biolayer interferometry. When biosensors carrying the immobilized peptide were titrated with increasing
336 concentrations of BacA, we observed specific binding with an apparent equilibrium dissociation constant
337 (K_D) of 4.9 µM (**Figure 8B** and **Figure 8–figure supplement 5**). A similar assay with the polymerization-
338 deficient F130R variant, by contrast, only yielded residual binding with very fast dissociation rates
339 (**Figure 8C**). These results confirm the direct interaction of BacA with region C1 of PbpC. Moreover, they
340 indicate that BacA polymerization is a prerequisite for efficient and stable PbpC binding, likely because
341 the high local accumulation of BacA within polymers leads to an increase in the avidity for its interaction
342 partner.

343 Having identified a direct association of BacA with the N-terminal peptide of PbpC, we set out to map the
344 PbpC-binding site of BacA using hydrogen-deuterium-exchange (HDX) mass spectrometry, a method that
345 allows the detection of local shifts in the accessibility of backbone amide hydrogens induced by conform-
346 ational changes and/or ligand binding (Konermann et al., 2011). When analyzed alone, the HDX profile of
347 BacA showed very fast HDX in the N- and C-terminal regions flanking the bactofilin domain, which indi-
348 cates a high degree of disorder and thus corroborates the predicted domain structure of BacA (**Figure 8–**
349 **figure supplement 6**). Upon incubation with the PbpC peptide, several peptides in the C-terminal half of
350 the central bactofilin domain exhibited a small, yet significant decrease in the HDX rate (**Figure 8C**), with
351 the most prominent changes observed in a region close to the C-terminal polymerization interface (**Figure**
352 **8D**). Although additional studies are required to precisely define the interface, this finding suggest that
353 inter-subunit interactions in the bactofilin polymer could potentially induce slight conformational changes
354 in BacA that promote PbpC binding.

355 To clarify whether the interaction with PbpC contributes to BacA assembly, we compared the localization
356 patterns of BacA-Venus in wild-type and Δ*pbpC* cells. The behavior of the fusion protein was unchanged
357 in the mutant background, suggesting that PbpC does not have a major role in BacA polymerization or
358 membrane binding (**Figure 8–figure supplement 1B**). Nevertheless, it was conceivable that PbpC binding
359 could contribute, at least to some extent, to the association of BacA polymers with the cytoplasmic mem-

360 brane, thereby complementing the activity of the MTS. To test this hypothesis, we set out to investigate
361 the effect of PbpC on the assembly of an MTS-free BacA-mVenus variant (Δ2-8). Our initial studies showed
362 that the mutant protein had a diffuse localization when analyzed in cells producing PbpC at native levels
363 (**Figure 1B**). However, it was possible that, in this setting, the affinity between the two proteins or the
364 number of PbpC molecules available for the interaction are not be high enough to compensate for the
365 loss of the endogenous membrane-targeting sequence. We therefore performed localization studies in
366 cells that overproduced a C-terminally truncated variant of PbpC in which the periplasmic region was
367 replaced by the fluorescent protein mCherry ($PbpC_{1-132}$-mCherry) (Kühn et al., 2010), thereby accumulate-
368 ing elevated levels of the PbpC membrane anchor. Control experiments showed that the truncated pro-
369 tein colocalized with wild-type BacA-mVenus and stabilized the polar bactofilin cluster under conditions
370 of BacA-mVenus overproduction (**Figure 9 and Figure 9–figure supplement 1**). Importantly, the increased
371 availability of the PbpC membrane anchor also fully restored polar localization for the MTS-free fusion
372 protein (Δ2-8), whereas it could not reverse the localization defect of the polymerization-deficient F130R
373 variant (**Figure 9**). These results support the notion that membrane binding and polymerization are co-
374 operative processes that are both required for efficient bactofilin assembly. Moreover, they suggest that
375 membrane-bound client proteins can contribute to the recruitment of bactofilins to the cytoplasmic mem-
376 brane, although the importance of this effect may vary between systems.

Discussion

377 Bactofilins are widespread among bacteria and mediate the spatial organization of diverse cellular pro-
378 cesses. Although the polymers they form are typically associated with the inner face of the cytoplasmic
379 membrane, the vast majority of bactofilin homologs lack transmembrane helices, suggesting the existence
380 of an alternative membrane-targeting mechanism. Consistent with this notion, the membrane-binding
381 activity of the bactofilin *Th*Bac from *T. thermophilus* has recently been localized to the unstructured N-
382 terminal tail of the protein, and a sequence alignment based on several well-characterized bactofilin hom-
383 ologs suggested the existence of a conserved N-terminal motif in this protein region (Deng et al., 2019).
384 Our work considerably extends these initial findings by determining the precise composition of the N-
385 terminal MTS, unraveling its mode of action and clarifying its conservation among bacteria. Moreover, we
386 reveal a mutual dependence of membrane-binding and bactofilin polymerization and provide first insights
387 into mode of interaction between the bactofilin scaffold and membrane-associated client proteins.

388 Mutational analysis of *C. crescentus* BacA identified residues F2 and K4/K7 as key components of the BacA
389 MTS (**Figure 1B**). This finding is explained by the results of our MD analysis, which show that the insertion
390 of F2 into the hydrophobic core of the lipid bilayer (**Figure 5**) is critical to ensure the proper positioning of
391 the MTS in the membrane, thereby promoting electrostatic interactions of the N-terminal NH_3^+ group with
392 the negatively charged lipid headgroups of phosphatidylglycerol. The positively charged ε-amino groups
393 of K4 and K7, by contrast, directly interact with these lipid headgroups, making the largest contribution
394 to the overall interaction energy (**Figure 6**). Notably, the N-terminal methionine residue of proteins is
395 usually removed after translation when followed by an amino acid with a short side chain (Hirel et al.,
396 1989). In the case of BacA, however, the bulky phenylalanine residue at position 2 presumably inhibits
397 this process, so that residue M1 may be a genuine, conserved part of the MTS. Although M1 interacts
398 most extensively with the lipid bilayer (**Figure 6**), its absence has only a moderate effect on the mem-
399 brane-binding activity of BacA *in vitro* (**Figure 3**). This observation supports the idea that most of its
400 interaction energy is contributed by the N-terminal NH_3^+ group, whereas the insertion of the M1 side chain
401 into the hydrophobic core of the membrane only serves to tune the positioning of the N-terminus at the
402 lipid-water interface. Consistent with the results of the MD simulations, the loss of either F2 or K4/K7
403 drastically impairs the membrane-binding activity of BacA (**Figures 2C and 3**). Moreover, it prevents the
404 assembly of BacA into distinct higher-order complexes (**Figure 1B**), suggesting a link between BacA mem-
405 brane association and polymerization. Indeed, a soluble BacA variant lacking the native MTS (Δ2-8) regains
406 the ability to form polymeric complexes when fused to a heterologous MTS from *E. coli* MreB (**Figure 4C**),

407 indicating that membrane binding is a prerequisite for efficient BacA polymerization. Conversely, poly-
408 merization appears to promote membrane binding, because the disruption of the BacA polymerization
409 interface (F130R) strongly increases the fraction of soluble protein (**Figure 4**). Comparable results were
410 obtained when a suitable membrane anchor was provided *in trans* (**Figure 9**). Together, these findings
411 point to a cooperative binding mechanism, whereby (i) membrane association leads to an increase in the
412 local concentration of BacA that facilitates its self-assembly into polymers, and (ii) polymerization closely
413 juxtaposes multiple MTS-containing N-terminal tails, thereby increasing the avidity of BacA for the mem-
414 brane and shifting the equilibrium to the membrane-bound state. This process may be particularly rele-
415 vant at the low bactofilin levels (~200 BacA molecules per cell) found in *C. crescentus* cells (Kühn et al.,
416 2010).

417 Interestingly, substitutions in the MTS that did not completely abolish BacA assembly often led to the
418 formation of multiple and/or mislocalized complexes (**Figure 1–figure supplement 4**). The mechanism
419 determining the polar localization of BacA is still unclear. However, it is conceivable that bactofilin poly-
420 mers are intrinsically curved and thus preferentially associate with membrane regions of positive or
421 negative Gaussian curvature (Kühn et al., 2010; Pöhl et al., 2024). Alternatively, the MTS may preferential-
422 ly bind certain lipid species that are enriched in the polar regions of the cell, with its modification leading
423 to a change in the lipid specificity that interferes with this recruitment pathway. Exchanges at less critical
424 positions of the MTS may affect these recruitment mechanisms by reducing the membrane-binding
425 affinity and thus, indirectly, also the dimensions of BacA polymers or by altering their lipid specificity.

426 To assess the conservation of mode of membrane association established for BacA, we conducted a com-
427 prehensive comparison of the N-terminal regions of all bactofilin homologs known to date. This analysis
428 revealed that amino acid sequences similar to the MTS of BacA are highly prevalent in a large number of
429 bactofilins from the phyla Pseudomonadota, Bacteroidota and Bacillota, strongly suggesting that MTS-
430 mediated membrane binding is a common theme among this group of proteins (**Figure 7**). Notably, how-
431 ever, most prevalent N-terminal motifs identified for the bactofilin homologs of Spirochaetota and
432 Thermodesulfobacteriota are fundamentally different from those of the other phyla investigated (**Figure**
433 **7 and Figure 7–figure supplement 2**), suggesting that they exhibit in a different mode of membrane bind-
434 ing or interact with factors other than the cytoplasmic membrane.

435 Notably, a small subset of bacterial bactofilin homologs contain an N-terminal transmembrane domain,
436 which mediates their stable insertion into the cytoplasmic membrane. While found in a diversity of bac-
437 terial phyla, these proteins are particularly abundant in the Pseudomonadota, especially in their gamma-

438 proteobacterial and alphaproteobacterial lineages (**Figure 7–figure supplement 1**). One of them, the
439 bactofilin CcmA of *P. mirabilis*, has been previously investigated (Hay et al., 1999). It was shown to under-
440 go proteolytic processing and exist in two forms, a full-length form that is an integral membrane protein
441 and a shorter form that lacks the N-terminal transmembrane segments. Intriguingly, the shorter form has
442 an N-terminal sequence (MFSRKTE) that corresponds to the predicted consensus sequence of the MTS in
443 Pseudomonadota and, indeed, behaves like a typical peripheral membrane protein. The specific functional
444 properties conferred by these two distinct modes of membrane association are still unclear. However, it
445 is tempting to speculate that the assembly of MTS-containing bactofilins may be dynamically regulated by
446 cell cycle-dependent changes in membrane curvature or composition, whereas homologs with trans-
447 membrane domains may form more static polymeric assemblies. Consistent with this idea, *C. crescentus*
448 BacA only assembles at the old cell pole once its cell envelope bulges outward to initiate stalk formation,
449 thereby establishing a region of positive Gaussian curvature, although its cytoplasmic levels remain con-
450 stant throughout the cell cycle (Kühn et al., 2010). Similarly, the BacA homolog of the stalked budding
451 bacterium *H. neptunium* consistently assembles in regions of positive Gaussian curvature, first localizing
452 to the stalked pole and then, once the terminal segment of the stalk starts to expand into a bud, to the
453 bud neck (Pöhl et al., 2024).

454 Instead of using an MTS or a transmembrane domain, some bactofilins may also interact with the mem-
455 brane indirectly by binding to other membrane-associated proteins, as recently suggested for the bacto-
456 filin homolog CcmA from *H. pylori* (Sichel et al., 2022). Our results suggest that the interaction with PbpC
457 contributes, at least to some extent, to the membrane association of *C. crescentus* BacA. We did not ob-
458 serve any obvious localization defects in a Δ*pbpC* mutant (**Figure 8–figure supplement 1A**), indicating that
459 PbpC binding is not critical for its proper assembly and positioning at native accumulation levels. However,
460 when produced in excess, the PbpC membrane anchor was able to fully compensate for the absence of
461 the N-terminal MTS, providing sufficient links with the cytoplasmic membrane to enable the assembly of
462 polar BacA clusters. Thus, client protein binding is an important factor to consider when analyzing the
463 assembly and localization dynamics of bactofilins *in vivo*.

464 The determinants mediating the interaction of membrane-associated bactofilin scaffolds with their client
465 proteins are still poorly understood. For *M. xanthus* BacP, the binding site for its soluble interactor PadC
466 was shown to be located in the exceptionally long unstructured C-terminal tail, which contains a
467 conserved KKKVVVKK motif potentially involved in the interaction (Anand et al., 2020; Lin et al., 2017).
468 However, most bactofilin homologs have much shorter C-terminal tails, which are typically poorly con-
469 served in sequence and, at least in certain cases, dispensable for function *in vivo* (Sichel et al., 2022),

470 suggesting a direct interaction of client proteins with the bactofilin core domain. Our results indeed
471 suggest that the cytoplasmic tail of PbpC associates with the C-terminal region of the bactofilin domain,
472 close to the polymerization interface. Importantly, BacA assembly appears to be critical for efficient PbpC
473 binding *in vitro* (**Figure 8C**), suggesting that inter-subunit contacts could potentially change the conform-
474 ation of BacA such as to increase its affinity for its client protein. In addition, polymers may have a higher
475 avidity for PbpC than BacA monomers. Polymerization thus acts as a trigger for both membrane associa-
476 tion and the recruitment of client proteins, ensuring the tight regulation of bactofilin function.

477 Collectively, our findings provide important new insights into the function of bactofilins and the nucleo-
478 tide-independent dynamics of their assembly. It will be interesting to analyze how cell cycle- or stress-
479 induced changes in membrane composition affect the membrane-binding and polymerization behavior of
480 MTS-containing bactofilin homologs. Moreover, it will be informative to shed light on the mode of mem-
481 brane binding for bactofilins with conserved N-terminal sequences that are clearly distinct from the global
482 consensus motif.

Material and methods

483 **Media and growth conditions**

484 *C. crescentus* CB15N and its derivates were grown aerobically at 28 °C in PYE rich medium (Poindexter,
485 1964), unless indicated otherwise. To induce stalk elongation, cells were cultivated for 24 h in M2G^{-P} medi-
486 um (Kühn et al., 2010) prior to analysis. When required, media were supplemented with antibiotics at the
487 following concentrations (mg ml^{-1} in liquid/solid media): kanamycin (5/25 µg/ml), streptomycin (5/5
488 µg/ml) or gentamicin (0.5/5 µg/ml). *E. coli* strains are grown aerobically at 37 °C in LB medium containing
489 antibiotics at the following concentrations (mg ml^{-1} in liquid/solid media): ampicillin (200/200), chloram-
490 phenicol (20/30), gentamicin (0.5/5), kanamycin (30/50). To induce gene expression, media were supple-
491 mented with 0.005% or 0.03% D-xylose, 0.5 mM sodium vanillate or 1 mM IPTG, when appropriate.

492 **Construction of plasmids and strains**

493 The bacterial strains and plasmids used in this work are described in **Supplementary files 5** and **6**. The
494 oligonucleotides used for their construction are listed in **Supplementary file 7**. All plasmids were verified
495 by DNA sequencing. *C. crescentus* was transformed by electroporation (Ely, 1991). Non-replicating plas-
496 mids were integrated into the chromosome by single homologous recombination at the *xylX* locus
497 (Thanbichler et al., 2007). Proper chromosomal integration was verified by colony PCR.

498 **Live-cell imaging**

499 To prepare samples for microscopy, overnight cultures were diluted to an OD_{600} of 0.1 and cultivated for
500 1 h prior to the addition of 0.005% D-xylose. After a further 1 h (BacA variants) or 2 h (PbpC variants) of
501 incubation, the cultures were diluted tenfold and samples (1.5 µl) of the suspensions were spotted on 1%
502 agarose pads prepared with double-deionized water. Images were taken with an Axio Observer.Z1 micros-
503 cope (Zeiss, Germany) equipped with a Plan Apochromat 100x/1.45 Oil DIC, a Plan Apochromat 100x/1.4
504 Oil Ph3 M27 objective and a pco.edge 4.2 sCMOS camera (PCO, Germany). An X-Cite® 120PC metal halide
505 light source (EXFO, Canada) and appropriate filter cubes (ET-CFP, ET-YFP or ET-TaxasRed; Chroma, USA)
506 were used for fluorescence detection. Images were recorded with VisiView 3.3.0.6 (Visitron Systems,
507 Germany) and processed with Fiji 2.14.0/1.54f (Schindelin et al., 2012) and Adobe Illustrator CS6 (Adobe
508 Systems, USA). The subcellular distribution of fluorescence signals was analyzed with BacStalk (Hartmann
509 et al., 2020).

510 **Transmission electron microscopy**

511 BacA or its mutant derivatives (2-3 mg/ml) were dialyzed overnight against TEM buffer (50 mM Tris-HCl
512 (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 5% glycerol). Subsequently, 5 µl samples of the solutions were
513 applied to glow-discharged carbon-coated grids (G2400C; Plano Wetzlar, Germany). After a brief incubi-
514 tion period, the grids were blotted with filter paper, washed with a droplet of double-distilled water and
515 then treated for ~1 min with 2% (w/v) uranyl acetate before being blotted dry. Micrographs were taken
516 with a JEM-2100 transmission electron microscope (JEOL, Japan) at an acceleration voltage of 120 kV.
517 Images were captured with a 2k x 2k fast scan CCD camera F214 (TVIPS, Germany). Fiji 2.14.0/1.54f
518 (Schindelin et al., 2012) was used for data analysis.

519 **Single-particle tracking**

520 For single-particle tracking, cells were cultivated in M2G minimal media at 28 °C. In the early exponential
521 growth phase, the expression of genes placed under the control of the *xylX* (P_{xyl}) promoter was induced
522 by the addition of 0.005% D-xylose. After another 3 h of incubation, cells were spotted on coverslips (25
523 mm diameter; Menzel Gläser, Germany) and covered with 1% agarose pads prepared with double-
524 destilled water. All coverslips were cleaned before use by sonication in 2% (v/v) Hellmanex II solution
525 (Hellma, Germany) for 30 min at 37 °C, followed by rinsing in distilled water and a second round of soni-
526 cation in double-distilled water. Images were taken at 20-ms intervals by slimfield microscopy (Plank et
527 al., 2009), using an Olympus IX-71 microscope equipped with a UAPON 100x/ NA 1.49 TIRF objective, a
528 back-illuminated electron-multiplying charge-coupled device (EMCCD) iXon Ultra camera (Andor Solis,
529 USA) in stream acquisition mode, and a LuxX 457-100 (457 nm, 100 mW) light-emitting diode laser
530 (Omicron-Laserage Laserprodukte GmbH, Germany) as an excitation light source. The laser beam was
531 focused onto the back focal plane and operated during image acquisition with up to 2 mW (60 W cm^{-2} at
532 the image plane). Andor Solis 4.21 software was used for camera control and stream acquisition. Each
533 single-particle tracking analysis was preceded by the acquisition of a phase contrast image. Subsequently,
534 ~500 frames were acquired to bleach most fluorescent proteins in the cell and thus reach the single-mole-
535 cule level. Subsequently, remaining and newly synthesized molecules were tracked over ~2,500 frames.
536 Prior to analysis, the frames recorded before reaching the single-molecule level were removed from the
537 streams using photobleaching curves as a reference, and the proper pixel size (100 nm) and time incre-
538 ment were adjusted in the imaging metadata using Fiji (Schindelin et al., 2012). Subsequently, cell meshes
539 were determined using Oufti (Paintdakhi et al., 2016) and single particles were tracked with u-track 2.2.0
540 (Jaqaman et al., 2008). After the removal of all trajectories that were shorter than five steps, the diffusion-
541 al behavior of the tracked particles was analyzed using SMTracker 2.0 (Oviedo-Bocanegra et al., 2021).

542 **Cell fractionation analysis**

543 Overnight cultures were diluted to an OD_{600} of 0.1 and grown for 2 h prior to the addition of D-xylose to a
544 final concentration of 0.03%. The cells were further cultivated for 1 h (*bacA-mVenus* alleles) or 2 h (*creS-*
545 *mNeonGreen*) to allow the expression of the genes of interest. Subsequently, cells from 5 ml culture were
546 harvested by centrifugation, washed with 0.2 M Tris-HCl (pH 8.0) and stored at -80 °C. For further pro-
547 cessing, the pelleted cells were resuspended in a buffer containing 60 mM Tris-HCl (pH 8.0), 0.2 M sucrose,
548 0.2 M EDTA, 100 µg ml^{-1} phenylmethylsulfonyl fluoride (PMSF) and 10 µg ml^{-1} DNase I and lysed by soni-
549 cation at 30% amplitude for 7.5 min with alternating 10-s on and off phases (Model 120 Sonic Dis-
550 membrator; Fisher Scientific, USA). The lysates were centrifuged for 10 min at 4,000 ×g (4 °C) to remove
551 incompletely lysed cells, followed by ultracentrifugation for 1 h at 133,000 ×g (4 °C) to separate the soluble
552 and membrane-containing insoluble fractions. The supernatants were carefully removed, and the pellets
553 were resuspended in an equal volume of 0.2 M Tris-HCl (pH 8.0). Both fractions were then subjected to
554 immunoblot analysis with anti-FlgH, anti-MipZ and anti-GFP or anti-mNeonGreen antibodies.

555 **Immunoblot analysis**

556 Immunoblot analysis was performed as described previously (Thanbichler and Shapiro, 2006). Proteins
557 were detected with a polyclonal anti-GFP antibody (Sigma, Germany; Cat. #: G1544; RRID: AB_439690), a
558 monoclonal anti-mNeonGreen antibody (Chromotek, Germany; Cat. #: 32f6; RRID: AB_2827566), anti-
559 MipZ antiserum (Thanbichler and Shapiro, 2006), anti-FlgH antiserum (Mohr et al., 1996) or a monoclonal
560 anti-RFP antibody (MBL Life Science, Germany; Cat. #: M155-3) at dilutions of 1:10,000, 1:1,000, 1:10,000,
561 1:10,000 and 1:10,000, respectively. Goat anti-rabbit immunoglobulin G conjugated with horseradish
562 peroxidase (Perkin Elmer, USA) or goat anti-mouse immunoglobulin G conjugated with horseradish per-
563 oxidase (Sigma, Germany) were used as secondary antibodies. Immunocomplexes were detected with the
564 Western Lightning Plus-ECL chemiluminescence reagent (Perkin Elmer, USA). The signals were recorded
565 with a ChemiDoc MP imaging system (BioRad, Germany) and analyzed using Image Lab software (BioRad,
566 Germany).

567 **Protein purification**

568 To overproduce His_6-SUMO-BacA or mutant variants thereof, *E. coli* Rosetta(DE3)/pLysS cells transformed
569 with the appropriate plasmids were grown overnight at 37 °C, diluted 100-fold into 3 l of LB medium
570 containing antibiotics and cultivated to an OD_{600} of ~0.6. Subsequently, the media were supplemented
571 with IPTG to a final concentration of 1 mM, and the cells were incubated for another 4 h before they were
572 harvested by centrifugation and stored at -80 °C until further use. To purify the fusion proteins, the

573 pelleted cells were resuspended in buffer B (50 mM Tris/HCl pH 8.0, 300 mM NaCl, 0.1 mM EDTA, 5%
574 glycerol and 20 mM imidazole) supplemented with 10 µg/ml DNase I and 100 µg/ml PMSF and lysed by
575 three passages through a French press at 16,000 psi to break cells. The crude cell extract was cleared by
576 centrifugation for 30 min at 16,000 ×rpm (4 °C) and loaded onto a 5 ml HisTrap™ HP affinity column (GE
577 Healthcare, USA) equilibrated with buffer B with an ÄKTA Pure FPLC system (Cytiva). After washing of the
578 column with 50 ml of buffer B, protein was eluted with a 50-ml linear gradient of imidazole (20-250 mM
579 imidazole in buffer B). Fractions that were highly enriched in the protein of interest were supplemented
580 with 200 µg Ulp1-His_6 protease (Marblestone et al., 2006) and dialyzed overnight at 4 °C against buffer C
581 (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.1 mM EDTA, 5% glycerol, 1 mM DTT) to cleave off the His_6-SUMO
582 tag and remove the imidazole. Subsequently, the protein solution was again applied to a 5 ml HisTrap™
583 HP affinity column to separate the untagged BacA protein from His_6-SUMO and Ulp1-His_6. The flow-
584 through fractions were collected and analyzed by SDS-PAGE. Fractions containing the desired protein in
585 high amount and purity were dialyzed against buffer D (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.1 mM
586 EDTA, 5% glycerol) ($BacA_{\Delta 2\text{-}8}$) or buffer E (50 mM MOPS-NaOH pH 7.0, 200 mM NaCl, 0.1 mM EDTA, 5%
587 glycerol) (all other BacA variants). The protein solutions were then concentrated using Amicon Ultra
588 Centrifugal Filter (10 kDa MWCO) ultrafiltration devices (Millipore, Germany) and stored in small aliquots
589 at -80 °C.

590 **Size-exclusion chromatography**

591 BacA or its F130R variant were diluted to a concentration of 1.5 mg/ml, applied to a Superdex 200 10/300
592 GL size-exclusion column (Cytiva, Germany) and eluted at a flow rate of 0.3 ml/min using an ÄKTA Pure
593 FPLC system (Cytiva). Protein was detected by photometry at a wavelength of 280 nm.

594 **Preparation of liposomes**

595 Liposomes were generated from 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (16:0-18:1
596 PG) (10 mg/mL in chloroform; Avanti Polar Lipids, USA). Chloroform was evaporated in a rotatory eva-
597 porator, resulting in a lipid film that was left to dry overnight. Subsequently, the lipids were resuspended
598 in liposome buffer (50 mM MOPS-NaOH pH 7.0, 200 mM NaCl), and the mixture was incubated at room
599 temperature for 1 h with periodic vigorous agitation. To prepare small unilamellar vesicles (SUVs) with an
600 average diameter of 100 nm, the final lipid solution (20 mg ml^{-1}) was extruded at least ten times through
601 a Mini Extruder (Avanti Polar Lipids, USA) equipped with polycarbonate membranes of 0.1 µm pore size,
602 until the solution was clear.

603 **Co-sedimentation assay**

604 Protein (20 µM) was incubated for 20 min at room temperature with or without liposomes (0.4 mg ml^{-1})
605 in binding buffer (50 mM MOPS-NaOH pH 7.0, 300 mM NaCl) in a total reaction volume of 100 µl. The
606 mixtures were then centrifuged for 20 min at 100,000 ×g (20 °C) in a TLA-55 rotor (Beckman Coulter,
607 Germany). After transfer of the supernatant to a reaction tube and resuspension of the pellet in 100 µl of
608 liposome buffer (50 mM MOPS-NaOH pH 7.0, 200 mM NaCl), samples of the two fractions were mixed
609 with SDS-PAGE sample buffer, heated at 95 °C for 10 min and loaded onto a 15% SDS-polyacrylamide gel.
610 After electrophoresis, protein was stained with Coomassie Brilliant Blue R-250. The gels were imaged in a
611 ChemiDoc MP imaging system (BioRad, Germany) using Image Lab software (BioRad, Germany), and the
612 intensity of protein bands was quantified using Fiji 2.14.0/1.54f (Schindelin et al., 2012).

613 **Bio-layer interferometry**

614 Bio-layer interferometry experiments were conducted using a BLItz system equipped with Octet® High
615 Precision Streptavidin 2.0 (SAX2) Biosensors (Satorius, Germany). In the initial step, an N-terminally bio-
616 tinylated $PbpC_{1-13}$ peptide (Biotin-Ahx-MNDWTLPPYKFDD; GenScript, USA) was immobilized on the sensor.
617 After the establishment of a stable baseline, association reactions were monitored with BacA at various
618 concentrations or with different BacA variants of the same concentration. At the end of each binding step,
619 the sensor was transferred into an analyte-free buffer to measure the dissociation kinetics. The extent of
620 non-specific binding was assessed by monitoring the interaction of the analyte with unmodified sensors.
621 All analyses were performed in BLItz binding buffer (50 mM MOPS/NaOH pH 7.0, 100 mM NaCl, 1 mM
622 EDTA, 5% glycerol, 10 µM BSA, 0.01 % Tween)

623 **Hydrogen-deuterium exchange mass spectrometry**

624 Samples were prepared using a two-arm robotic autosampler (LEAP technologies, Denmark). 7.5 µl of
625 BacA (25 µM) or a mixture of BacA (25 µM) and $PbpC_{aa1-13}$ (100 µM) were mixed with 67.5 µl of D_2O-
626 containing buffer (20 mM HEPES-NaOH pH 8.0, 300 mM NaCl) to start the exchange reaction. After 10,
627 100, 1,000 and 10,000 sec of incubation at 25 °C, 55 µl samples were taken from the reaction and mixed
628 with an equal volume of quench buffer (400 mM KH_2PO_4/H_3PO_4, 2 M guanidine-HCl, pH 2.2) kept at 1 °C.
629 95 µl of the resulting mixture were immediately injected into an ACQUITY UPLC M-class system with HDX
630 technology (Waters™, USA) (Wales et al., 2008). Undeuterated samples of BacA and a mixture of BacA
631 and $PbpC_{1-13}$ were prepared similarly by 10-fold dilution into H_2O-containing buffer. Proteins were di-
632 gested online on an Enzymate BEH Pepsin column (300 Å, 5 µm, 2.1 mm × 30 mm; Waters™, USA) at 12 °C
633 with a constant flow (100 µl min^{-1}) of 0.1 % (v/v) formic acid in water, and the resulting peptic peptides

634 were collected on a trap column (2 mm × 2 cm) that was filled with POROS 20 R2 material (Thermo Fisher
635 Scientific, USA) and kept at 0.5 °C. After 3 min, the trap column was placed in line with an ACQUITY UPLC
636 BEH C18 1.7 µm 1.0 × 100 mm column (Waters™, USA), and the peptides were eluted at 0.5 °C using a
637 gradient of 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in acetonitrile (B) at a flow rate
638 of 30 µl/min generated as follows: 0-7 min/95-65 % A, 7-8 min/65-15 % A, 8-10 min/15 % A, 10-11 min/5 %
639 A, 11-16 min/95 % A. Peptides were ionized with an electrospray ionization source operated at 250 °C
640 capillary temperature and a spray voltage of 3.0 kV. Mass spectra were acquired over a range of 50 to
641 2,000 m/z on a G2-Si HDMS mass spectrometer with ion mobility separation (Waters™, USA) in Enhanced
642 High Definition MS ($HDMS^E$) or High Definition MS (HDMS) mode for undeuterated and deuterated sam-
643 ples, respectively. A [Glu1]-Fibrinopeptide B standard (Waters™, USA) was employed for lock mass correc-
644 tion. After each run, the pepsin column was washed three times with 80 µl of 4 % (v/v) acetonitrile and
645 0.5 M guanidine hydrochloride, and blanks were performed between each sample. All measurements
646 were carried out in triplicate.

647 Peptides from the non-deuterated samples (acquired with $HDMS^E$) were identified with the ProteinLynx
648 Global SERVER (PLGS, Waters™, USA), employing low energy, elevated energy and intensity thresholds of
649 300, 100 and 1000 counts, respectively. Peptides were matched using a database containing the amino
650 acid sequences of the proteins of interest, pepsin and their reversed sequences. The search parameters
651 were as follows: peptide tolerance = automatic; fragment tolerance = automatic; min fragment ion match-
652 es per peptide = 1; min fragment ion matches per protein = 7; min peptide matches per protein = 3;
653 maximum hits to return = 20; maximum protein mass = 250,000; primary digest reagent = non-specific;
654 missed cleavages = 0; false discovery rate = 100. Deuterium incorporation was quantified with DynamX
655 3.0 (Waters™, USA), using peptides that fulfilled the following criteria: minimum intensity = 5,000 counts;
656 maximum length = 40 amino acids; minimum number of products = 2; maximum mass error = 25 ppm;
657 retention time tolerance = 0.5 min. After automated data processing with DynamX, all spectra were manu-
658 ally inspected and, if necessary, peptides were omitted (e.g. in case of a low signal-to-noise ratio or the
659 presence of overlapping peptides).

660 **Bioinformatic analysis**

661 Nucleotide and protein sequences were obtained from the National Center for Biotechnology Information
662 (NCBI) or UniProt (UniProt Consortium, 2023) databases, respectively. Sequences were compared and
663 analyzed using the blastn, blastp or PSI-blast algorithm as implemented on the NCBI website. The presence
664 of putative amphipathic helices was assessed using AMPHIPASEEK (Sapay et al., 2006). Multiple sequence

665 alignments were generated with MUSCLE v3.8.31 (Edgar, 2004) and viewed and edited with Jalview v2
666 (Waterhouse et al., 2009).

667 To identify conserved motifs in the N-terminal regions of bactofilins, all known bactofilin homologs were
668 retrieved from the UniProt database (UniProt Consortium, 2023). Subsequently, a Python-based pre-
669 processing procedure was used to exclude non-bacterial entries, remove sequences with predicted trans-
670 membrane helices and eliminate sequences from species with unclear phylogeny. In addition, highly
671 similar sequences (with over 90% similarity) from the same species were filtered out to correct biases due
672 to over-sequencing. The N-terminal region of each protein was determined using hmmscan (HMMER 3.3.2)
673 (Eddy, 2011) with the hidden Markov profile constructed from the seed alignment of the bactofilin domain
674 downloaded from the Pfam database (Paysan-Lafosse et al., 2022). Subsequently, sequences with N-
675 terminal regions shorter than 8 amino acids were eliminated. The resulting set of sequences was categor-
676 ized by phylum, and MEME (Bailey and Elkan, 1994) was used to detect the 10 most probable motifs. The
677 analysis employed the following settings: classical objective function, with zero or one occurrence per
678 sequence, motif width ranging from 5 to 50 amino acids, and a 0th-order background Markov model.

679 **Molecular dynamics simulations**

680 All molecular dynamics (MD) simulations were carried out with Gromacs (version 2021.1) (Abraham et al.,
681 2015). The simulation systems were set up and prepared for MD simulation with the Charmm-GUI (Jo et
682 al., 2008; Jo et al., 2009; Lee et al., 2016; Wu et al., 2014),using the Charmm36m force field (Huang et al.,
683 2017) for the peptide and lipids together with the Charmm-specific TIP3P water model. The lipid composi-
684 tion of the simulated bilayer (**Supplementary file 2**) was chosen such that it mimics as closely as possible
685 the *C. crescentus* membrane (Chow and Schmidt, 1974; De Siervo and Homola, 1980). Symmetric bilayers
686 with 128 lipids in each monolayer were constructed, that is, the same number and type of lipids was
687 present in the two leaflets. At the beginning of the simulations, a single bactofilin 10-mer peptide (either
688 the wild-type sequence MFSKQAKSNN, or the K4SK7S or F2Y mutants) were modeled as extended con-
689 formations and placed in the bulk water phase, located at least 3 nm away from the lipid headgroups. The
690 net charge of the simulation box was neutralized with 150 mM KCl. After energy minimization (with
691 steepest descent), MD simulations were carried out with periodic boundary conditions in the NpT
692 ensemble at a constant temperature of 310 K, maintained via the Bussi velocity-rescaling thermostat
693 (Bussi et al., 2007), and constant 1 bar pressure, maintained by semi-isotropic pressure coupling of the
694 lateral (x,y) and normal (z) dimensions of the simulation box to a weak coupling barostat (Berendsen et
695 al., 1984). Short-range Coulomb and Lennard-Jones 6,12 interactions were described with a buffered pair

696 list (Páll and Hess, 2013) with potentials smoothly shifted to zero at a 1.2 nm distance, with forces
697 switched to zero between 1.0 and 1.2 nm. The long-range electrostatic interactions were described with
698 the particle mesh Ewald (PME) method with 0.12 nm grid spacing (Darden et al., 1993). The LINCS and
699 SETTLE algorithms were used to constrain all protein bonds involving H-atoms and to keep the water
700 molecules rigid, respectively, allowing to integrate the equations of motion with 2 fs time steps using the
701 leap-frog integrator. The final production MD simulations were 500 ns long.

702 **Data analysis**

703 Data were analyzed in Excel 2019 (Microsoft) or Python 3.10.12 and mainly visualized using the Python
704 Matplotlib v3.6.2 (Hunter, 2007) and Seaborn v0.12.2 (Waskom, 2021) libraries. The plots obtained were
705 edited with Adobe Illustrator CS6 (Adobe Systems) to generate the final figures.

706 **Statistics and reproducibility**

707 All experiments were performed at least twice independently with similar results. No data were excluded
708 from the analyses. To quantify imaging data, multiple images were analyzed per condition. The analyses
709 included all cells in the images or, in the case of high cell densities, all cells in a square portion of the
710 images. The selection of the images and fields of cells analyzed was performed randomly.

711 **Availability of biological material**

712 The plasmids and strains used in this study are available from the corresponding author upon request.

713 **Data availability**

714 All data generated in this study are included in the manuscript and the supplementary material.

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Data availability

All relevant data generated in this study are included in the manuscript, the supplemental information, and the Source Data file.

Competing interests

The authors declare no competing interests.

Author contributions

Ying Liu: Conceptualization, methodology, validation, investigation, formal analysis, visualization, writing–original draft, writing–review and editing; constructed strains, performed cell biological and biochemical studies and conducted the bioinformatic analyses. **Rajani Karmakar**: investigation, formal analysis, visualization, writing–review and editing; conducted the MD simulations. **Maria Billini**: investigation, formal analysis, visualization, writing–review and editing; constructed strains and performed cell biological and biochemical studies. **Wieland Steinchen**: investigation, formal analysis, visualization, writing–review and editing; performed the HDX mass spectrometry analysis. **Saumyak Mukherjee**: supervision, formal analysis, writing–review and editing; contributed to the analysis of the MD simulations. **Rogelio Hernández-**

Tamayo: investigation, formal analysis, visualization, writing–review and editing; performed the single-particle tracking analysis. **Thomas Heimerl**: investigation, formal analysis, visualization, writing–review and editing; conducted the transmission electron microscopy analysis. **Gert Bange**: supervision, funding acquisition, writing–review and editing. **Lars V. Schäfer**: supervision, formal analysis, funding acquisition, writing–original draft, writing–review and editing; supervised the MD simulations and contributed to the analysis of the data obtained. **Martin Thanbichler**: conceptualization, supervision, formal analysis, visualization, funding acquisition, writing–original draft, writing–review and editing.

Figure legends

734 **Figure 1. Identification of residues critical for the membrane-binding activity of BacA *in vivo*. (A)** Sche-
735 matic representation of the BacA-mVenus fusion protein used in this study. The proposed membrane-
736 targeting sequence is highlighted in red. The sequence at the bottom shows that result of an amphipathic
737 helix prediction for BacA using the AMPHIPASEEK software (Sapay et al., 2006). Residues predicted to be
738 located in an unstructured, randomly coiled region are labeled with "c". **(B)** Localization patterns of
739 mutant BacA-mVenus variants. Δ*bacAB* cells producing BacA-mVenus or mutant variants thereof (strains
740 LY84, LY89, LY90, LY97, LY111, LY112, LY113, LY119) were analyzed by phase contrast and fluorescence
741 microscopy. The outlines of the cells are shown in the fluorescence images. Demographs summarizing the
742 single-cell fluorescence profiles obtained from random subpopulations of cells are given next to the
743 respective fluorescence images. The numbers of cells analyzed are: WT (130), Δ2-8 (292), F130R (156),
744 F2Y (138), F2E (194), K4S-K7S (151), K4E-K7E (382), F2E-K4E-K7E (130). The vertical red line indicates the
745 junction between cell body and the stalk. Scale bar: 2 µm. **(C)** Helical wheel diagram of the first eight
746 amino acids of BacA. Residues are colored by properties: hydrophobic (gray), basic (blue), uncharged
747 (yellow).

748 **Figure 1–figure supplement 1. Ultrastructure of different BacA variants.** Wild-type BacA, its MTS-free
749 Δ2-8 variant and its polymerization-deficient F130R variant (xx mg/ml) were stained with uranyl acetate
750 and visualized by transmission electron microscopy. Bar: 100 nm.

751 **Figure 1–figure supplement 2. Size-exclusion chromatography analysis of wild-type BacA and its F130R**
752 **variant.** The indicated proteins were applied to a Superdex 200 size-exclusion column and detected photo-
753 metrically at a wavelength of 280 nm. The following standard proteins were analyzed as a reference to
754 calibrate the column: Thyroglobulin (669 kDa), Ferritin (440 kDa), Aldolase (158 kDa), Conalbumin (75 kDa),
755 Ovalbumin (44 kDa), Ribonuclease A (14 kDa).

756 **Figure 1–figure supplement 3. Stability of different BacA-mVenus variants.** Derivates of strain JK5
757 (Δ*bacAB*) carrying the indicated alleles of *bacA-mVenus* under the control of the xylose-inducible P_{xyl}
758 promoter were grown overnight, diluted to an OD_{600} of ~ 0.1 and incubated for another hour. The strains
759 were then induced with 0.03% xylose for 1 h and subjected to immunoblot analysis with an anti-GFP
760 antibody. Strain JK5 was analyzed as a negative control (NC). The positions of standard proteins (in kDa)
761 are indicated on the left side of the images.

762 **Figure 1–figure supplement 4. Localization patterns of different BacA-mVenus variants.** Δ*bacAB* cells
763 producing the indicated BacA-mVenus variants (strains LY95, LY88, LY96, LY91, LY92) were analyzed by
764 phase contrast and fluorescence microscopy. The outlines of the cells are shown in the fluorescence
765 images. Demographs summarizing the single-cell fluorescence profiles obtained from random subpopula-
766 tions of cells are given next to the respective fluorescence images. The numbers of cells analyzed are: S3A
767 (109), K4S (150), Q5A (121), A6S (184), K7S (128). The vertical red line indicates the junction between cell
768 body and the stalk. Scale bar: 2 µm.

769 **Figure 2. Verification of residues M1, F2 and K4/K7 as critical components of the BacA MTS. (A)** Mobility
770 of the indicated BacA-mVenus fusion proteins. Shown is the average mean-squared displacement (MSD)
771 (± SD) as a function of time, based on single-particle tracking analysis. The fitted lines were obtained by
772 linear regression analysis. **(B)** Cell fractionation experiment investigating the membrane-binding activity
773 of BacA-mVenus (WT) or a mutant variant lacking the predicted MTS (Δ2-8). Whole-cell lysates (W) as well
774 as the soluble (S) and pellet (P) fractions of cells producing the indicated proteins were subjected to
775 immunoblot analysis with an anti-GFP antibody, detecting the BacA-mVenus fusion protein. As controls,
776 the same samples were probed with antibodies raised against the soluble cell division regulator MipZ
777 (Thanbichler and Shapiro, 2006) or the membrane-bound flagellar L-ring subunit FlgH (Mohr et al., 1996)
778 from *C. crescentus*. **(C)** As in panel B, but for cells producing mutant BacA-mVenus variants with single or
779 multiple amino-acid exchanges in the predicted MTS. Shown are representative images (n=3 independent
780 replicates). The strains used are given in the legend to Figure 1B.

781 **Figure 2–figure supplement 1. Subcellular localization of the single-molecule tracks obtained for differ-**
782 **ent BacA-mVenus variants.** The single-particle tracks determined for wild-type BacA-mVenus, free
783 mVenus and the indicated BacA-mVenus variants were mapped onto phase contrast images of the cells
784 in which they were recorded. The images show the results obtained from representative cells. Red lines
785 indicate slow-moving particles, green lines indicate fast-moving particles (as defined in **Supplementary**
786 **file 1**). Bar: 1 µm.

787 **Figure 2–video 1. Single-particle dynamics of different BacA-mVenus variants.** The movies show the
788 single-particle dynamics of wild-type BacA-mVenus, free mVenus and the indicated BacA-mVenus variants
789 in representative cells. The outlines of the cells are indicated in white. Red lines show the tracks of indivi-
790 dual particles. Images were acquired at 20-ms intervals. Bar: 1 µm.

791 **Figure 3. Co-sedimentation analysis of the association of various BacA variants with liposomes.** The
792 indicated proteins (20 µM) were incubated without (-) or with (+) liposomes (0.4 mg/mL) prior to ultra-
793 centrifugation. The supernatant and pellet fractions of each mixture were analyzed by SDS gel electro-
794 phoresis. Shown are scans of representative gels and a quantification of the average relative signal
795 intensities (± SD) obtained for the different fractions (n=3 independent replicates).

796 **Figure 4. Interplay between BacA assembly and membrane binding. (A)** Cell fractionation experiment
797 investigating the membrane-binding activity of the polymerization-deficient F130R variant of BacA-
798 mVenus *in vivo* (LY119). The analysis was performed as described for **Figure 2B**. **(B)** Co-sedimentation
799 analysis of the association of BacA-F130R with liposomes *in vitro*, performed as described for **Figure 3**. **(C)**
800 Role of polymerization in the membrane association of a BacA-mVenus variant carrying the membrane-
801 targeting sequence of *E. coli* MreB. Shown are phase contrast and fluorescence images of Δ*bacAB* mutants
802 (LY103, LY123) producing either a BacA-mVenus variant in which the MTS is replaced by two tandem
803 copies of the N-terminal amphiphilic helix of *E. coli* MreB (^{MreB}WT) or a polymerization-deficient variant
804 thereof (^{MreB}F130R). Demographs summarizing the single-cell fluorescence profiles obtained from random
805 subpopulations of cells are given next to the respective fluorescence images. The numbers of cells anal-
806 yzed are: ^{MreB}WT (126), ^{MreB}F130R (169). The vertical red line indicates the junction between cell body and
807 the stalk. Scale bar: 2 µm. **(D)** Mobility of the indicated BacA-mVenus fusion proteins. Shown is the average
808 mean-squared displacement (MSD) (± SD) as a function of time, based on single-particle tracking analysis.
809 The mVenus, Δ2-8 and WT data are taken from **Figure 2A** and shown for comparison. **(E)** Cell fractionation
810 experiment investigating the membrane-binding activity of ^{MreB}BacA-mVenus (^{MreB}WT) and its polymeriza-
811 tion-deficient F130R variant (^{MreB}F130R) *in vivo* (LY103, LY123). The analysis was performed as described
812 for **Figure 2B.**

813 **Figure 4–figure supplement 1. Solubility of different CreS-mNeonGreen variants. (A)** Schematics show-
814 ing the CreS-mNeonGreen variants analyzed in this study. The membrane-targeting regions of CreS and
815 BacA are shown in blue and red, respectively. **(B)** Cell fractionation experiment investigating the mem-
816 brane-binding activity of CreS-mNeonGreen (WT) or mutant variants lacking the predicted membrane-
817 targeting region (Δ27) or containing the BacA MTS instead of the native membrane-targeting region
818 (^{BacA}CreS). Whole-cell lysates (W) as well as the soluble (S) and pellet (P) fractions of cells producing the
819 indicated proteins were subjected to immunoblot analysis with an anti-mNeonGreen antibody. As
820 controls, the same samples were probed with antibodies raised against the soluble cell division regulator
821 MipZ (Thanbichler and Shapiro, 2006) or the membrane-bound flagellar L-ring subunit FlgH (Mohr et al.,

822 1996) from *C. crescentus*. Shown are representative images (n=3 independent replicates). **(C)** Quantifica-
823 tion of the relative amounts of CreS-mNeonGreen or its mutant variants in the soluble and pellet fractions.
824 The intensities of the signals obtained for the soluble and pellet fractions in the analysis described in panel
825 B were quantified and normalized to the total intensity in these two fractions. Data represent the average
826 (± SD) of three independent replicates.

827 **Figure 5. Molecular dynamics simulation of the interaction between the BacA MTS and a model mem-**
828 **brane. (A)** Snapshot of the molecular dynamics (MD) simulation system showing the 10-mer peptide
829 MFSKQAKSNN ($BacA_{1-10}$; red) after binding to the lipid bilayer. The water is shown in surface representa-
830 tion. K^+ and Cl^- counterions are not shown. **(B)** Close-up view of a representative snapshot from the MD
831 simulation visualizing the binding mode of the peptide on the membrane surface. **(C)** Structural overlay
832 of 40 snapshots from the MD simulation, taken after constant time intervals from the trajectory. **(D)**
833 Density profiles of individual residues in the wild-type peptide along the membrane normal, i.e. the z-
834 component of the distance vector from the center-of-mass (COM) of the bilayer, with the membrane
835 midplane located at zero. The vertical dashed black line indicates the maximum of the density distribution
836 of the lipid headgroup phosphates.

837 **Figure 5–figure supplement 1. Density profiles for different MTS variants determined by molecular**
838 **dynamics simulation.** Density profiles of individual residues in the **(A)** wild-type, **(B)** F2Y and **(C)** K4S-K7S
839 peptides along the membrane normal. The vertical dashed black line indicates the maximum of the density
840 distribution of the lipid headgroup phosphates. The data shown in panel A are reproduced from **Figure**
841 **5D** for comparison.

842 **Figure 6. Contact numbers and interaction energies for different peptide-lipid bilayer interactions. (A)**
843 The graph shows the total number of contacts between individual residues in the wild-type, K4S-K7S and
844 F2Y peptides and the lipid bilayer as well as the number of contacts with PG lipids and GLY lipids. A contact
845 between a peptide residue and a lipid was defined to exist if any two non-hydrogen atoms of the residue
846 and a lipid molecule were within a distance of 0.5 nm to each other. Contacts were counted for each
847 frame of the MD trajectories and averaged. Multiple contacts between a peptide and a lipid molecule
848 were treated as a single contact, so that the number of contacts counted was either 1 or 0. The statistical
849 errors plotted were obtained from the difference between the two different sets of 500-ns simulations,
850 starting with peptides in an unfolded or α-helical conformation, respectively. **(B)** Energies of the inter-
851 actions between individual residues in the wild-type, K4S-K7S and F2Y peptides and the lipid bilayer. The

852 interaction energies plotted are the combined interaction energies of all Coulomb and van-der-Waals
853 interactions in the force field averaged over the simulation trajectories.

854 **Figure 7. Conservation of the N-terminal regions of bactofilin homologs in different bacterial phyla.** The
855 pie chart in the middle shows the relative distribution of the 14337 unique bactofilin homologs analyzed
856 among the indicated bacterial phyla. The sequence logos give the most widespread N-terminal motifs
857 obtained either by a global analysis of all 14337 bactofilin sequences (global consensus) or by an analysis
858 of subsets of these sequences from specific phyla.

859 **Figure 7–figure supplement 1. Assessment of the conservation of the bactofilin membrane-targeting**
860 **sequence. (A)** Analysis pipeline used to analyze the conservation of the N-terminal regions among bacter-
861 ial bactofilin homologs. **(B)** Relative abundance of annotated bactofilin homologs in the different domains
862 of life. **(C)** Abundance of bactofilins with predicted transmembrane helices in different bacterial lineages.
863 **(D)** Distribution of bactofilin homologs without predicted transmembrane helices among different bacter-
864 ial phyla. Small phyla contributing less than three percent of the total number of sequences have been
865 aggregated in the category 'Others'.

866 **Figure 7–figure supplement 2. Conserved N-terminal motifs in bactofilin homologs from different phyla.**
867 The sequence logos give the most frequent N-terminal motifs obtained either by a global analysis of all
868 14,337 bactofilin sequences (global consensus) or by an analysis of subsets of these sequences from
869 specific phyla. The number of bactofilin homologs that show N-terminal sequences corresponding to a
870 specific motif and the likelihood for the existence of this motif are indicated on the left side of each
871 sequence logo.

872 **Figure 8. Interaction of BacA with its client protein PbpC. (A)** Localization patterns of different PbpC
873 variants. Δ*bacB* Δ*pbpC* cells producing mVenus-PbpC (LY75) or mutant variants thereof lacking region C1
874 (LY76) or carrying an unstructured region from *C. crescentus* DipM in place of the unstructured region
875 connecting region C1 and the transmembrane helix (LY77) were analyzed by phase contrast and fluores-
876 cence microscopy. The outlines of the cells are shown in the fluorescence images. Demographs summariz-
877 ing the single-cell fluorescence profiles obtained from random subpopulations of cells are given next to
878 the respective fluorescence images. The numbers of cells analyzed are: LY75 (158), LY76 (253), LY77 (119).
879 Scale bar: 2 µm. **(B)** Biolayer interferometric analysis of the interaction between $PbpC_{1\text{-}13aa}$ and BacA. A
880 synthetic peptide comprising the first 13 amino acids of PbpC ($PbpC_{1\text{-}13}$) was immobilized on a biosensor
881 and probed with increasing concentrations of BacA. After the association step, the sensor was transferred

882 to a protein-free buffer to monitor the dissociation reaction. The graph shows a representative titration
883 series (n=3 independent replicates). **(C)** Comparison of the interaction of $PbpC_{aa1-13}$ with BacA and its poly-
884 merization deficient F130R variant, performed as described in panel B. **(D)** Mapping of the PbpC binding
885 site on BacA by hydrogen-deuterium exchange (HDX) mass spectrometry. The plots show the extent of
886 deuterium uptake by three representative peptides obtained after peptic digestion of BacA protein (2.5
887 µM) that had been incubated in the absence or presence of the $PbpC_{1-13}$ peptide (10 µM) for the indicated
888 time periods (see **Supplementary file 4** for the full set of peptides). **(E)** Mapping of the differences in
889 deuterium uptake observed at t=1000 s onto the solid-state NMR structure of BacA (Shi et al., 2015).

890 **Figure 8–figure supplement 1. Localization patterns of mVenus-PbpC and BacA-Venus in different strain**
891 **backgrounds. (A)** Localization of mVenus-PbpC in the Δ*bacB* Δ*pbpC* (LY75) and the Δ*bacA* Δ*pbpC* (LY72)
892 backgrounds. **(B)** Localization of BacA-Venus in the wild-type (MT256) and Δ*pbpC* (JK136) backgrounds.
893 Cells were analyzed by phase contrast and fluorescence microscopy. The outlines of the cells are shown
894 in the fluorescence images. Demographs summarizing the single-cell fluorescence profiles obtained from
895 random subpopulations of cells are given next to the respective fluorescence images. The numbers of cells
896 analyzed are: LY75 (158), LY72 (697), MT256 (264), JK136 (222). Scale bar: 1 µm.

897 **Figure 8–figure supplement 2. Sequence alignment of the cytoplasmic tail of PbpC homologs.** The
898 schematic at the top shows the structure of PbpC. Conserved domains are shown in different colors. The
899 cytoplasmic tail of PbpC comprises conserved Region C1 (aa 1-13), a proline-rich region (aa 14-62), con-
900 served Region C2 (aa 63-70), and a region rich in positively charged amino acids (aa 71-83), located
901 adjacent to the transmembrane helix. Abbreviations: TG: transglycosylase domain, TP: transpeptidase
902 domain. The NCBI identifiers of the proteins analyzed are given on the left.

903 **Figure 8–figure supplement 3. Stability of the mVenus-PbpC fusion proteins used in this study.** Δ*bacB*
904 Δ*pbpC* cells producing wild-type mVenus-PbpC (WT; LY75), an mVenus-PbpC variant lacking the conserved
905 region C1 (Δ2-13; LY76) or an mVenus-PbpC variant containing an unstructured region from *C. crescentus*
906 DipM in place of the unstructured region in between regions C1 and C2 (chimera; LY77) were grown over-
907 night, diluted to an OD_{600} of ~0.1 and incubated for another hour. Subsequently, the cells were induced
908 for 1.5 h with 0.3 % xylose and subjected to immunoblot analysis with an anti-GFP antibody. A Δ*bacAB*
909 mutant producing BacA-mVenus and the wild-type strain CB15N (NC) were analyzed as positive and
910 negative controls, respectively. The positions of standard proteins (in kDa) are indicated on the left of
911 image.

912 **Figure 8–figure supplement 4. Relevance of BacA binding for the localization and functionality of PbpC**
913 **under phosphate-limiting conditions. (A)** Phase contrast and fluorescence images of the *C. crescentus*
914 wild-type (WT), a Δ*bacB* Δ*pbpC* mutant (LY71) and a Δ*bacB* Δ*pbpC* mutant producing either mVenus-PbpC
915 (LY75) or an N-terminally truncated variant thereof lacking region C1 (Δ2-13; LY76) under the control of a
916 xylose-inducible promoter after 24 h of cultivation in phosphate-limited ($M2G^{-P}$) medium containing 0.3%
917 xylose. The demographs at the bottom show the fluorescence profiles of a representative subpopulation
918 of cells stacked on top of each other and sorted according to cell length. The numbers of cells analyzed
919 are: 428 (WT), 415 (LY71), 659 (LY75), 753 (LY76). Bar: 3 µm. **(B)** Quantification of stalk lengths in the
920 cultures described in panel A. Shown are bee swarm plots of the data. The red dot indicates the median,
921 the lines indicate the standard deviation. **(C)** Stability of the indicated mVenus-PbpC variants under phos-
922 phate starvation. The indicated strains were cultivated for 24 h in phosphate-limited medium in the
923 absence (uninduced) or presence (induced) of 0.3% xylose and subjected to immunoblot analysis with an
924 anti-GFP antibody. The positions of mVenus-PbpC and free mVenus are indicated on the right.

925 **Figure 8–figure supplement 5. Biolayer interferometry analysis of the interaction between $PbpC_{aa1-13}$**
926 **and BacA. (A)** Control showing the interaction of wild-type BacA (50 µM) with an unmodified biosensor.
927 **(B)** Affinity of BacA for the immobilized $PbpC_{1-13}$ peptide. The final wavelength shifts measured for the
928 different association curves in **Figure 8B** were plotted against the corresponding BacA concentrations.
929 Data represent the average (± SD) of three independent replicates.

930 **Figure 8–figure supplement 6. Mapping of the PbpC-binding site of BacA by hydrogen-deuterium-**
931 **exchange (HDX) analysis. (A)** Schematic showing the domain structure of BacA. **(B)** HDX analysis of BacA.
932 Purified BacA (2.5 µM) was incubated in deuterated buffer for the indicated time intervals either alone
933 (Apo) or in the presence of $PbpC_{1-13}$ peptide (10 µM). Shown is the degree of HDX along the primary
934 sequence of BacA in the indicated conditions. The color scale is given on the right. The schematic at the
935 top displays the predicted secondary structure of BacA. The black bars represent peptides of BacA that
936 were analyzed for HDX. Residue-specific HDX information was obtained from these overlapping peptides
937 by using the shortest peptide covering a given residue. Gaps indicate amino acid sequences not covered
938 by any peptide. **(C)** HDX difference map. Shown are the residue-specific differences in HDX between BacA
939 in the presence of $PbpC_{1-13}$ peptide and BacA alone that were obtained after the indicated incubation
940 times. The data are projected onto the primary sequence of BacA. The color code is given on the right.
941 Blue color denotes regions showing reduced HDX in the presence of $PbpC_{1-13}$.

942 **Figure 9. Contribution of PbpC to BacA membrane association.** *C. crescentus* Δ*bacAB* Δ*pbpC* cells
943 producing the indicated BacA-mVenus variants (WT, Δ2-8, F130R) under the control of a xylose-inducible
944 promoter and $PbpC_{1-132}$-mCherry under the control of a vanillate-inducible promoter (strains MAB575,
945 MAB576 and MAB577) were grown in the presence of xylose (left) or both xylose and vanillate (right) prior
946 to microscopic analysis. The images show representative fluorescence micrographs, with the cell outlines
947 indicated in white. Arrowheads indicate polar $PbpC_{1-132}$-mCherry foci. Demographs summarizing the
948 single-cell BacA-mVenus fluorescence profiles obtained from random subpopulations of cells are provided
949 next to the respective fluorescence images. The number of cells analyzed is shown in the top left-hand
950 corner of each graph. The schematics on top illustrate the protein constructs used for the analysis (not to
951 scale). Bar: 3 µm.

952 **Figure 9–figure supplement 1. Levels and stability the fluorescent fusion proteins used in colocalization**
953 **studies.** *C. crescentus* Δ*bacAB* Δ*pbpC* cells producing the indicated BacA-mVenus variants under the
954 control of a xylose-inducible promoter and $PbpC_{1-132}$-mCherry under the control of a vanillate-inducible
955 promoter (strains MAB575, MAB576 and MAB577) were grown in the presence of xylose (xyl) and/or
956 vanillate (van) and subjected to immunoblot analysis with anti-GFP and anti-mCherry antibodies. The cells
957 analyzed were from the same cultures as those in **Figure 9**. The positions of the fusion proteins and of the
958 corresponding free fluorescent proteins are shown on the right.

Supplementary file legends

959 **Supplementary file 1. Diffusion constants of different BacA-mVenus variants.** The table shows the num-
960 ber of cells and tracks analyzed in the single-particle tracking studies as well as the diffusion coefficients
961 obtained for each of the proteins investigated (Microsoft Word, DOCX file).

962 **Supplementary file 2. Composition of the lipid bilayer in the MD simulations.** The table shows the type,
963 charge, fatty acid composition, percentage share and number count of the lipids in each of the two leaflets
964 that constitute the lipid bilayer in the molecular dynamics (MD) simulations (Microsoft Word, DOCX file).

965 **Supplementary file 3. Representative snapshot from an MD simulation visualizing the interaction of the**
966 **wild-type BacA$_{1\text{-}10}$ peptide with a model lipid bilayer.** The file gives the structural coordinates of the
967 snapshot shown in **Figure 5B** (PDB file).

968 **Supplementary file 4. Detailed description of the HDX data.** The spreadsheets give a summary of the
969 conditions used for the HDX analysis and a full list of the peptides obtained in the different experiments
970 (Microsoft Excel, XLSX file).

971 **Supplementary file 5. Strains used in this study.** The table gives the genotypes, mode of construction and
972 source of all strains used in this study (Microsoft Word, DOCX file).

973 **Supplementary file 6. Plasmids used in this study.** The table provides descriptions of all the plasmids used
974 in this study, including details of their construction or source (Microsoft Word, DOCX file).

975 **Supplementary file 7. Oligonucleotides used in this study.** The table shows the sequences of all synthetic
976 oligonucleotides used in this study (Microsoft Word, DOCX file).

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Figures

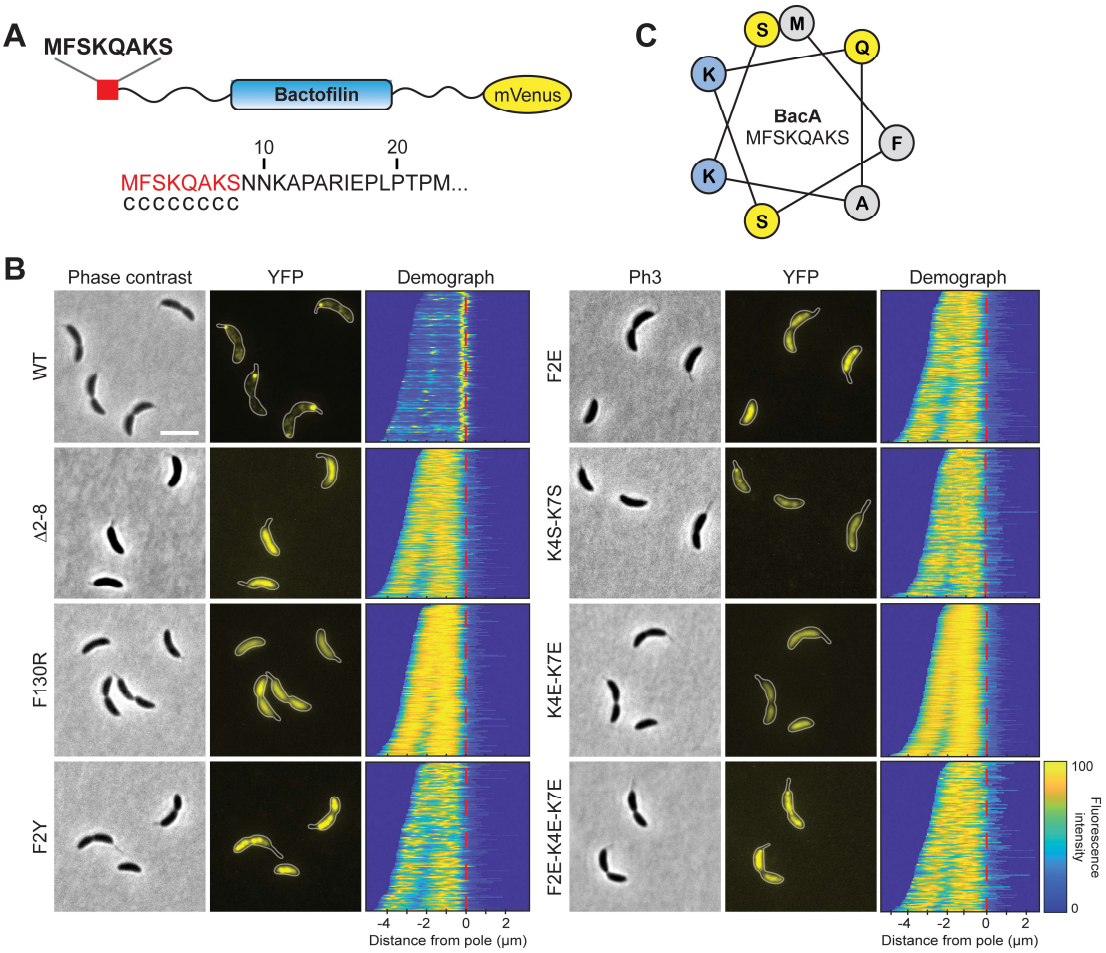
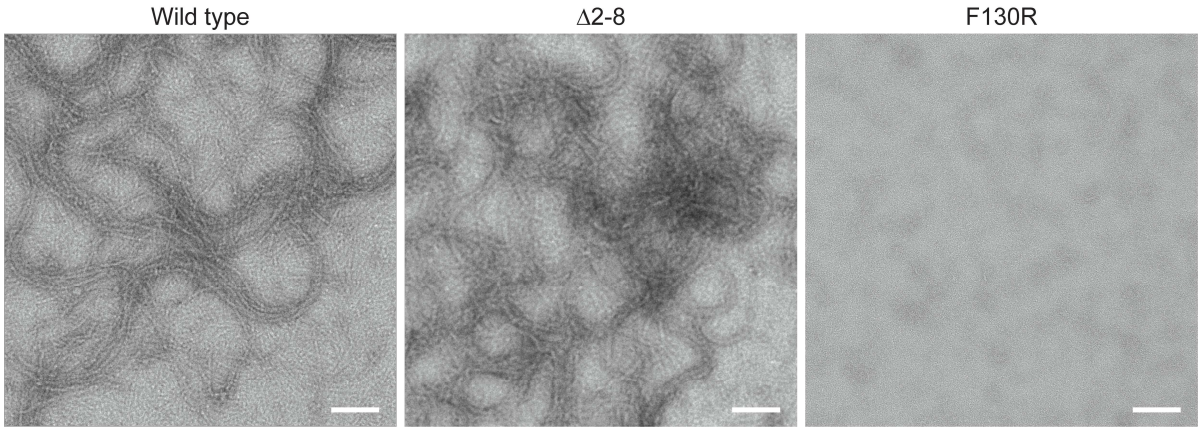
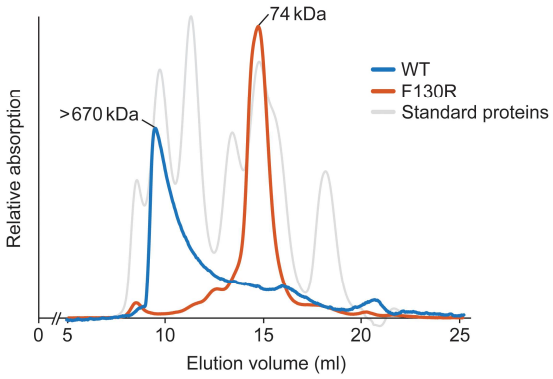


Figure 3. Identification of residues critical for the membrane-binding activity of BacA *in vivo*. (A) Schematic representation of the BacA-mVenus fusion protein used in this study. The proposed membrane-targeting sequence is highlighted in red. The sequence at the bottom shows that result of an amphipathic helix prediction for BacA using the AMPHIPASEEK software (Sapay et al., 2006). Residues predicted to be located in an unstructured, randomly coiled region are labeled with "c". **(B)** Localization patterns of mutant BacA-mVenus variants. Δ*bacAB* cells producing BacA-mVenus or mutant variants thereof (strains LY84, LY89, LY90, LY97, LY111, LY112, LY113, LY119) were analyzed by phase contrast and fluorescence microscopy. The outlines of the cells are shown in the fluorescence images. Demographs summarizing the single-cell fluorescence profiles obtained from random sub-populations of cells are given next to the respective fluorescence images. The numbers of cells analyzed are: WT (130), Δ2-8 (292), F130R (156), F2Y (138), F2E (194), K4S-K7S (151), K4E-K7E (382), F2E-K4E-K7E (130). The vertical red line indicates the junction between cell body and the stalk. Scale bar: 2 µm. **(C)** Helical wheel diagram of the first eight amino acids of BacA. Residues are colored by properties: hydrophobic (gray), basic (blue), uncharged (yellow).



1165 **Figure 1–figure supplement 1. Ultrastructure of different BacA variants.** Wild-type BacA, its MTS-free Δ2-8 variant and its
1166 polymerization-deficient F130R variant (xx mg/ml) were stained with uranyl acetate and visualized by transmission electron
1167 microscopy. Bar: 100 nm.



1168 **Figure 1–figure supplement 2. Size-exclusion chromatography analysis of wild-type BacA and its F130R variant.** The indicated
1169 proteins were applied to a Superdex 200 size-exclusion column and detected photometrically at a wavelength of 280 nm. The
1170 following standard proteins were analyzed as a reference to calibrate the column: Thyroglobulin (669 kDa), Ferritin (440 kDa),
1171 Aldolase (158 kDa), Conalbumin (75 kDa), Ovalbumin (44 kDa), Ribonuclease A (14 kDa).

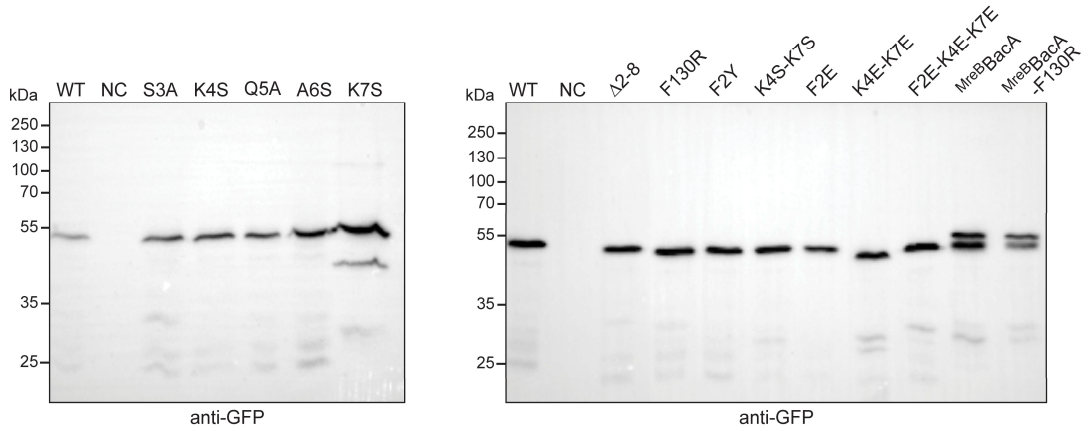


Figure 1–figure supplement 3. Stability of different BacA-mVenus variants. Derivates of strain JK5 (Δ*bacAB*) carrying the indicated alleles of *bacA-mVenus* under the control of the xylose-inducible P_{xyl} promoter were grown overnight, diluted to an OD_{600} of ~ 0.1 and incubated for another hour. The strains were then induced with 0.03% xylose for 1 h and subjected to immunoblot analysis with an anti-GFP antibody. Strain JK5 was analyzed as a negative control (NC). The positions of standard proteins (in kDa) are indicated on the left side of the images.

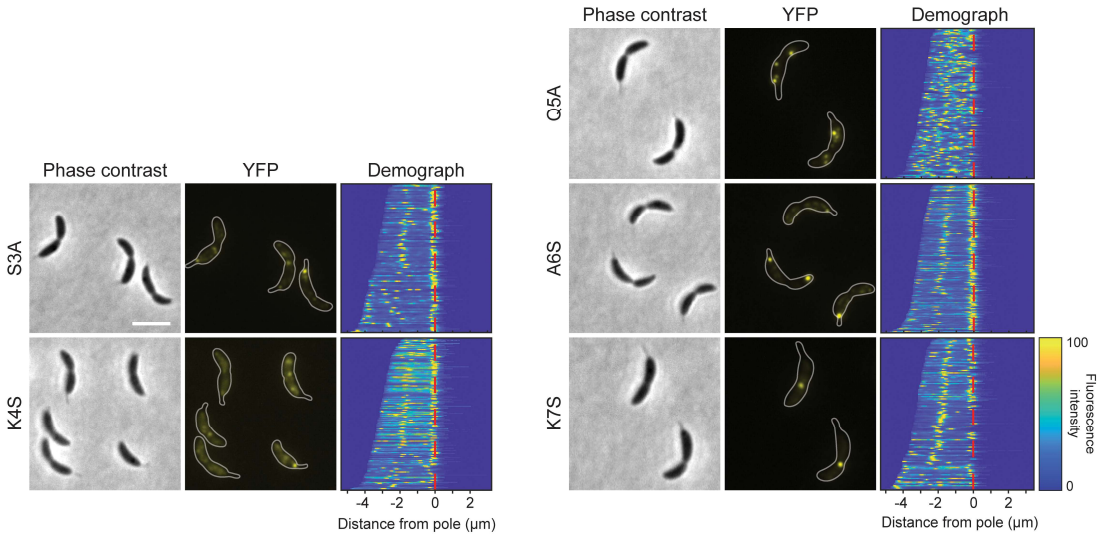


Figure 1–figure supplement 4. Localization patterns of different BacA-mVenus variants. Δ*bacAB* cells producing the indicated BacA-mVenus variants (strains LY95, LY88, LY96, LY91, LY92) were analyzed by phase contrast and fluorescence microscopy. The outlines of the cells are shown in the fluorescence images. Demographs summarizing the single-cell fluorescence profiles obtained from random subpopulations of cells are given next to the respective fluorescence images. The numbers of cells analyzed are: S3A (109), K4S (150), Q5A (121), A6S (184), K7S (128). The vertical red line indicates the junction between cell body and the stalk. Scale bar: 2 µm.

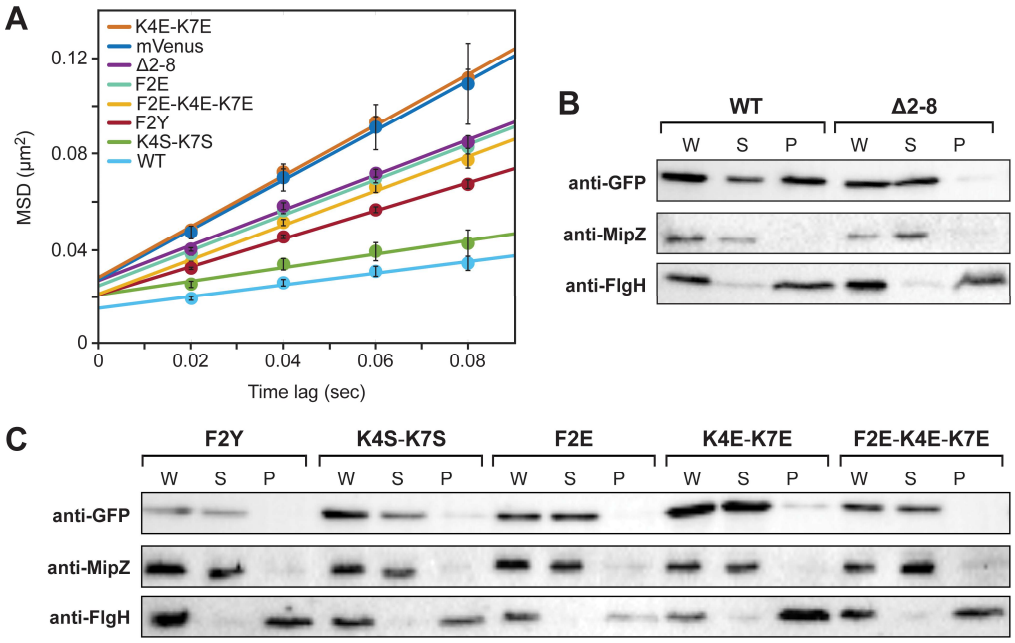
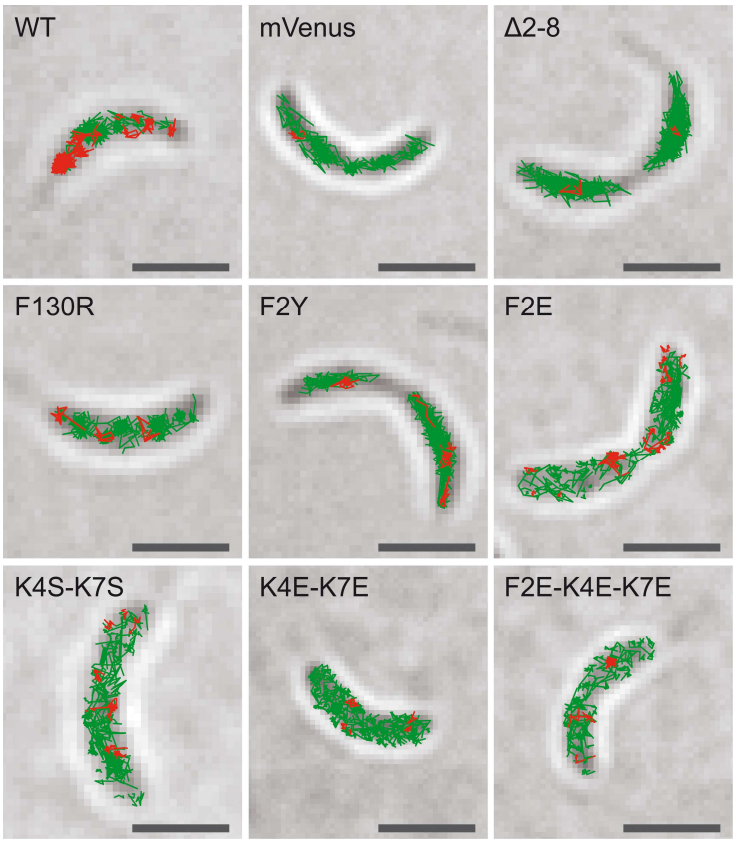
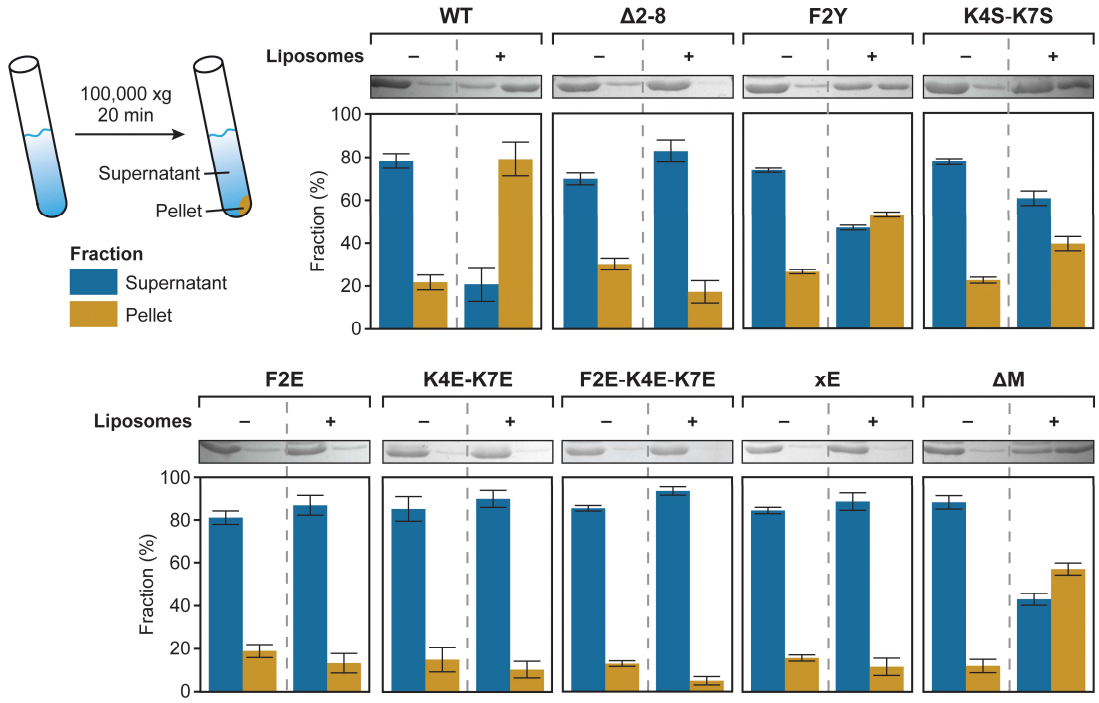


Figure 4. Verification of residues M1, F2 and K4/K7 as critical components of the BacA MTS. (A) Mobility of the indicated BacA-mVenus fusion proteins. Shown is the average mean-squared displacement (MSD) (± SD) as a function of time, based on single-particle tracking analysis. The fitted lines were obtained by linear regression analysis. **(B)** Cell fractionation experiment investigating the membrane-binding activity of BacA-mVenus (WT) or a mutant variant lacking the predicted MTS (Δ2-8). Whole-cell lysates (W) as well as the soluble (S) and pellet (P) fractions of cells producing the indicated proteins were subjected to immunoblot analysis with an anti-GFP antibody, detecting the BacA-mVenus fusion protein. As controls, the same samples were probed with antibodies raised against the soluble cell division regulator MipZ (Thanbichler and Shapiro, 2006) or the membrane-bound flagellar L-ring subunit FlgH (Mohr et al., 1996) from *C. crescentus*. **(C)** As in panel B, but for cells producing mutant BacA-mVenus variants with single or multiple amino-acid exchanges in the predicted MTS. Shown are representative images (n=3 independent replicates). The strains used are given in the legend to Figure 1B.



1193 **Figure 2–figure supplement 1. Subcellular localization of the single-molecule tracks obtained for different BacA-mVenus**
1194 **variants.** The single-particle tracks determined for wild-type BacA-mVenus, free mVenus and the indicated BacA-mVenus variants
1195 were mapped onto phase contrast images of the cells in which they were recorded. The images show the results obtained from
1196 representative cells. Red lines indicate slow-moving particles, green lines indicate fast-moving particles (as defined in **Supple-**
1197 **mentary file 1**). Bar: 1 µm.

1198 **Figure 2–video 1. Single-particle dynamics of different BacA-mVenus variants.** The movies show the single-particle dynamics of
1199 wild-type BacA-mVenus, free mVenus and the indicated BacA-mVenus variants in representative cells. The outlines of the cells
1200 are indicated in white. Red lines show the tracks of individual particles. Images were acquired at 20-ms intervals. Bar: 1 µm.



1201 **Figure 3. Co-sedimentation analysis of the association of various BacA variants with liposomes.** The indicated proteins (20 µM)
1202 were incubated without (-) or with (+) liposomes (0.4 mg/mL) prior to ultracentrifugation. The supernatant and pellet fractions
1203 of each mixture were analyzed by SDS gel electrophoresis. Shown are scans of representative gels and a quantification of the
1204 average relative signal intensities (± SD) obtained for the different fractions (n=3 independent replicates).

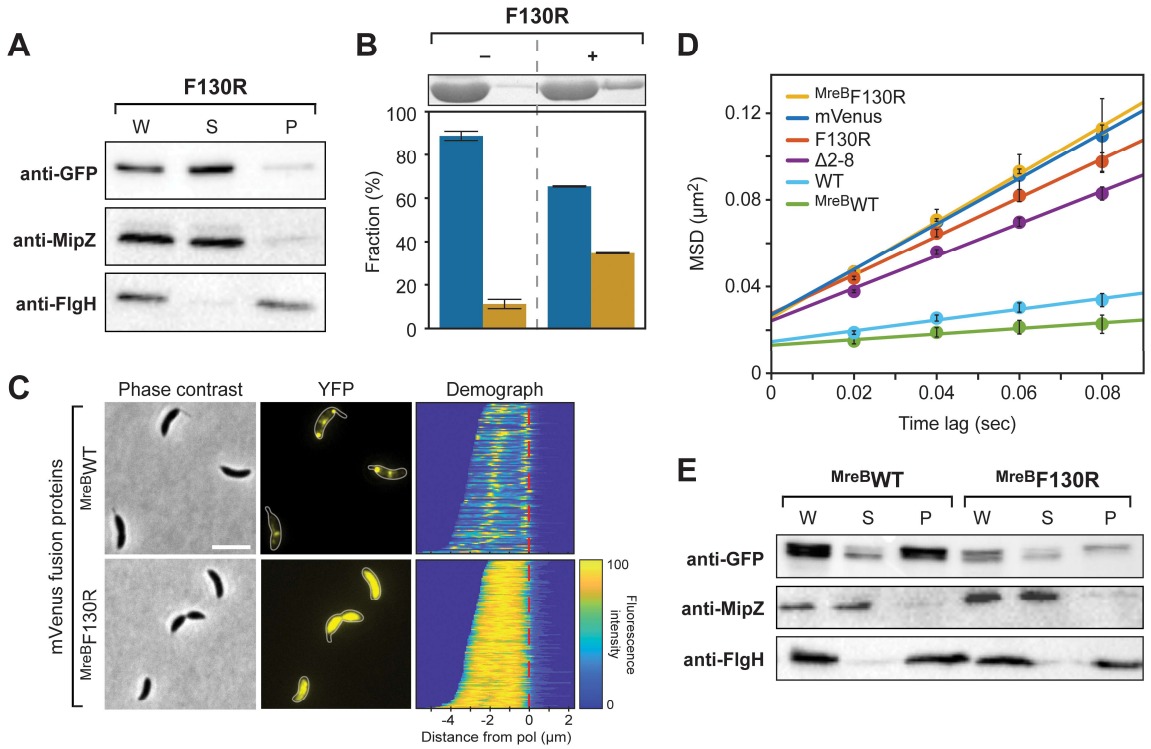
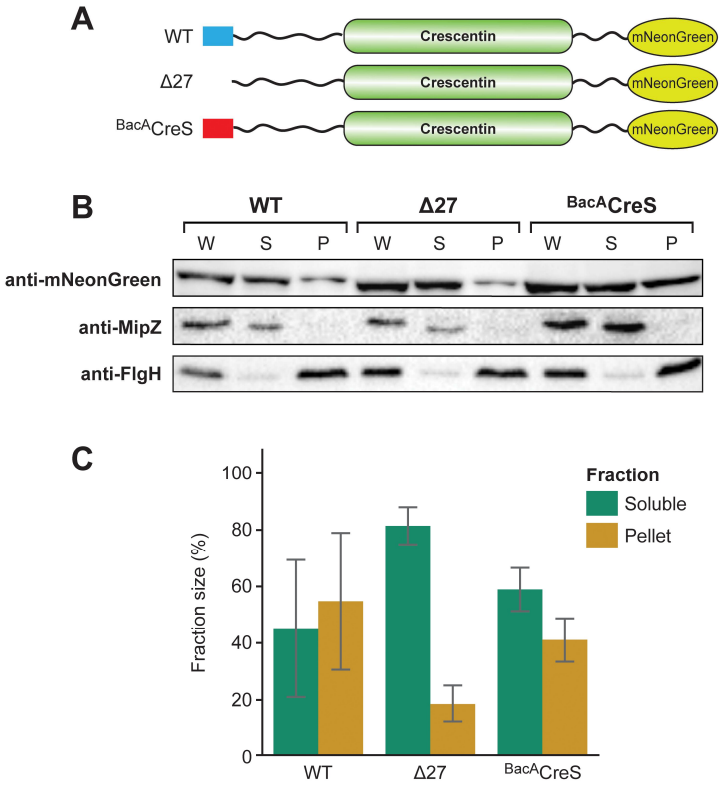


Figure 4. Interplay between BacA assembly and membrane binding. (A) Cell fractionation experiment investigating the membrane-binding activity of the polymerization-deficient F130R variant of BacA-mVenus *in vivo* (LY119). The analysis was performed as described for **Figure 2B**. **(B)** Co-sedimentation analysis of the association of BacA-F130R with liposomes *in vitro*, performed as described for **Figure 3**. **(C)** Role of polymerization in the membrane association of a BacA-mVenus variant carrying the membrane-targeting sequence of *E. coli* MreB. Shown are phase contrast and fluorescence images of Δ*bacAB* mutants (LY103, LY123) producing either a BacA-mVenus variant in which the MTS is replaced by two tandem copies of the N-terminal amphiphilic helix of *E. coli* MreB (^{MreB}WT) or a polymerization-deficient variant thereof (^{MreB}F130R). Demographs summarizing the single-cell fluorescence profiles obtained from random subpopulations of cells are given next to the respective fluorescence images. The numbers of cells analyzed are: ^{MreB}WT (126), ^{MreB}F130R (169). The vertical red line indicates the junction between cell body and the stalk. Scale bar: 2 µm. **(D)** Mobility of the indicated BacA-mVenus fusion proteins. Shown is the average mean-squared displacement (MSD) (± SD) as a function of time, based on single-particle tracking analysis. The mVenus, Δ2-8 and WT data are taken from **Figure 2A** and shown for comparison. **(E)** Cell fractionation experiment investigating the membrane-binding activity of ^{MreB}BacA-mVenus (^{MreB}WT) and its polymerization-deficient F130R variant (^{MreB}F130R) *in vivo* (LY103, LY123). The analysis was performed as described for **Figure 2B.**



1219 **Figure 4–figure supplement 1. Solubility of different CreS-mNeonGreen variants. (A)** Schematics showing the CreS-mNeonGreen
1220 variants analyzed in this study. The membrane-targeting regions of CreS and BacA are shown in blue and red, respectively. **(B)**
1221 Cell fractionation experiment investigating the membrane-binding activity of CreS-mNeonGreen (WT) or mutant variants lacking
1222 the predicted membrane-targeting region (Δ27) or containing the BacA MTS instead of the native membrane-targeting region
1223 (^{BacA}CreS). Whole-cell lysates (W) as well as the soluble (S) and pellet (P) fractions of cells producing the indicated proteins were
1224 subjected to immunoblot analysis with an anti-mNeonGreen antibody. As controls, the same samples were probed with anti-
1225 bodies raised against the soluble cell division regulator MipZ (Thanbichler and Shapiro, 2006) or the membrane-bound flagellar
1226 L-ring subunit FlgH (Mohr et al., 1996) from *C. crescentus*. Shown are representative images (n=3 independent replicates). **(C)**
1227 Quantification of the relative amounts of CreS-mNeonGreen or its mutant variants in the soluble and pellet fractions. The
1228 intensities of the signals obtained for the soluble and pellet fractions in the analysis described in panel B were quantified and
1229 normalized to the total intensity in these two fractions. Data represent the average (± SD) of three independent replicates.

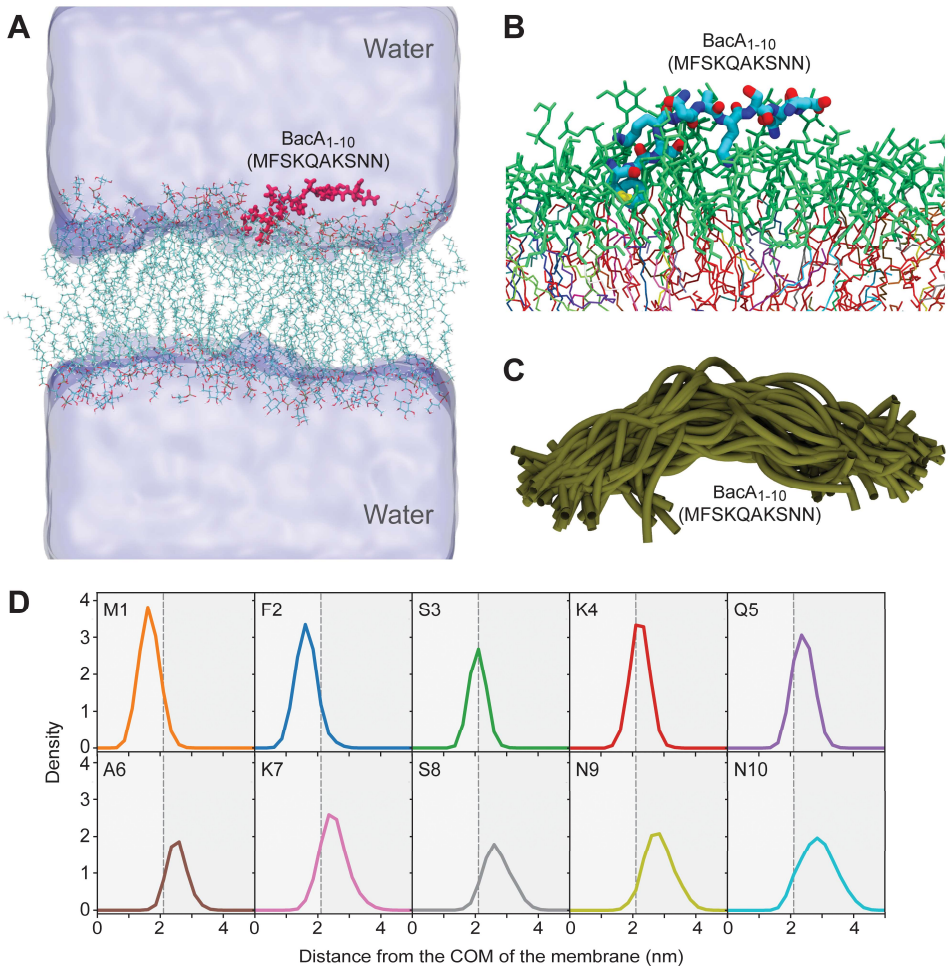


Figure 5. Molecular dynamics simulation of the interaction between the BacA MTS and a model membrane. (A) Snapshot of the molecular dynamics (MD) simulation system showing the 10-mer peptide MFSKQAKSNN ($BacA_{1-10}$; red) after binding to the lipid bilayer. The water is shown in surface representation. K^+ and Cl^- counterions are not shown. **(B)** Close-up view of a representative snapshot from the MD simulation visualizing the binding mode of the peptide on the membrane surface. **(C)** Structural overlay of 40 snapshots from the MD simulation, taken after constant time intervals from the trajectory. **(D)** Density profiles of individual residues in the wild-type peptide along the membrane normal, i.e. the z-component of the distance vector from the center-of-mass (COM) of the bilayer, with the membrane midplane located at zero. The vertical dashed black line indicates the maximum of the density distribution of the lipid headgroup phosphates.

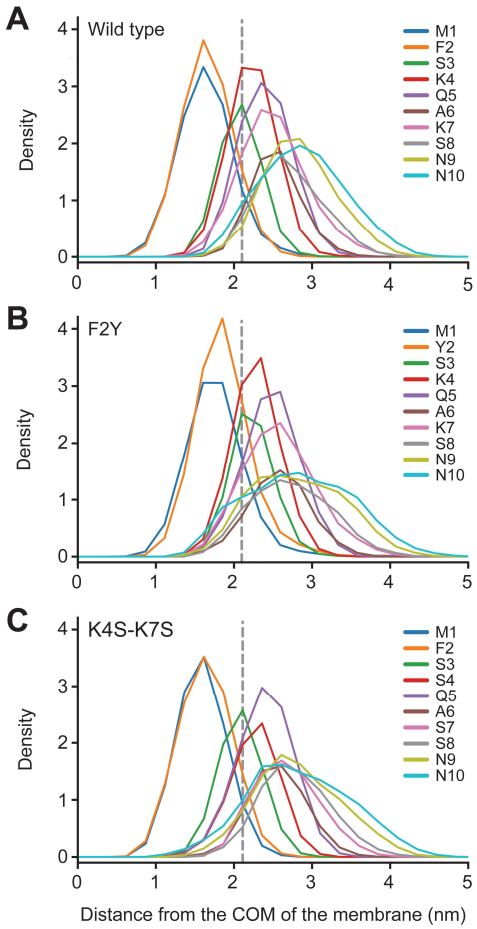
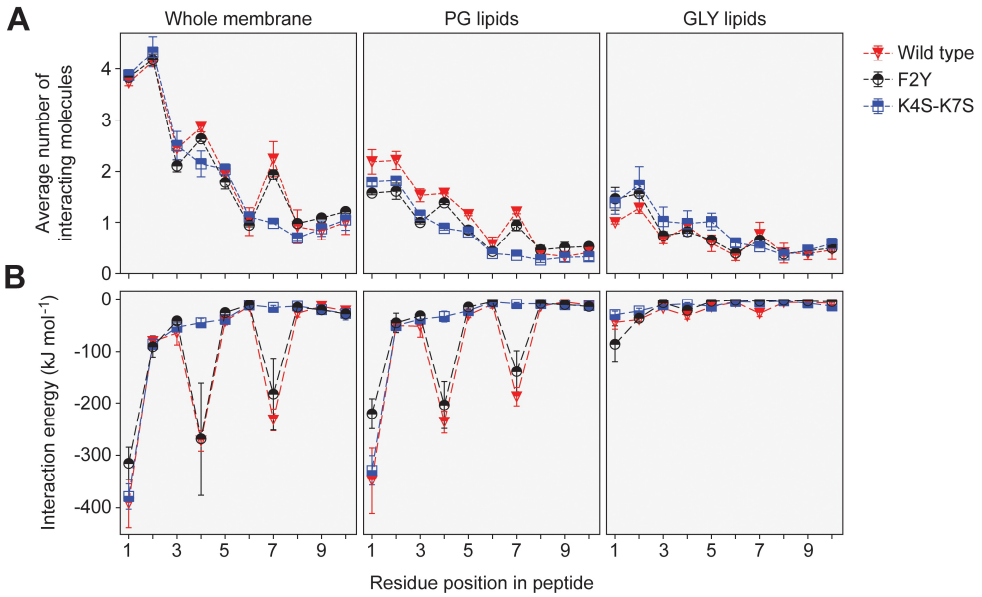


Figure 5–figure supplement 1. Density profiles for different MTS variants determined by molecular dynamics simulation. Density profiles of individual residues in the **(A)** wild-type, **(B)** F2Y and **(C)** K4S-K7S peptides along the membrane normal. The vertical dashed black line indicates the maximum of the density distribution of the lipid headgroup phosphates. The data shown in panel A are reproduced from **Figure 5D** for comparison.



1242 **Figure 6. Contact numbers and interaction energies for different peptide-lipid bilayer interactions. (A)** The graph shows the
1243 total number of contacts between individual residues in the wild-type, K4S-K7S and F2Y peptides and the lipid bilayer as well as
1244 the number of contacts with PG lipids and GLY lipids. A contact between a peptide residue and a lipid was defined to exist if any
1245 two non-hydrogen atoms of the residue and a lipid molecule were within a distance of 0.5 nm to each other. Contacts were
1246 counted for each frame of the MD trajectories and averaged. Multiple contacts between a peptide and a lipid molecule were
1247 treated as a single contact, so that the number of contacts counted was either 1 or 0. The statistical errors plotted were obtained
1248 from the difference between the two different sets of 500-ns simulations, starting with peptides in an unfolded or α-helical
1249 conformation, respectively. **(B)** Energies of the interactions between individual residues in the wild-type, K4S-K7S and F2Y
1250 peptides and the lipid bilayer. The interaction energies plotted are the combined interaction energies of all Coulomb and van-
1251 der-Waals interactions in the force field averaged over the simulation trajectories.

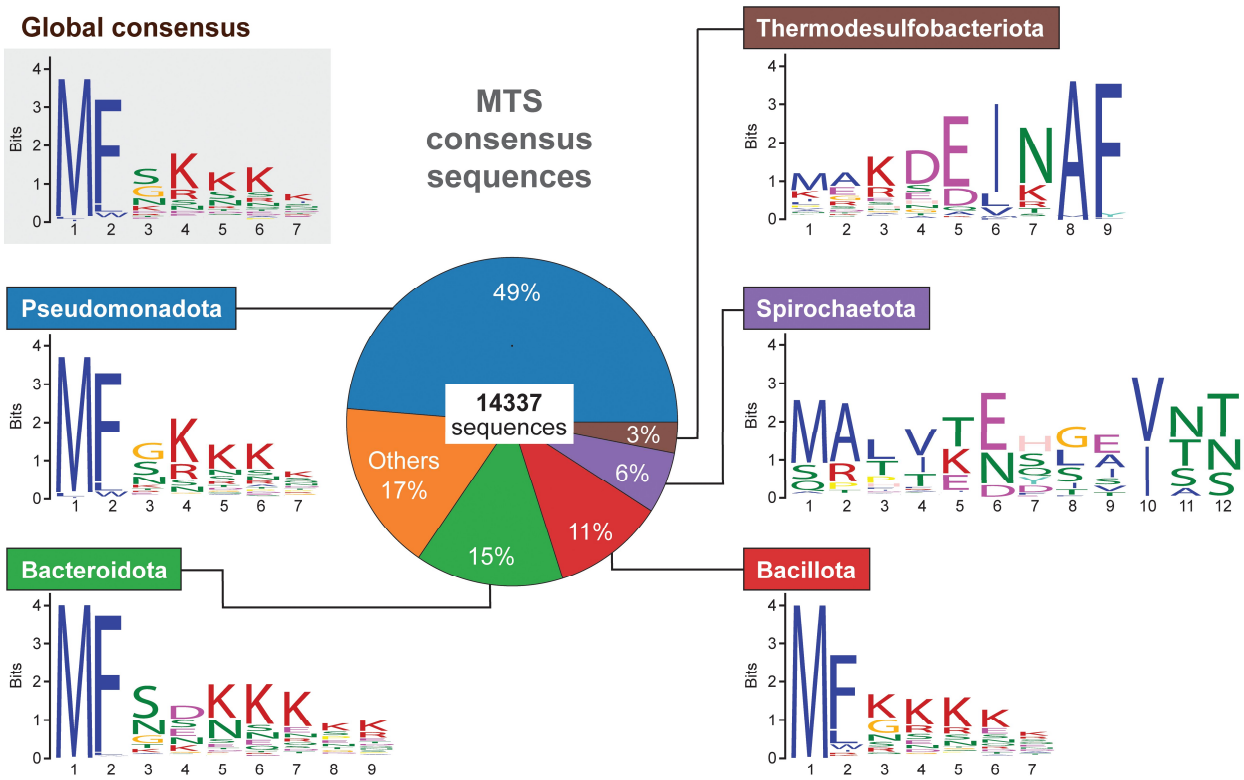


Figure 7. Conservation of the N-terminal regions of bactofilin homologs in different bacterial phyla. The pie chart in the middle shows the relative distribution of the 14337 unique bactofilin homologs analyzed among the indicated bacterial phyla. The sequence logos give the most widespread N-terminal motifs obtained either by a global analysis of all 14337 bactofilin sequences (global consensus) or by an analysis of subsets of these sequences from specific phyla.

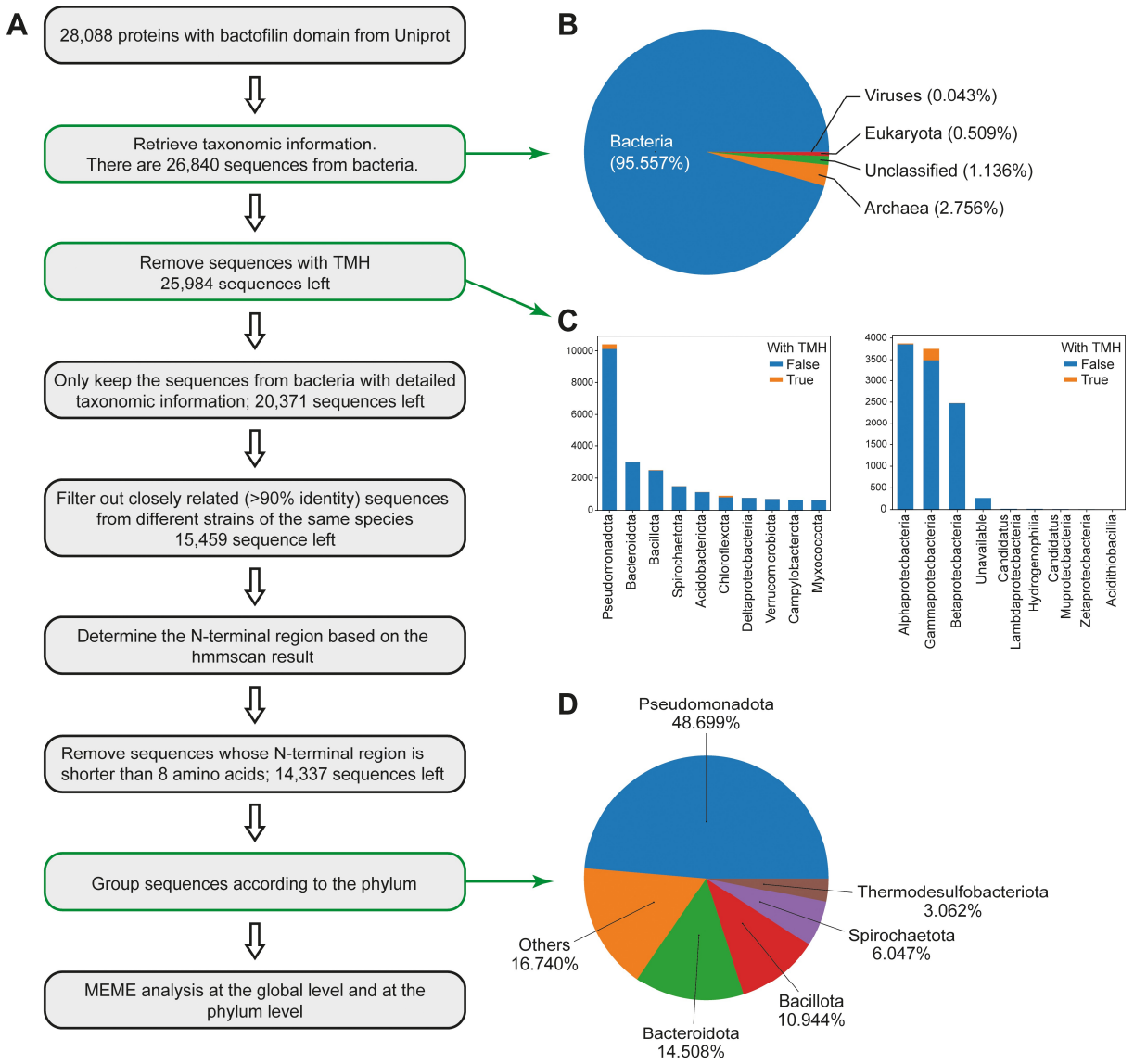


Figure 7–figure supplement 1. Assessment of the conservation of the bactofilin membrane-targeting sequence. (A) Analysis pipeline used to analyze the conservation of the N-terminal regions among bacterial bactofilin homologs. **(B)** Relative abundance of annotated bactofilin homologs in the different domains of life. **(C)** Abundance of bactofilins with predicted transmembrane helices in different bacterial lineages. **(D)** Distribution of bactofilin homologs without predicted transmembrane helices among different bacterial phyla. Small phyla contributing less than three percent of the total number of sequences have been aggregated in the category 'Others'.

Figure 7–figure supplement 2. Conserved N-terminal motifs in bactofilin homologs from different phyla. The sequence logos give the most frequent N-terminal motifs obtained either by a global analysis of all 14,337 bactofilin sequences (global consensus) or by an analysis of subsets of these sequences from specific phyla. The number of bactofilin homologs that show N-terminal sequences corresponding to a specific motif and the likelihood for the existence of this motif are indicated on the left side of each sequence logo.

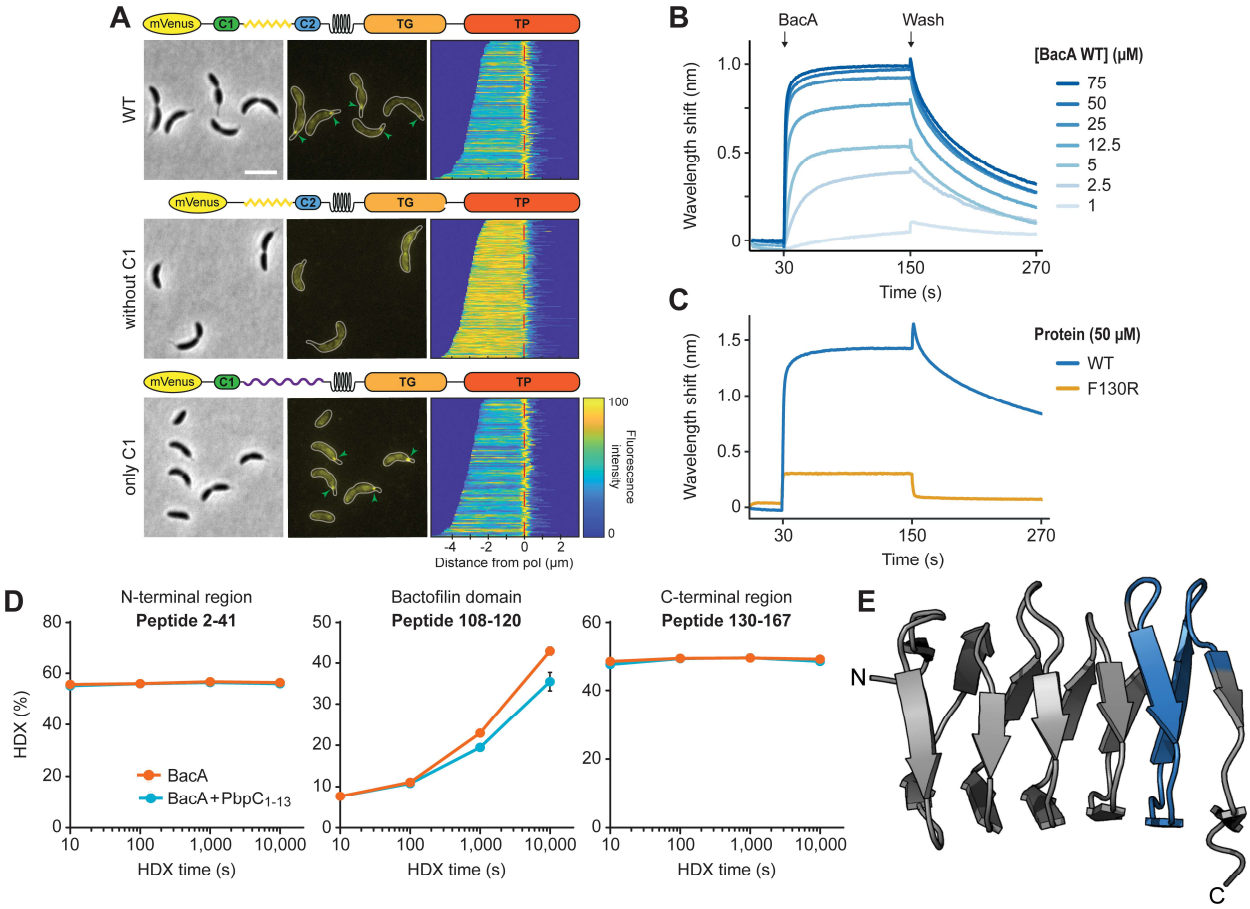
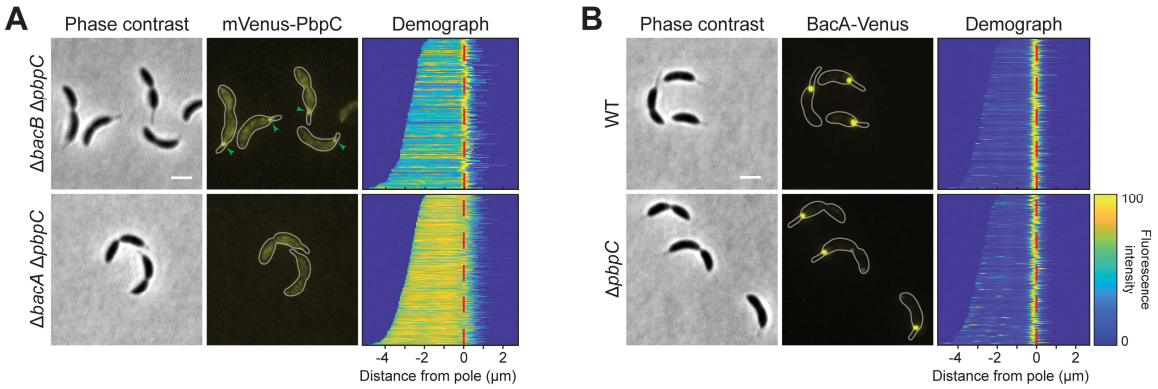


Figure 8. Interaction of BacA with its client protein PbpC. (A) Localization patterns of different PbpC variants. Δ*bacB* Δ*pbpC* cells producing mVenus-PbpC (LY75) or mutant variants thereof lacking region C1 (LY76) or carrying an unstructured region from *C. crescentus* DipM in place of the unstructured region connecting region C1 and the transmembrane helix (LY77) were analyzed by phase contrast and fluorescence microscopy. The outlines of the cells are shown in the fluorescence images. Demographs summarizing the single-cell fluorescence profiles obtained from random subpopulations of cells are given next to the respective fluorescence images. The numbers of cells analyzed are: LY75 (158), LY76 (253), LY77 (119). Scale bar: 2 µm. **(B)** Biolayer interferometric analysis of the interaction between $PbpC_{1-13aa}$ and BacA. A synthetic peptide comprising the first 13 amino acids of PbpC ($PbpC_{1-13}$) was immobilized on a biosensor and probed with increasing concentrations of BacA. After the association step, the sensor was transferred to a protein-free buffer to monitor the dissociation reaction. The graph shows a representative titration series (n=3 independent replicates). **(C)** Comparison of the interaction of $PbpC_{aa1-13}$ with BacA and its polymerization deficient F130R variant, performed as described in panel B. **(D)** Mapping of the PbpC binding site on BacA by hydrogen-deuterium exchange (HDX) mass spectrometry. The plots show the extent of deuterium uptake by three representative peptides obtained after peptic digestion of BacA protein (2.5 µM) that had been incubated in the absence or presence of the $PbpC_{1-13}$ peptide (10 µM) for the indicated time periods (see **Supplementary file 4** for the full set of peptides). **(E)** Mapping of the differences in deuterium uptake observed at t=1000 s onto the solid-state NMR structure of BacA (Shi et al., 2015).



1283 **Figure 8–figure supplement 1. Localization patterns of mVenus-PbpC and BacA-Venus in different strain backgrounds. (A)**
1284 Localization of mVenus-PbpC in the Δ*bacB* Δ*pbpC* (LY75) and the Δ*bacA* Δ*pbpC* (LY72) backgrounds. **(B)** Localization of BacA-Venus
1285 in the wild-type (MT256) and Δ*pbpC* (JK136) backgrounds. Cells were analyzed by phase contrast and fluorescence microscopy.
1286 The outlines of the cells are shown in the fluorescence images. Demographs summarizing the single-cell fluorescence profiles
1287 obtained from random subpopulations of cells are given next to the respective fluorescence images. The numbers of cells
1288 analyzed are: LY75 (158), LY72 (697), MT256 (264), JK136 (222). Scale bar: 1 μm.

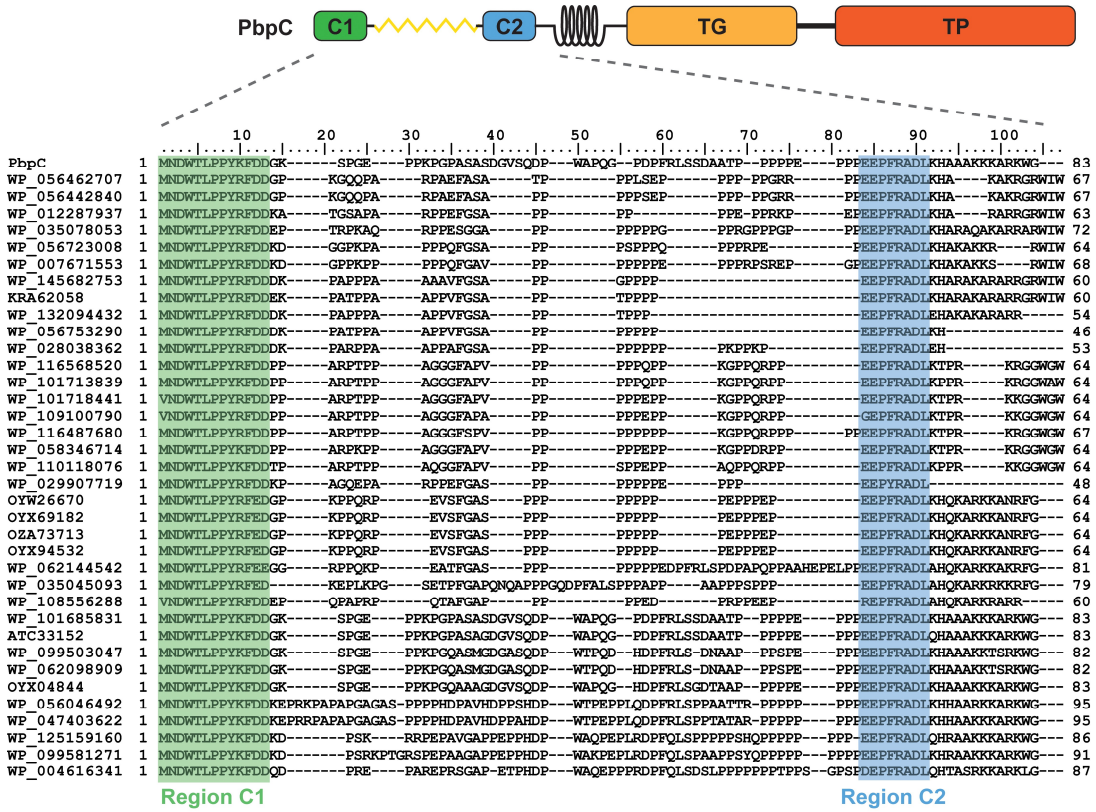
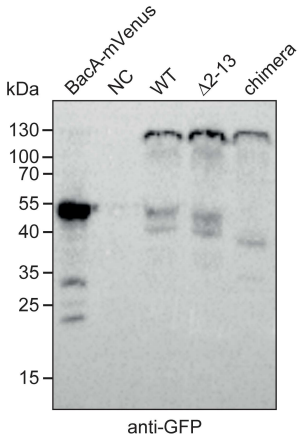


Figure 8–figure supplement 2. Sequence alignment of the cytoplasmic tail of PbpC homologs. The schematic at the top shows the structure of PbpC. Conserved domains are shown in different colors. The cytoplasmic tail of PbpC comprises conserved Region C1 (aa 1-13), a proline-rich region (aa 14-62), conserved Region C2 (aa 63-70), and a region rich in positively charged amino acids (aa 71-83), located adjacent to the transmembrane helix. Abbreviations: TG: transglycosylase domain, TP: transpeptidase domain. The NCBI identifiers of the proteins analyzed are given on the left.



1294 **Figure 8–figure supplement 3. Stability of the mVenus-PbpC fusion proteins used in this study.** Δ*bacB* Δ*pbpC* cells producing
1295 wild-type mVenus-PbpC (WT; LY75), an mVenus-PbpC variant lacking the conserved region C1 (Δ2-13; LY76) or an mVenus-PbpC
1296 variant containing an unstructured region from *C. crescentus* DipM in place of the unstructured region in between regions C1 and
1297 C2 (chimera; LY77) were grown overnight, diluted to an OD_{600} of ~0.1 and incubated for another hour. Subsequently, the cells
1298 were induced for 1.5 h with 0.3 % xylose and subjected to immunoblot analysis with an anti-GFP antibody. A Δ*bacAB* mutant
1299 producing BacA-mVenus and the wild-type strain CB15N (NC) were analyzed as positive and negative controls, respectively. The
1300 positions of standard proteins (in kDa) are indicated on the left of image.

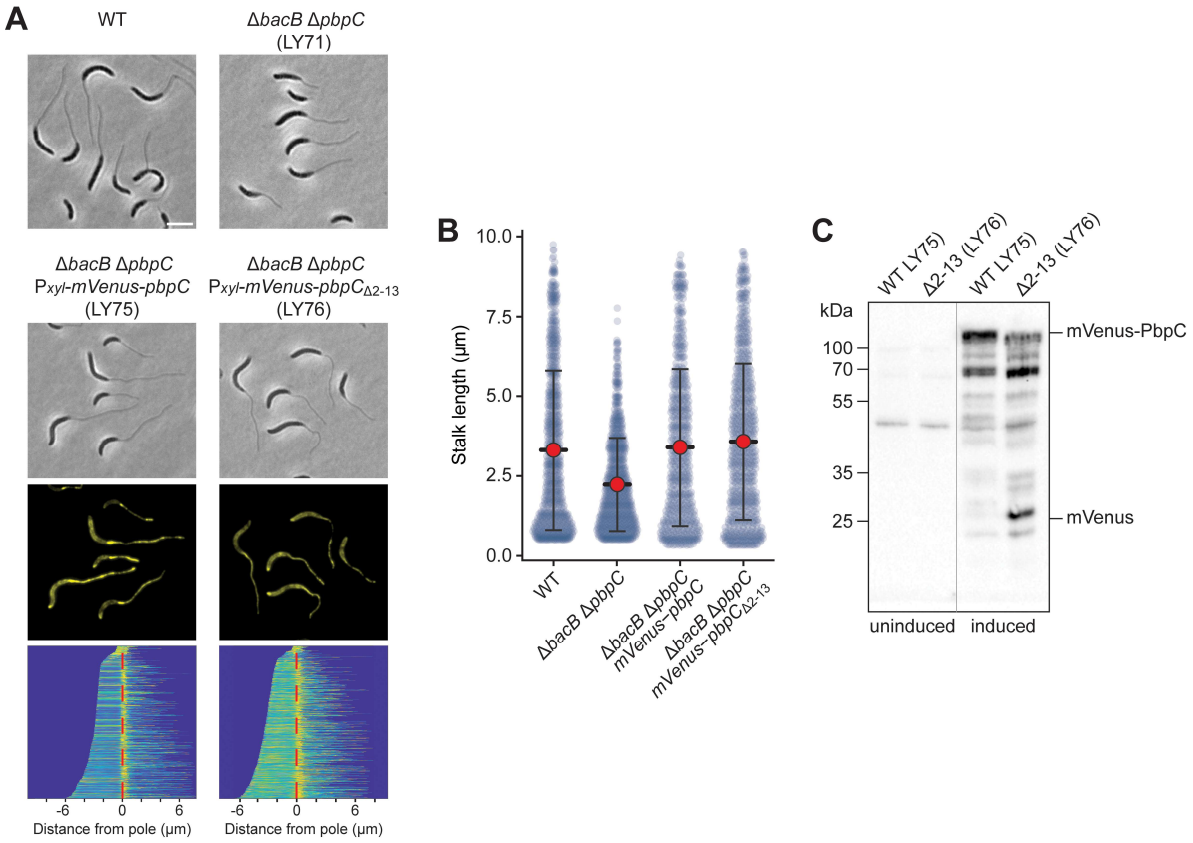
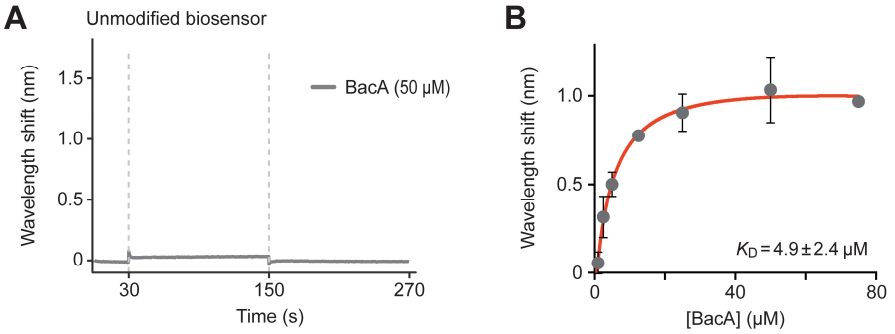
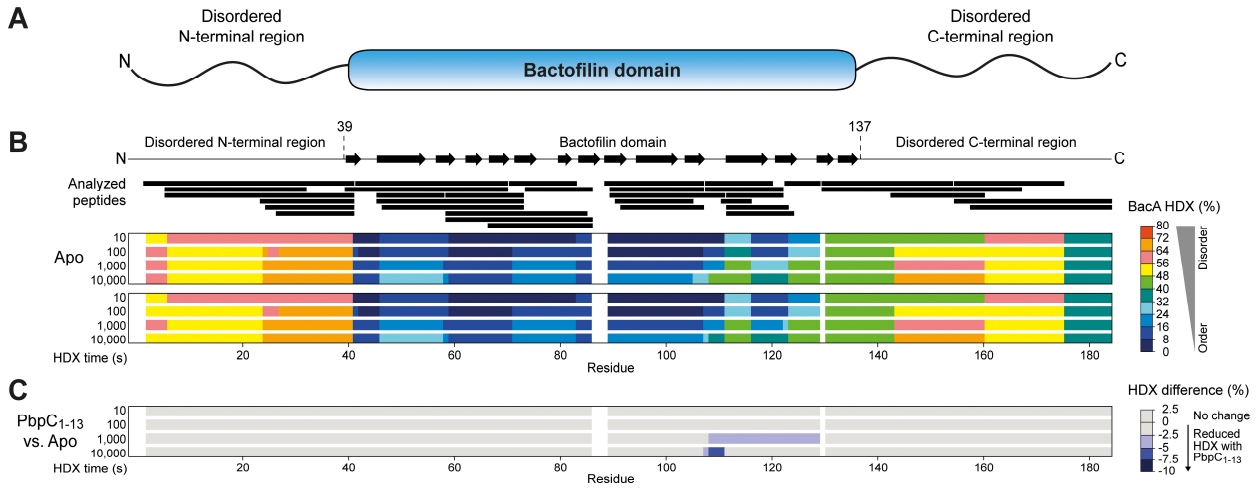


Figure 8–figure supplement 4. Relevance of BacA binding for the localization and functionality of PbpC under phosphate-limiting conditions. (A) Phase contrast and fluorescence images of the *C. crescentus* wild-type (WT), a Δ*bacB* Δ*pbpC* mutant (LY71) and a Δ*bacB* Δ*pbpC* mutant producing either mVenus-PbpC (LY75) or an N-terminally truncated variant thereof lacking region C1 (Δ2-13; LY76) under the control of a xylose-inducible promoter after 24 h of cultivation in phosphate-limited ($M2G^{-P}$) medium containing 0.3% xylose. The demographs at the bottom show the fluorescence profiles of a representative subpopulation of cells stacked on top of each other and sorted according to cell length. The numbers of cells analyzed are: 428 (WT), 415 (LY71), 659 (LY75), 753 (LY76). Bar: 3 µm. **(B)** Quantification of stalk lengths in the cultures described in panel A. Shown are bee swarm plots of the data. The red dot indicates the median, the lines indicate the standard deviation. **(C)** Stability of the indicated mVenus-PbpC variants under phosphate starvation. The indicated strains were cultivated for 24 h in phosphate-limited medium in the absence (uninduced) or presence (induced) of 0.3% xylose and subjected to immunoblot analysis with an anti-GFP antibody. The positions of mVenus-PbpC and free mVenus are indicated on the right.



1312 **Figure 8–figure supplement 5. Biolayer interferometry analysis of the interaction between PbpC$_{aa1-13}$ and BacA. (A)** Control
1313 showing the interaction of wild-type BacA (50 µM) with an unmodified biosensor. **(B)** Affinity of BacA for the immobilized PbpC$_{1-}$
1314 $_{13}$ peptide. The final wavelength shifts measured for the different association curves in **Figure 8B** were plotted against the cor-
1315 responding BacA concentrations. Data represent the average (± SD) of three independent replicates.



1316 **Figure 8–figure supplement 6. Mapping of the PbpC-binding site of BacA by hydrogen-deuterium-exchange (HDX) analysis. (A)**
1317 Schematic showing the domain structure of BacA. **(B)** HDX analysis of BacA. Purified BacA (2.5 µM) was incubated in deuterated
1318 buffer for the indicated time intervals either alone (Apo) or in the presence of PbpC$_{1-13}$ peptide (10 µM). Shown is the degree of
1319 HDX along the primary sequence of BacA in the indicated conditions. The color scale is given on the right. The schematic at the
1320 top displays the predicted secondary structure of BacA. The black bars represent peptides of BacA that were analyzed for HDX.
1321 Residue-specific HDX information was obtained from these overlapping peptides by using the shortest peptide covering a given
1322 residue. Gaps indicate amino acid sequences not covered by any peptide. **(C)** HDX difference map. Shown are the residue-specific
1323 differences in HDX between BacA in the presence of PbpC$_{1-13}$ peptide and BacA alone that were obtained after the indicated
1324 incubation times. The data are projected onto the primary sequence of BacA. The color code is given on the right. Blue color
1325 denotes regions showing reduced HDX in the presence of PbpC$_{1-13}$.

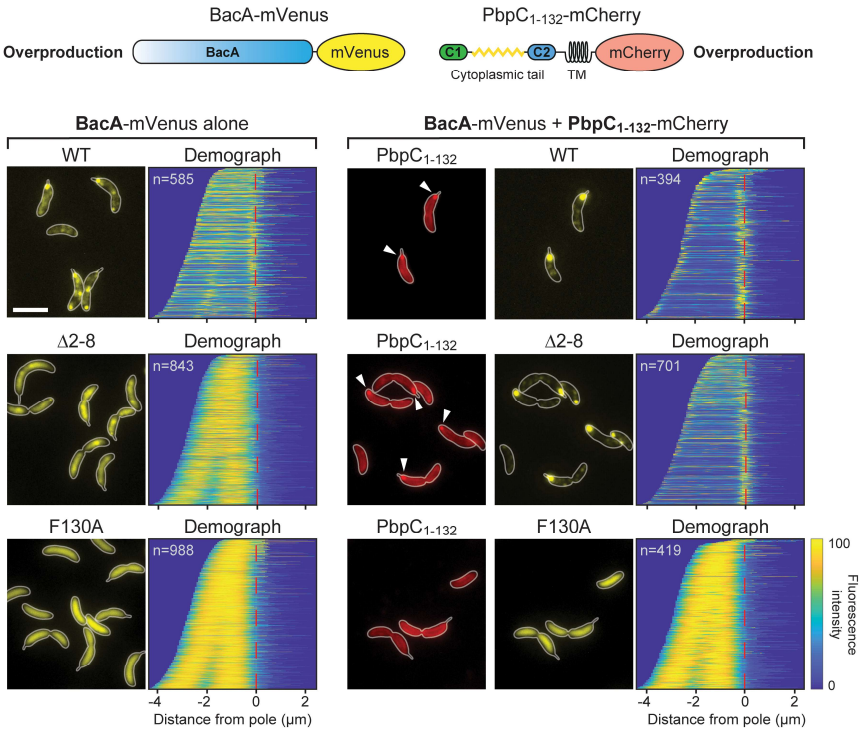


Figure 9. Contribution of PbpC to BacA membrane association. *C. crescentus* Δ*bacAB* Δ*pbpC* cells producing the indicated BacA-mVenus variants (WT, Δ2-8, F130R) under the control of a xylose-inducible promoter and $PbpC_{1-132}$-mCherry under the control of a vanillate-inducible promoter (strains MAB575, MAB576 and MAB577) were grown in the presence of xylose (left) or both xylose and vanillate (right) prior to microscopic analysis. The images show representative fluorescence micrographs, with the cell outlines indicated in white. Arrowheads indicate polar $PbpC_{1-132}$-mCherry foci. Demographs summarizing the single-cell BacA-mVenus fluorescence profiles obtained from random subpopulations of cells are provided next to the respective fluorescence images. The number of cells analyzed is shown in the top left-hand corner of each graph. The schematics on top illustrate the protein constructs used for the analysis (not to scale). Bar: 3 µm.

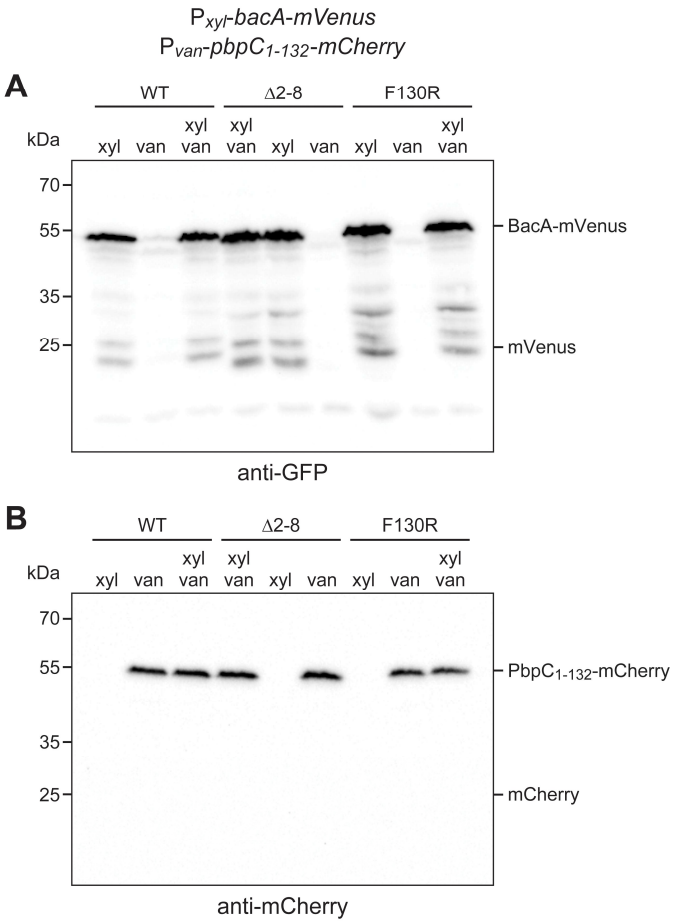


Figure 9–figure supplement 1. Levels and stability the fluorescent fusion proteins used in colocalization studies. *C. crescentus* Δ*bacAB* Δ*pbpC* cells producing the indicated BacA-mVenus variants under the control of a xylose-inducible promoter and $PbpC_{1-132}$-mCherry under the control of a vanillate-inducible promoter (strains MAB575, MAB576 and MAB577) were grown in the presence of xylose (xyl) and/or vanillate (van) and subjected to immunoblot analysis with anti-GFP and anti-mCherry antibodies. The cells analyzed were from the same cultures as those in **Figure 9**. The positions of the fusion proteins and of the corresponding free fluorescent proteins are shown on the right.