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A repurposed, non-canonical cytochrome c, chaperones calcium binding by PilY1 for type IVa pili formation

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33

34 **Abstract**

35 Type IVa pili (T4aP) are versatile bacterial cell surface structures that undergo
36 extension/adhesion/retraction cycles powered by the cell envelope-spanning T4aP machine. In
37 this machine, a complex composed of four minor pilins and PilY1 primes T4aP extension and is
38 also present at the pilus tip mediating adhesion. Similar to many other bacteria, *Myxococcus*
39 *xanthus* contains multiple minor pilins/PilY1 sets that are incompletely understood. Here, we
40 report that minor pilins and PilY1 (PilY1.1) of cluster_1 form priming and tip complexes
41 contingent on a non-canonical cytochrome *c* (TfcP) with an unusual His/Cys heme ligation and
42 calcium. We provide evidence that TfcP is unlikely to participate in electron transport and has
43 been repurposed to promote calcium binding by PilY1.1 at low calcium concentrations, thereby
44 stabilising PilY1.1 and enabling T4aP function in a broader range of calcium concentrations.
45 These results identify a novel function of cytochromes *c* and illustrate how incorporating an
46 accessory factor expands the environmental range under which the T4aP system functions.

47

48 Introduction

49 In bacteria, motility is important for virulence, promotes colonisation of habitats of diverse
50 composition, and stimulates biofilm formation¹. Type IVa pili (T4aP) are filamentous cell surface
51 structures that enable cell translocation across surfaces and also have critical functions in
52 surface adhesion, surface sensing, host cell interaction, biofilm formation, predation, virulence,
53 and DNA uptake²⁻⁴. The versatility of T4aP is based on their ability to undergo cycles of
54 extension, surface adhesion, and retraction^{5,6}. Retractions generate a force up to 150 pN per
55 pilus and pull cells across surfaces⁷.

56 In Gram-negative bacteria, the extension/retraction cycles of T4aP are driven by the T4aP
57 machine (T4aPM), which consists of 15 conserved proteins that form a complex that spans from
58 the outer membrane (OM) across the periplasm and inner membrane (IM) to the cytoplasm⁸⁻¹⁰
59 (Fig. 1a). Pilus extension and retraction are powered by the PilB and PilT ATPases,
60 respectively, that bind in a mutually exclusive manner to the cytoplasmic base of the T4aPM^{8,11-}
61 ¹³. All 15 proteins are essential for T4aP extension except for PilT, which is only important for
62 retraction⁴. The so-called priming complex is an integral part of the T4aPM, is composed of the
63 major pilin, four minor pilins and the PilY1 protein, and is incorporated into the machine
64 independently of the PilB ATPase^{10,14} (Fig. 1a). The five pilins interact directly to form a short
65 pilus that is capped by PilY1, which interacts directly with the minor pilins¹⁰. Pilus extension is
66 initiated by the incorporation of additional major pilin subunits from a reservoir in the IM to the
67 base of the priming complex in a process stimulated by PilB^{6,10,14}. Conversely, during retraction,
68 major pilin subunits are removed from the base of the pilus and reinserted into the IM in a
69 process stimulated by PilT^{12,15}. Because the major pilin is added to the priming complex during
70 the initiation of the extension process, the priming complex remains at the tip of the extended
71 pilus^{10,14,16}. Consistently, PilY1 is involved in surface adhesion, surface-sensing, specificity in
72 host cell recognition during infections, and virulence^{14,16-19}.

73 Among the 15 proteins of the T4aPM, nine are generally encoded by single copy genes²⁰. Some
74 species contain multiple PilT paralogs that enable retractions with different characteristics²¹. The
75 genes for the four minor pilins and PilY1 are also often present in multiple copies^{10,22-24}. The
76 multiplicity of minor pilins and PilY1 proteins has been suggested to allow individual species to
77 assemble priming complexes and tip complexes of different composition and with different
78 properties, thereby allowing the formation of T4aP that can function in a variety of different
79 habitats^{10,14,25}. Minor pilins are low abundance proteins that share overall structure and
80 sequence homology with the major pilin and have a prepilin signal peptide, a hydrophobic N-

81 terminal α -helix, and a C-terminal globular domain, which is less conserved²⁶. PilY1 proteins
82 have a type I signal peptide, are secreted to the periplasm, and are composed of two domains.
83 The conserved C-terminal PilY1-domain adopts a beta-propeller fold²⁷ that interacts with the
84 minor pilins in the priming and tip complex¹⁰ (Fig. 1a). The N-terminal domain is much less
85 conserved and is the domain that mediates host cell recognition, adhesion and surface-
86 sensing^{10,17,28}.

87 The soil-dwelling δ -proteobacterium *Myxococcus xanthus* uses T4aP-dependent motility
88 (T4aPdM) and gliding motility to move on surfaces to generate spreading colonies in the
89 presence of nutrients and spore-filled fruiting bodies in the absence of nutrients^{29,30}. The *M.*
90 *xanthus* genome contains three gene clusters (from here on cluster_1, _2 and _3; proteins
91 labelled with suffix 1, 2 and 3), each encoding four minor pilins and a PilY1 protein^{8,10}. Cluster_1
92 alone and cluster_3 alone support T4aPdM under standard conditions¹⁰. While the four
93 respective minor pilins share overall sequence homology, the three PilY1 proteins are highly
94 divergent in their N-terminal domains¹⁰. Thus, *M. xanthus* has the potential to generate at least
95 two, and possibly three, different T4aPM and T4aP that differ in their priming and tip complexes,
96 respectively.

97 To understand the functional range of the three minor pilin/PilY1 protein sets, we focused on the
98 proteins of cluster_1. Here, we provide evidence that these proteins form priming and tip
99 complexes in a calcium-dependent manner. We identify the TfcP protein and show that it is a
100 non-canonical cytochrome *c* with an unusual His/Cys heme ligation that is conditionally
101 essential for cluster_1-based T4aP formation and T4aPdM. Specifically, TfcP is important for
102 PilY1.1 stability under low calcium conditions; PilY1.1, in turn, is important for the stability of the
103 cluster_1 minor pilins. The effect of TfcP on PilY1.1 stability depends on calcium binding by
104 PilY1.1 and is bypassed at high calcium concentrations. Our data support a model whereby
105 TfcP is a repurposed cytochrome *c* that promotes calcium binding by PilY1.1 at low calcium
106 concentrations, thereby, allowing cluster_1 to support T4aP function in a broader range of
107 environmental conditions.

108

109 Results

110 TfcP is a non-canonical cytochrome c important for cluster 1-based T4aP formation

111 In addition to encoding four minor pilins (PilX1, PilW1, PilV1 and FimU1) and PilY1.1, cluster_1
112 contains an additional open reading frame (ORF) (Locus tag=*mxan_0363*) (Fig. 1b), for which
113 no homolog is present in cluster_2 and cluster_3. This ORF is conserved in gene clusters
114 encoding minor pilins and PilY1 in other Myxococcales genomes (Fig. S1a). Sequence analysis
115 of *Mxan_0363* homologs revealed a type I signal peptide followed by a cytochrome c domain
116 that includes a single cytochrome c signature motif CxxCH (ref.³¹), and a C-terminal extension
117 enriched in Pro residues and charged amino acids (Fig. 1cd). C-type cytochromes are secreted
118 to the periplasm in a Sec-dependent manner where they acquire the heme, which is covalently
119 attached to the two Cys residues in the signature motif by thioether bonds, while the His residue
120 is the proximal axial ligand of the heme iron³². ~90% of cytochromes c, the so-called canonical
121 cytochromes c, have a Met or His residue ~60 residues downstream of the signature motif that
122 serves as the second axial ligand of the heme iron^{31,33,34}. Interestingly, in *Mxan_0363* and
123 homologs, this is a Cys residue (Cys⁹¹ in *Mxan_0363*) (Fig. 1cd), which is rarely found as the
124 second axial ligand in c type cytochromes^{34,35}. In the vicinity of Cys⁹¹, no conserved Met or His
125 residues are present. All *Mxan_0363* homologs except CYFUS_005206 contain the C-terminal
126 extension (Fig. 1cd), which lacks in canonical cytochromes c. Thus, *Mxan_0363* has features in
127 common with canonical cytochromes c but also distinct features. *Mxan_0363* homologs were
128 not identified in species other than the listed Myxococcales (Fig. 1cd). From here on, we refer to
129 *Mxan_0363* as TfcP for T4aP formation cytochrome c protein.

130 Consistent with the overlap of or short distances between stop and start codons for
131 neighbouring genes (Fig. 1b), fragments were amplified in RT-PCR for all consecutive genes of
132 cluster_1 supporting that they constitute an operon (Fig. S1b).

133 To test whether TfcP is important for T4aP-formation or function, we generated in-frame
134 deletions of *tfcP* and the remaining five cluster_1 genes. The deletions were generated in a
135 strain in which cluster_2 and cluster_3 had been deleted ($\Delta 2\Delta 3$ _cluster strain) because
136 cluster_1 and _3 in the wild-type (WT) strain DK1622 function redundantly to support T4aP
137 formation and T4aPdM¹⁰. From here on, we used the $\Delta 2\Delta 3$ _cluster strain as a reference strain
138 and refer to it as the WT $_{\Delta 2\Delta 3}$ strain.

139 In motility assays for T4aPdM on 0.5% agar supplemented with 0.5% Casitone broth (CTT),
140 WT $_{\Delta 2\Delta 3}$ generated the flares at the colony edge characteristic of T4aPdM, while the $\Delta pIIA$

141 mutant, which lacks the major pilin PilA and served as a negative control, did not (Fig. 2a). As
142 previously shown for cluster_3 genes¹⁰, T4aPdM was abolished in the $\Delta pilX1$, $\Delta pilV1$, $\Delta pilW1$
143 and $\Delta pilY1.1$ mutants and reduced in the $\Delta fimU1$ mutant. Strikingly, T4aPdM was also abolished
144 in the $\Delta tfcP$ mutant. T4aPdM was restored in all six in-frame deletion mutants by ectopic
145 expression of the relevant gene from a plasmid integrated in a single copy at the Mx8 *attB* site.

146 To pinpoint the mechanism causing the T4aPdM defect in the cluster_1 mutants, we assessed
147 T4aP formation in the six in-frame deletion mutants using a shearing assay. In this assay, T4aP
148 are sheared off the cell surface, and the level of the major pilin PilA in the sheared fraction
149 quantified by immuno-blot analysis (Fig. 2b). None of the five non-motile mutants formed
150 detectable T4aP, while the $\Delta fimU1$ mutant still assembled T4aP at a much-reduced level
151 compared to the parent strain. For all six in-frame deletion mutants, the total cellular level of PilA
152 was similar or slightly lower than in the parent WT $_{\Delta 2\Delta 3}$ strain. As expected, T4aP-formation in the
153 $\Delta tfcP$ mutant was complemented by ectopic expression of *tfcP*.

154 To distinguish whether the defect in T4aP-formation was caused by lack of extension or by
155 hyper-retractions, we examined T4aP-formation in the six in-frame deletion mutants additionally
156 containing a $\Delta pilT$ mutation and, thus, lacking the PilT retraction ATPase (Fig. 2c). The
157 WT $_{\Delta 2\Delta 3}\Delta pilT$ strain formed T4aP at a highly increased level compared to WT $_{\Delta 2\Delta 3}$ consistent with
158 previous observations for the WT $\Delta pilT$ strain¹². In the absence of PilT, T4aP-formation was
159 partially restored in the $\Delta tfcP$ mutant, but at a much-reduced level compared to the WT $_{\Delta 2\Delta 3}\Delta pilT$
160 strain. By contrast, T4aP-formation in the $\Delta pilX1$, $\Delta pilV1$, $\Delta pilW1$ and $\Delta pilY1.1$ mutants was not
161 restored. For all in-frame deletion mutants except for the $\Delta fimU1$ mutant, the total cellular level
162 of PilA was lower than in WT $_{\Delta 2\Delta 3}\Delta pilT$ strain. We conclude that TfcP is important but not
163 essential for cluster_1-dependent T4aP extension while the three minor pilins PilX1, -V1 and -
164 W1 as well as PilY1.1 are essential for T4aP-formation, and FimU1 plays a less important role.
165 The observations are in agreement with similar experiments involving minor pilins and PilY1.3 of
166 cluster_3¹⁰.

167

168 TfcP is important for PilY1.1 stability

169 To understand how TfcP might be involved in T4aP extension, we used proteomics on whole-
170 cell extracts to quantify the accumulation of T4aPM components in WT $_{\Delta 2\Delta 3}$ and WT $_{\Delta 2\Delta 3}\Delta tfcP$
171 strains. To increase sensitivity, we used targeted proteomics in which protein abundance is
172 quantified relative to heavy labelled reference peptides of the proteins of interest (Methods). In

173 absence of TfcP, accumulation of 10 T4aPM components was largely unaffected, while the
174 accumulation of the four minor pilins and PilY1.1 was strongly reduced (Fig. 3a). Because PilY1
175 of cluster_3 is important for the stability of cluster_3 minor pilins¹⁰, we performed targeted
176 proteomics on the WT_{Δ2Δ3}Δ*pilY1.1* strain. In this strain, accumulation of the four minor pilins was
177 also strongly reduced, while TfcP accumulation was increased (Fig. 3a). In immuno-blot
178 analysis, we observed that in the absence of individual cluster_1 minor pilins, accumulation of
179 TfcP was increased and PilY1.1 unchanged (Fig. 3b). Immuno-blot analysis also confirmed that
180 PilY1.1 accumulation was strongly reduced in the absence of TfcP while TfcP accumulation was
181 increased in the absence of PilY1.1 (Fig. 3b). Altogether, these observations support that TfcP
182 is important for accumulation of PilY1.1, which, in turn, is important for minor pilin accumulation.

183 To resolve whether the effect of the Δ*tfcP* mutation on PilY1.1 and the Δ*pilY1.1* mutation on
184 minor pilin accumulation was due to altered transcription of the relevant genes or altered protein
185 stability, we performed qRT-PCR analysis on total RNA from the WT_{Δ2Δ3}, WT_{Δ2Δ3}Δ*tfcP* and
186 WT_{Δ2Δ3}Δ*pilY1.1* strains. Transcript levels of the cluster_1 genes were increased in the Δ*tfcP* and
187 the Δ*pilY1.1* mutants (Fig. 3c), suggesting negative feedback regulation of cluster_1 genes.
188 While the mechanism involved in this regulation remains unresolved, these results do not
189 support that the reduced levels of PilY1.1/minor pilins and minor pilins in the absence of TfcP
190 and PilY1.1, respectively are caused by reduced synthesis. Rather they support that TfcP
191 stabilises PilY1.1, which, in turn, stabilises the four minor pilins. Accumulation dependencies
192 have also been reported for the cluster_3 proteins in which PilY1.3 and minor pilins interact
193 directly to mutually stabilise each other¹⁰.

194 In *M. xanthus*, the T4aPM assembles at the two poles^{10,36-38}. To exclude that deletion of *tfcP*
195 affects assembly of the T4aPM, we used the bipolar localisation of the cytoplasmic protein PilM
196 as proxy for T4aPM assembly³⁷. We observed bipolar localisation of an active mCherry-PilM
197 fusion in most cells of the WT_{Δ2Δ3} and WT_{Δ2Δ3}Δ*tfcP* strains (Fig. 3d), supporting that TfcP is not
198 important for assembly of the remaining proteins into rudimentary T4aPM.

199

200 TfcP is a periplasmic protein

201 To understand how TfcP stabilises PilY1.1, we determined its subcellular localisation using
202 active TfcP-FLAG and TfcP-sfGFP fusions expressed at native or slightly above native levels
203 from the endogenous locus (Fig. 4ab). Cells of WT_{Δ2Δ3} synthesizing TfcP-FLAG were
204 fractionated into fractions enriched for soluble, IM and OM proteins. TfcP-FLAG was present in

205 the soluble fraction while the control proteins fractionated as described^{12,36} (Fig. 4c). Because
206 sequence analysis of TfcP predicted a type I signal peptide, this experiment supports that TfcP
207 localises to the periplasm similarly to PilY1 proteins¹⁰. In agreement with these observations, in
208 fluorescence microscopy, TfcP-sfGFP localised along the entire cell periphery and foci
209 formation was not observed (Fig. 4d).

210 To determine whether TfcP is present in pili, we purified pili from the WT_{Δ2Δ3}Δ*pilT* mutant (Fig.
211 S2a) and used label-free quantitative proteomics to quantify cluster_1 proteins. All cluster_1
212 proteins except TfcP were detected in low amounts relative to the PilA major pilin (Fig. 4e) as
213 described for cluster_3 proteins¹⁰.

214 Altogether, these observations support that the minor pilins and PilY1.1 of cluster_1 form a
215 priming complex in the T4aPM for T4aP extension as well as a pilus tip complex. We also
216 conclude that TfcP is a soluble, periplasmic protein that stabilises PilY1.1, and that TfcP is
217 incorporated into neither the T4aPM nor T4aP. These findings together with previous results
218 that all proteins that are incorporated into the T4aPM localise (bi)polarly^{10,36-38}, suggest that the
219 stabilising effect of TfcP on PilY1.1 occurs in the periplasm and before PilY1.1 incorporation into
220 the T4aPM.

221

222 TfcP is a non-canonical cytochrome c with a low redox potential and heme binding is important
223 for TfcP stability *in vivo*.

224 We overexpressed and purified a MalE-tagged TfcP variant (MalE-TfcP) from *Escherichia coli* to
225 assess its heme-binding and redox characteristics (Fig. S2b). The fusion for overexpression
226 contains the MalE type I signal peptide and lacks the TfcP signal peptide. Purified MalE-TfcP
227 exhibited a distinct red colour indicating that it binds heme (Fig 5a). Oxidised MalE-TfcP had
228 strong peroxidase activity (Fig. 5a) in agreement with heme-containing proteins having intrinsic
229 peroxidase activity³⁹. Importantly, peroxidase activity was inhibited when MalE-TfcP was
230 reduced by dithiothreitol (DTT), supporting that this activity results from oxidised heme bound to
231 MalE-TfcP⁴⁰.

232 To assess the heme-binding properties of TfcP, we used UV-Vis spectroscopy. MalE-TfcP has a
233 cytochrome c-like spectrum with a strong Soret-peak in the oxidised form and after reduction
234 with sodium-dithionite (Fig. 5b). In the spectrum of reduced TfcP, the α - and β -peak become
235 visible in the 550 nm region. This fits well to spectra of canonical cytochromes c. Interestingly,
236 we did not observe a red-shift of the Soret-peak from the oxidised to the reduced spectrum. For

237 canonical cytochromes *c* with His/His or His/Met coordination a 10 nm bathochromic shift
238 typically observed, while a semisynthetic cytochrome *c* with His/Cys coordination of the heme
239 iron did not exhibit this shift^{35,41,42}, suggesting that Cys⁹¹ (Fig. 1cd) is the second axial ligand in
240 TfcP and responsible for the lack of the red-shift. This is also supported by the presence of a
241 peak at 360 nm in the oxidised spectrum, which has been reported for His/Cys ligation⁴³. The
242 presence of cysteine-to-Fe³⁺ charge transfer bands at ~630 nm and ~730 nm in the oxidised
243 form, which disappear upon dithionite reduction (Fig. 5b, inset), are also in full agreement with
244 spectral properties of His/Cys coordinated *c*-type cytochromes³⁵. To further support that the
245 special spectral properties are due to Cys⁹¹, we purified the MalE-TfcP^{C91M} variant (Fig. S2b). In
246 this variant, a red-shift was observed upon reduction of the protein (Fig 5c). In addition, the
247 360 nm peak was not detected in the oxidised form. We conclude that Cys⁹¹ is the second axial
248 ligand of the heme iron in TfcP and that TfcP is an unusual cytochrome *c*.

249 We used electron paramagnetic resonance (EPR)-spectroscopy to investigate the environment
250 of the heme-center. We obtained *g*-values of 2.51, 2.26 and 1.88 (Fig. 5d) that fit well to the *g*-
251 values observed for multiple heme-containing proteins with a cysteine thiolate ligated heme
252 iron⁴⁴. Cytochromes *c* with a distal Cys were reported to have a very low midpoint potential in
253 the range of -350 mV while canonical cytochromes *c* have a potential of approximately
254 +250 mV^{35,41,42,45}. To determine whether TfcP has a similarly low redox-potential, we used UV-
255 Vis and EPR monitored redox titrations. For the UV-Vis redox-titration, MalE-TfcP was
256 incubated with a redox-mediator cocktail. Spectra and potentials were recorded in five-minute
257 intervals after addition of sodium-dithionite. After plotting the absorbance change at 550 nm
258 versus the potential and fitting to the Nernst equation, the midpoint potential was determined as
259 $E_m = -304 \pm 8$ mV, where 8 mV represent the intrinsic fitting error in one experiment (Fig. 5e). In
260 the EPR-monitored redox titration, we followed the change of *g*=2.26 EPR signal in frozen
261 samples obtained by sequential reduction with sodium-dithionite in the presence of mediators
262 and found a midpoint potential of $E_m = -320 \pm 15$ mV, where 15 mV represent the intrinsic fitting
263 error in one experiment. Overall, both experiments support that TfcP has a very low redox-
264 potential. The slight difference between the two experiments is likely due to pH changes, which
265 occur during freezing. The low redox potential (approx. -312 mV) indicates that TfcP is not likely
266 to be part of a respiratory chain in *M. xanthus* (see Discussion).

267 To clarify whether the heme-binding characteristics of TfcP is important for activity *in vivo*, we
268 substituted the two Cys residues to Ala in the C³¹xxCH heme-binding motif and Cys⁹¹ to His or
269 Met (Fig. 1cd). The variants were synthesised ectopically as FLAG-tagged proteins in the $\Delta tfcP$

270 mutant from the strong *pilA* promoter. None of the variants complemented the motility defect in
271 the $\Delta tfcP$ mutant, while the WT protein did (Fig. S3a). However, all three mutant variants
272 accumulated at much-reduced levels compared to TfcP and TfcP-FLAG expressed from the
273 native site, and none of the three mutant variants supported PilY1.1 accumulation (Fig. S3b).
274 These observations demonstrate that heme-binding and distal coordination of the heme iron are
275 important for TfcP stability.

276 Also, a FLAG-tagged TfcP $\Delta^{118-153}$ variant lacking the C-terminal extension was not able to
277 complement the T4aPdM defect in the $\Delta tfcP$ mutant (Fig. S3a), accumulated at a reduced level,
278 and did not support PilY1.1 accumulation (Fig. S3b), providing experimental support for the
279 functional relevance of this C-terminal extension.

280

281 Added calcium restores T4aP-formation in the absence of TfcP

282 Several PilY1 proteins have been shown to bind calcium using an EF-hand-like motif in the C-
283 terminal domain, and calcium binding is important for function^{27,46,47}. PilY1.1 and PilY1.2 also
284 contain the consensus EF-hand-like calcium binding Dx[DN]xDGxxD motif in the C-terminal
285 PilY1 domain, while PilY1.3 has two calcium binding motifs in the N-terminal domain (Fig. 6a).
286 In a homology model of the C-terminal domain of PilY1.1, the D¹¹⁶⁵xDxDNxxD¹¹⁷³ motif is
287 located in a surface exposed loop between two β -strands as described for the PilY1 domain of
288 *Pseudomonas aeruginosa*²⁷ (Fig. 6b).

289 To address the effect of calcium on T4aPdM, we first considered that the previous experiments
290 were performed either in 1.0% CTT (targeted proteome analyses and qRT-PCR), which has a
291 calcium concentration of $\sim 30 \mu\text{M}$ according to the manufacturer, or on 0.5% agar supplemented
292 with 0.5% CTT (motility assays) or 1.5% agar supplemented with 1.0% CTT (T4aP purification).
293 The estimated calcium concentration of 0.5% agar is $\sim 0.15 \text{ mM}$ ⁴⁸. Next, we repeated the assays
294 for T4aPM in the presence of additional CaCl_2 . To ensure that the effect of added CaCl_2 could
295 be attributed to T4aP, we used the $\text{WT}_{\Delta 2\Delta 3}\Delta aglQ$ strain, which lacks the AglQ motor for
296 gliding^{49,50}. In the presence of $\geq 0.25 \text{ mM}$ added CaCl_2 , $\text{WT}_{\Delta 2\Delta 3}\Delta aglQ$ exhibited a dramatic
297 change of motility pattern from expansion in flares to a radial film-like expansion (Fig. 6c).
298 Intriguingly, the $\text{WT}_{\Delta 2\Delta 3}\Delta aglQ\Delta tfcP$ mutant also responded to increasing calcium concentrations.
299 At added CaCl_2 concentrations $\geq 0.5 \text{ mM}$, this mutant regained T4aPdM and at 1.0 mM
300 displayed a motility pattern similar to that of the parent strain. By contrast, added CaCl_2 did not
301 restore T4aPdM in the $\text{WT}_{\Delta 2\Delta 3}\Delta pilY1.1$ mutant even at 10 mM (Fig. 6c; Fig. S4ab). Likewise,

302 10 mM CaCl₂ did not restore T4aPdM in the $\Delta pilX1$, $\Delta pilV1$, $\Delta pilW1$ and $\Delta pilA$ mutants while the
303 $\Delta fimU1$ mutant displayed the same radial motility pattern as the parent strain (Fig. S4a). In
304 additional experiments, we observed that WT _{$\Delta 2\Delta 3$} $\Delta aglQ$ responded to added CaCl₂
305 concentrations as low as 0.025 mM while the WT _{$\Delta 2\Delta 3$} $\Delta aglQ\Delta tfcP$ only responded at ≥ 0.5 mM
306 (Fig. S4b). In control experiments, we observed that neither 5 mM NaCl nor 5 mM MgCl₂
307 restored motility in the WT _{$\Delta 2\Delta 3$} $\Delta tfcP$ mutant. We also observed that a strain only containing
308 cluster_3 responded to added CaCl₂ with an altered expansion pattern; however, this pattern
309 was only evident at added CaCl₂ concentrations ≥ 0.25 mM (Fig. S4b). We conclude that CaCl₂
310 at an added concentration of 1.0 mM restores T4aPdM in the WT _{$\Delta 2\Delta 3$} $\Delta tfcP$ strain. From here on,
311 we used an added CaCl₂ concentration of 1.0 mM.

312 Consistent with the effect of added CaCl₂ on motility, the WT _{$\Delta 2\Delta 3$} $\Delta tfcP$ mutant formed T4aP in
313 the presence of 1.0 mM CaCl₂ based on the shearing assay, while the WT _{$\Delta 2\Delta 3$} $\Delta pilY1.1$ mutant
314 did not (Fig. 6d). Added CaCl₂ also increased the amount of shearable pili in WT _{$\Delta 2\Delta 3$} . The level
315 of T4aP in the WT _{$\Delta 2\Delta 3$} $\Delta tfcP$ mutant was lower than in WT _{$\Delta 2\Delta 3$} (Fig. 6d). Calcium also increased
316 the amount of total cellular PilA in all strains (Fig. 6d). The retraction deficient strains
317 WT _{$\Delta 2\Delta 3$} $\Delta pilT$ and WT _{$\Delta 2\Delta 3$} $\Delta tfcP\Delta pilT$ assembled more T4aP in the presence of added CaCl₂ than
318 in the absence supporting that calcium stimulates T4aP-formation rather than counteracting
319 retractions (Fig. 6d). We conclude that 1.0 mM added CaCl₂ can substitute for TfcP function in
320 T4aP-formation and T4aPdM.

321

322 TfcP enhances calcium-dependent stabilisation of PilY1.1

323 To understand how a high concentration of calcium compensates for lack of TfcP, we used
324 targeted proteomics. In WT _{$\Delta 2\Delta 3$} , 1.0 mM CaCl₂ caused an increase in PilA abundance and a
325 decrease in TfcP abundance while accumulation of other T4aPM components including the
326 remaining cluster_1 proteins was largely unaffected (Fig. 6e). In the WT _{$\Delta 2\Delta 3$} $\Delta tfcP$ mutant, added
327 CaCl₂ not only caused an increase in PilA abundance but also increased the abundance of all
328 remaining cluster_1 proteins including PilY1.1 (Fig. 6e). In the WT _{$\Delta 2\Delta 3$} $\Delta pilY1.1$ mutant, extra
329 CaCl₂ also caused increased PilA abundance, but a reduction in TfcP abundance as in the
330 WT _{$\Delta 2\Delta 3$} parent strain. PilW1 and FimU1 abundance was unaffected by added CaCl₂ in
331 WT _{$\Delta 2\Delta 3$} $\Delta pilY1.1$, while PilV1 and PilX1 abundance increased. We conclude that a high
332 concentration of CaCl₂ causes increased PilY1.1 accumulation in the absence of TfcP. In
333 addition, extra CaCl₂ also caused (1) increased PilA accumulation independently of TfcP and

334 PilY1.1, (2) decreased accumulation of TfcP independently of PilY1.1, and (3) increased
335 accumulation of the minor pilins PilX1 and PilV1 independently of PilY1.1.

336 Because changes in extracellular calcium can cause altered gene expression⁵¹, we performed
337 qRT-PCR analyses to discriminate whether added CaCl₂ affects transcription or protein stability.
338 We observed significant changes in the transcription of all cluster_1 genes as well as of *pilA* in
339 response to added CaCl₂ (Fig. 6f); however, generally, these changes did not correlate with the
340 altered protein accumulation profiles. For instance, in WT_{Δ2Δ3}, 1.0 mM added CaCl₂ caused (1)
341 increased PilA accumulation but *pilA* transcription was decreased, and (2) decreased *pilY1.1*
342 transcription but PilY1.1 abundance remained unchanged, and in the WT_{Δ2Δ3}Δ*tfcP* mutant,
343 added CaCl₂ caused decreased *pilY1.1* transcription but PilY1.1 abundance increased. We
344 conclude that added CaCl₂ at 1.0 mM can substitute for TfcP in stabilising PilY1.1.

345 Label-free quantitative proteomics of purified pili from WT_{Δ2Δ3}Δ*pilT* and WT_{Δ2Δ3}Δ*pilT*Δ*tfcP* (Fig.
346 S2a) revealed a strong increase in the abundance of cluster_1 minor pilins and PilY1.1 relative
347 to PilA in the presence of calcium (Fig. 6g) suggesting that calcium also stabilises minor pilins
348 and PilY1.1 in the tip complex. Of note, TfcP was also not detected in purified pili from
349 WT_{Δ2Δ3}Δ*pilT* grown in the presence of added calcium (Fig. 6g).

350 To determine whether the effect of calcium on PilY1.1 stability depends on its binding to PilY1.1,
351 we attempted to purify full-length PilY1.1 or its C-terminal domain but were unsuccessful, thus,
352 precluding *in vitro* analyses of the calcium binding properties of PilY1.1. Therefore, to assess
353 calcium binding by PilY1.1 *in vivo*, we introduced the Asp¹¹⁷³ to Ala substitution in the EF-hand-
354 like calcium binding D¹¹⁶⁵xDxDNxxD¹¹⁷³ motif in PilY1.1 (Fig. 6b) and expressed the protein from
355 the native site in WT_{Δ2Δ3} strains. The homologous substitution in other PilY1 proteins disrupts
356 calcium binding without affecting the overall structure of the C-terminal beta-propeller
357 domain^{27,46,47}.

358 The *pilY1.1*^{D1173A}*tfcP*⁺ mutant was strongly reduced in T4aPdM in the absence of added CaCl₂
359 (Fig. 7a); however, this strain regained T4aPdM and was indistinguishable from the parent
360 strain at ≥0.25 mM added CaCl₂. By contrast, the *pilY1.1*^{D1173A}Δ*tfcP* strain was non-motile even
361 at 10 mM added CaCl₂. These observations support that calcium binding is important for PilY1.1
362 function and that PilY1.1^{D1173A} is fully functional at elevated calcium concentrations but only if
363 TfcP is present.

364 Consistent with the observations for T4aPdM, PilY1.1^{D1173A} accumulation was reduced in the
365 *pilY1.1*^{D1173A}*tfcP*⁺ mutant in the absence of added CaCl₂, and 1.0 mM CaCl₂ at least partially

366 restored its accumulation (Fig. 7b). By contrast, in the *pilY1.1^{D1173A}ΔtfcP* strain, PilY1.1^{D1173A} was
367 detected at very low levels in the absence of added CaCl₂ and did not increase upon addition of
368 CaCl₂. Thus, PilY1.1^{D1173A} depends on TfcP for stability, and responds to added calcium only in
369 the presence of TfcP. By comparison, PilY1.1^{WT}, is fully functional at ≥1.0 mM added CaCl₂ in
370 the absence of TfcP (Fig. 6c).

371 Finally, to determine whether TfcP can stabilise PilY1.1^{WT} independently of calcium, we
372 analysed PilY1.1 accumulation in the presence of the highly specific calcium chelator BAPTA
373 (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid). In WT_{Δ2Δ3} cells expressing TfcP-
374 FLAG from the endogenous site and grown in 1.0% CTT, PilY1.1 was still detected in the
375 presence of 40 μM but not in the presence of 80 μM BAPTA while TfcP was detected under all
376 conditions and increased upon BAPTA addition (Fig. 7c). These observations strongly support
377 that TfcP can only stabilise PilY1.1 in the presence of calcium. Because CaCl₂ can stabilise
378 PilY1.1 in the absence of TfcP, these observations suggest that the primary function of TfcP is
379 to chaperone calcium binding by PilY1.1 at low calcium concentrations.

380

381 Discussion

382 Here, we identify TfcP, a repurposed, non-canonical cytochrome *c*, as a novel protein important
383 for cluster_1-dependent T4aP formation in *M. xanthus* at low calcium concentrations. We
384 demonstrate that TfcP stabilises PilY1.1 at low calcium concentrations. PilY1.1, in turn,
385 stabilises the four minor pilins of cluster_1 in that way enabling the formation of the cluster_1-
386 based priming complex in the T4aPM and, thus, T4aP formation. Bacteria in their natural
387 habitats experience large fluctuations in environmental conditions and depend on adaptive
388 strategies to endure such changes. TfcP expands the range of calcium concentrations under
389 which cluster_1 encoded minor pilins and PilY1.1 can support T4aPdM thereby increasing
390 fitness of *M. xanthus* under changing environmental conditions and enabling colonisation of
391 habitats with low calcium concentrations (Fig. 8).

392 Several lines of evidence support that that the effect of TfcP on PilY1.1 stability is calcium-
393 dependent. First, under standard conditions, *M. xanthus* is exposed to ~30 μ M calcium in CTT
394 suspension culture and ~0.15 mM calcium on 0.5% agar-plates for motility assays. Under these
395 conditions, TfcP is important for PilY1.1 stability. However, at concentrations \geq 1 mM added
396 CaCl₂, calcium alone is sufficient to stabilise PilY1.1 independently of TfcP. Second, in the
397 complete absence of calcium, i.e. after addition of the calcium specific chelator BAPTA, TfcP
398 does not stabilise PilY1.1 while TfcP still accumulates. Third, the PilY1.1^{D1173A} variant, which is
399 predicted to bind calcium with reduced affinity, depends on TfcP for stability at 1.0 mM added
400 CaCl₂, and even at 10 mM CaCl₂, this protein is non-functional in the absence of TfcP. Thus,
401 TfcP and calcium both function to stabilise PilY1.1. However, while high calcium concentrations
402 alone can stabilise PilY1.1, TfcP cannot stabilise PilY1.1 in the absence of calcium. Altogether,
403 these findings support a model whereby calcium binding by PilY1.1 is the primary determinant
404 for its stability, and in which TfcP stabilises PilY1.1 at low calcium concentrations by
405 chaperoning calcium binding by PilY1.1. The functional outcome of this TfcP-dependent
406 stimulation of calcium binding by PilY1.1 is that PilY1.1 accumulates at low calcium
407 concentrations and is able to support cluster_1-dependent T4aP formation and T4aPdM. Many
408 myxobacteria including *M. xanthus* are found in terrestrial habitats in which calcium
409 concentrations are described to vary from 0.1-1.0 mM at root-soil interfaces, 3.4-14 mM in some
410 soils and as low as 10-150 μ M in other soils⁵². We suggest that TfcP is key to enabling PilY1.1-
411 dependent T4aP formation and T4aPdM in the lower range of calcium concentrations.
412 Interestingly, *M. stipitatus* and *Coralloccoccus coralloides* only have one gene cluster for minor
413 pilins and PilY1, and this cluster encodes a TfcP ortholog (Fig. 1c; Fig. S1a) emphasising the
414 importance of TfcP in T4aPdM in myxobacteria.

415 TfcP is a periplasmic protein and contains a non-canonical cytochrome *c* domain in which the
416 second axial heme ligand is a Cys residue rather than the more common His and Met residues
417 in canonical cytochromes *c*. Accordingly, TfcP has a very low redox potential of -304 to -320 mV
418 based on two methods. Moreover, TfcP variants unable to bind heme or with altered heme-
419 binding properties are unstable *in vivo*. *M. xanthus* is strictly aerobic and the genome encodes
420 complex I-IV of the electron transport chain⁵³. Thus, the low redox-potential of TfcP supports
421 that it is not part of the respiratory chain, which starts with a potential of -320 mV for the redox
422 pair NAD/NADH⁵⁴. Some cytochromes *c* are involved in electron transport across the OM to
423 external electron acceptors; however, these proteins are canonical cytochromes *c*⁵⁵ suggesting
424 that TfcP also does not engage in this type of electron transport. Some *c*-type cytochromes with
425 His/Cys ligation, e.g. the triheme DsrJ of *Allochromatium vinosum*, are involved in dissimilatory
426 sulfur metabolism in which sulfate is used as terminal electron acceptor⁵⁶. Other *c*-type
427 cytochromes with His/Cys ligation have been suggested to have a role in signalling^{35,57}.
428 Because *M. xanthus* does not respire on sulfate, it is unlikely that TfcP would be involved in
429 dissimilatory sulfur metabolism. While we cannot rule out a function of TfcP in signalling, our
430 data support a scenario in which TfcP is a repurposed cytochrome *c* that is no longer involved in
431 electron transport, and in which the covalently bound heme serves a structural function to
432 stabilise TfcP. This “inert” cytochrome *c* then chaperones calcium binding by PilY1.1 at low
433 calcium concentrations. TfcP also differs from canonical cytochromes *c* by having a C-terminal
434 extension. This extension is important for TfcP stability; however, its precise function remains to
435 be uncovered.

436 PilY1 proteins of *P. aeruginosa*, *Kingella kingae* and *Neisseria gonorrhoeae* bind calcium using
437 an EF-hand-like calcium binding motif in their C-terminal PilY1 domain (Fig. 6b). An Asp to Ala
438 substitution of the C-terminal Asp residue in this motif abolishes calcium binding and renders
439 the proteins non-functional while overall still folding correctly^{27,46,47}. The corresponding
440 substitution in the D¹¹⁶⁵xDxDNxxD¹¹⁷³ motif in PilY1.1 increased its dependency on TfcP and
441 calcium for stability supporting that PilY1.1 binds calcium as described for other PilY1 proteins.
442 However, while the PilY1 variants of *P. aeruginosa*, *K. kingae* and *N. gonorrhoeae* deficient in
443 calcium binding accumulate, PilY1.1 depends on TfcP for stability at low calcium levels. Also,
444 PilY1.1^{D1173A} only accumulates at very low levels even in the presence of calcium and TfcP,
445 suggesting that PilY1.1 binds calcium with a lower affinity than the three other PilY1 proteins.
446 The observation that PilY1.1 is unstable in the absence of TfcP at low calcium concentrations
447 suggest that the two proteins interact directly. However, such an interaction remains to be
448 shown and will be addressed in future experiments. PilY1.1 as well as the four minor pilins of

449 cluster_1 were detected in purified pili as previously shown for the minor pilins and PilY1.3 of
450 cluster_3¹⁰. By contrast, we did not detect TfcP in purified pili. We previously showed that
451 sfGFP-tagged PilY1.3 and the sfGFP-tagged minor pilin PilW3 of cluster_3 localise polarly, are
452 incorporated into the T4aPM but do not support pilus extension likely because the sfGFP-tag
453 jams the machine by precluding passage of PilY1.3-sfGFP and PilW3-sfGFP through the
454 secretin channel in the OM¹⁰. sfGFP-tagged TfcP was fully active and did not localise polarly.
455 These observations strongly support that TfcP is neither part of the T4aPM nor of the pilus.
456 They also strengthen the hypothesis that the suggested direct interaction between PilY1.1 and
457 TfcP is transient and occurs in the periplasm before PilY1.1 incorporation into the T4aPM (Fig.
458 8). The observation that added calcium stabilises PilY1.1 in the absence of TfcP supports that
459 TfcP does not act as a metallochaperone to deliver calcium to PilY1.1. Altogether, we suggest
460 that TfcP transiently interacts with PilY1.1, thereby stimulating folding and efficient calcium
461 binding by PilY1.1. Subsequently, PilY1.1 with bound calcium is incorporated into the priming
462 complex of the T4aPM to support T4aP formation (Fig. 8). This mechanism of protein
463 stabilisation is reminiscent to that of periplasmic chaperones, which in an ATP-independent
464 manner transiently interact with their periplasmic clients to enable folding⁵⁸, except that the
465 TfcP/PilY1.1 interaction is suggested to promote calcium binding by PilY1.1, which then
466 stabilises PilY1.1. Altogether, these findings also provide evidence for a novel cytochrome *c*
467 function in protein folding and/or stabilisation.

468 In the presence of added calcium at 1.0 mM, more T4aP are formed and the ratio between
469 minor pilins and PilY1.1 to PilA is increased. These observations support that calcium not only
470 helps to stabilise PilY1.1, but may also stabilise the pilus including the minor pilin/PilY1.1 tip
471 complex in extracellular space. In this context, it is interesting to note that calcium binding has
472 been reported to stabilise the interactions between major pseudopilin subunits in the
473 pseudopilus of the type II secretion system of *Klebsiella oxytoca*⁵⁹. In future experiments, this
474 effect of calcium will be addressed.

475 In addition to the conserved proteins of the T4aPM, T4aP extension in several species depends
476 on accessory factors that are much less conserved. For instance, the c-di-GMP binding protein
477 FimX in *P. aeruginosa* and SgmX in *M. xanthus* stimulate T4aP extension⁶⁰⁻⁶². TfcP adds to be
478 list of such regulators and also acts at the level of extension; however, in contrast to these
479 cytoplasmic regulators, TfcP acts in the periplasm.

480

481 **Methods**

482 **Bacterial strains and growth media.** All *M. xanthus* strains are derivatives of DK1622⁶³ and
483 listed in Supplementary Table 1. All plasmids are listed in Supplementary Table 2. In-frame
484 deletion mutants were generated using double homologous recombination using a *galk*-
485 containing plasmid⁶⁴. Genes were ectopically expressed from the *pilA*-promoter in plasmids
486 integrated by site-specific recombination at the *attB* site. All plasmids were verified by
487 sequencing. All strains were confirmed by PCR. Oligonucleotides are listed in Supplementary
488 Table 3. *M. xanthus* liquid cultures were grown in 1% CTT broth (1% Bacto Casitone (Gibco),
489 10 mM Tris-HCl pH8.0, 1 mM KPO₄ pH7.6, 8 mM MgSO₄) or on 1% CTT 1.5% agar plates.
490 When required media were supplemented with kanamycin (50 µg ml⁻¹) or oxytetracyclin (10 µg
491 ml⁻¹)⁶⁵. *E. coli* strains were grown in LB broth⁶⁶. Plasmids were propagated using *E. coli* NEB-
492 Turbo.

493 **Bioinformatics.** Homologs of TfcP were searched using BlastP⁶⁷. Pairwise sequence
494 alignments were calculated using EMBOSS-Needle⁶⁸. Protein domains were identified using
495 InterPro⁶⁹. Alignments of TfcP and homologs were computed using MUSCLE⁶⁸. The homology
496 model of PilY1.1 was generated using the Phyre2 server⁷⁰.

497 **Motility assay.** T4aPdM was assayed as described⁷¹. Briefly, exponentially growing *M. xanthus*
498 cultures were harvested and concentrated in 1% CTT to a density of 7×10⁹ cells ml⁻¹. 5 µl of the
499 concentrated cell suspension were spotted on soft-agar CTT plates (0.5% CTT, 10 mM Tris-HCl
500 pH 8.0, 1 mM KPO₄ pH 7.6, 8 mM MgSO₄, 0.5% select-agar (Invitrogen)) and incubated at 32°C
501 for 24 hrs. Colonies were imaged using a Leica MZ75 stereomicroscope equipped with a Leica
502 MC120 HD camera.

503 **T4aP shearing assay.** T4aP were sheared off *M. xanthus* cells as described⁷². Briefly, cells
504 were grown on CTT 1.5% agar plates at 32°C for three days, then scraped off, and
505 resuspended in pili resuspension buffer (100 mM Tris-HCl pH 7.6, 150 mM NaCl) (1 ml per
506 60 mg cells). Cell suspensions were vortexed 10 min at maximum speed. A 100 µl aliquot was
507 harvested and resuspended in 200 µl sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-
508 HCl pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, 1.5 mM EDTA, 0.001% Bromophenol Blue),
509 and denatured at 95°C for 10 min and used to determine the cellular PilA amount. The
510 remaining cell suspension was cleared three times by 20 min centrifugation at 20,000 *g* at 4°C.
511 Pili in the cleared supernatant were precipitated by adding 10× pili-precipitation buffer (final
512 concentration: 100 mM MgCl₂, 2% PEG 6000, 100 mM Tris-HCl pH 7.6, 150 mM NaCl),
513 incubation on ice for 4 hrs and centrifugation at 20,000 *g* for 30 min, 4°C. The pellet was

514 resuspended in 1 μ l SDS lysis buffer per mg cells and boiled for 10 min at 95°C. The samples
515 were separated by SDS-PAGE and analysed for PilA accumulation by immuno-blot using PilA
516 antibodies.

517 **Immuno-blot and peroxidase staining.** Immuno-blot analysis was carried out as described⁶⁶.
518 Samples were prepared by harvesting exponentially growing *M. xanthus* cells and subsequently
519 resuspension in SDS lysis buffer to an equal concentration of cells. Immuno-blot was done
520 using as primary antibodies α -PilB, α -PilC, α -PilQ³⁶, α -PilA, α -LonD¹⁰, α -FLAG (Rockland; 600-
521 401-383), α -GFP (Roche; 11814460001), α -MalE (New England Biolabs), α -PilY1.1⁷³ and α -
522 TfcP. Antibodies against TfcP were generated by Eurogentec against TfcP Δ ¹⁻¹⁸-His₆ purified from
523 *E. coli* Rosetta 2(DE3) containing plasmid pMH6 using native Ni-NTA affinity purification. As
524 secondary antibodies, goat α -rabbit immunoglobulin G peroxidase conjugate (Sigma-Aldrich,
525 A8275) and sheep α -mouse immunoglobulin G peroxidase conjugate (Amersham, NXA931)
526 were used. Antibodies and conjugates were used in the following dilutions: 1:500 α -TfcP;
527 1:1000 α -PilY1.1; 1:3000 α -PilB; α -PilC; 1:5000 α -PilQ; α -PilA; 1:6000 α -LonD; 1:2000 α -GFP,
528 α -MalE, α -FLAG and α -mouse peroxidase conjugate; and, 1:10,000 α -rabbit peroxidase
529 conjugate. Blots were developed using Luminata™Western HRP substrate (Millipore). Unless
530 otherwise noted, protein from 3×10^8 cells were loaded per lane. For peroxidase staining, protein
531 was separated by SDS-PAGE, blotted on a nitrocellulose membrane and developed using
532 Luminata™Western HRP substrate.

533 **Fractionation of *M. xanthus*.** *M. xanthus* was fractionated into fractions enriched for soluble,
534 IM and OM proteins as described⁷⁴. Briefly, an exponentially growing *M. xanthus* culture was
535 harvested and the pellet resuspended in lysis buffer (50 mM Tris-HCl pH7.6, Protease inhibitor
536 cocktail (Roche)) (1 ml per 80 mg cells). A 75 μ l aliquot was taken as the whole cell sample,
537 suspended with SDS-lysis buffer and boiled 10 min at 95°C. Cells were lysed using sonication
538 and lysates cleared by centrifugation at 8000 g for 1 min. The cleared lysate was subjected to
539 ultra-centrifugation using an Air-Fuge (Beckman) at $\sim 150,000$ g for 1 hr. The resulting
540 supernatant contains soluble proteins and was mixed with SDS-lysis buffer. The pellet was
541 resuspended in detergent-lysis buffer (50 mM Tris-HCl pH 7.6, 2% Triton X-100) and subjected
542 to ultra-centrifugation as described. The resulting supernatant is enriched for IM proteins while
543 the pellet is enriched for OM proteins. The supernatant was mixed with SDS lysis buffer and the
544 pellet resuspended in SDS-lysis buffer. The samples were analysed by SDS-PAGE and
545 immuno-blot.

546 **Fluorescence microscopy.** Exponentially growing *M. xanthus* cells were spotted on 1%
547 agarose pads supplemented with TPM (10 mM Tris-HCl pH 8.0, 1 mM KPO₄ pH 7.6, 8 mM
548 MgSO₄) and incubated for 30 min at 32°C before microscopy. Cells were imaged using a Leica
549 DMI600B microscope with a Hamamatsu Flash 4.0 camera. Images were recorded with Leica
550 MM AF software and processed with Metamorph.

551 **Targeted proteomics.** To identify peptides of T4aPM proteins suitable for targeted-mass
552 spectrometry (MS) analysis, we performed sample preparation on *M. xanthus* cell pellets for
553 total proteome analysis as described¹⁰. Briefly, proteins were extracted from cell pellets by heat
554 exposure in the presence of 2% sodium-lauroylsarcosinate. Extracts were then reduced,
555 alkylated and digested overnight using trypsin (Promega). Peptides were purified using C18
556 solid phase extraction and analysed on a Q-Exactive Plus mass spectrometer connected to an
557 Ultimate 3000 RSLC and a nanospray flex ion source (all Thermo Scientific). The peptides were
558 analysed using data dependent acquisition with settings as described¹⁰. MS raw data were
559 searched using Mascot (Matrix Science) and loaded into Scaffold 4 (Proteome software) for
560 further data evaluation. Peptides considered most amenable for targeted MS were chosen for
561 reference peptide synthesis (JPT Peptide Technologies, Berlin) containing heavy labelled (¹³C
562 and ¹⁵N) C-terminal Lys or Arg residues with a resulting mass shift of +8 Da and +10 Da,
563 respectively. Sequences of reference peptides are listed in Supplementary Table 4. For targeted
564 MS experiments, reference peptides and iRT retention calibration peptides (Biognosys) were
565 spiked into the *M. xanthus* total proteome peptide samples (generated as described), and
566 analysed by liquid chromatography (LC)-MS.

567 Peptides were separated on a 90 min gradient from 2-50% acetonitrile at a flow rate of 300 nl
568 min⁻¹, and analysed by MS in targeted parallel reaction monitoring (PRM) mode. The mass
569 spectrometer first acquired a full MS-Selected Ion Monitoring (SIM) scan with an MS1 resolution
570 of 70,000, AGC (automatic gain control) target setting of 1e⁶ and 100 ms max injection time.
571 Then PRM scans were carried out with a MS2 resolution of 35,000, AGC target setting of 2e⁵,
572 100 ms maximum injection time with a quadrupole isolation window of 1.6 m/z. Normalised
573 collision energy was set to 27%. All stages of targeted MS data analysis was carried out in
574 Skyline (20.2.1.384)⁷⁵. Results with dot-product <0.85 or ratio_{heavy/light}<0.005 were excluded from
575 the analysis.

576 **Proteome analysis of T4aP.** Label-free quantification (LFQ) MS of the pili proteome was
577 carried out as described¹⁰. Briefly, pili were purified following the shearing assay protocol with
578 the modification that after precipitation, pili were resuspended in pili-resuspension buffer and re-

579 precipitated with pili-precipitation buffer three times. Pili were resuspended in pili-resuspension
580 buffer to 1 μ l buffer per 1 mg cells. 25% of the pili sample was mixed with SDS-lysis buffer and
581 analysed by SDS-PAGE and subsequent staining with Coomassie Blue. The remaining 75%
582 were precipitated with acetone. The dried acetone pellets were resuspended, reduced, alkylated
583 and digested with trypsin as described¹⁰. Pili LFQ proteomics analysis was carried out on an
584 Exploris 480 mass spectrometer (Thermo Scientific), connected to an Ultimate 3000 RSLC.
585 Peptides were separated on a 60 min gradient from 2-50% acetonitrile at a flow rate of 300 nl
586 min^{-1} . The Exploris 480 mass spectrometer first acquired a full MS scan with an MS1 resolution
587 of 60,000, AGC target setting of $3e^6$ and 60 ms max injection time, followed by MS/MS scans of
588 Top-20 most abundant signals. For MS/MS scans a resolution of 7,500 was set, with an AGC of
589 $2e^5$ and 30 ms max. injection time. Normalised collision energy was set to 27% and the isolation
590 window of the quadrupole was 1.6 m/z. All MS raw data was analysed by MaxQuant (1.6.17.0).
591 iBAQ values were calculated as described¹⁰ as the sum of all peptide intensities for a given
592 protein divided by the number of theoretically MS observable peptides. Following MaxQuant
593 analysis, the iBAQ values were normalised by the total iBAQ sum independently of the highly
594 abundant PilA.

595 **Purification of MalE-TfcP.** For purification of MalE-TfcP/MalE-TfcP^{C91M}, gene expression was
596 done in *E. coli* strain BL21 containing the helper plasmid pEC86, which encodes the *ccm* genes
597 for cytochrome *c* maturation of *E. coli*, as well as pMH31 (MalE-TfcP) or pMH39 (MalE-TfcP^{C91M})
598 using auto-induction in buffered 5052-Terrific-Broth (0.5% glycerol, 0.05% glucose, 0.2%
599 lactose, 2.4% yeast extract, 2% tryptone, 25 mM Na_2HPO_4 , 25 mM KH_2PO_4 , 50 mM NH_4Cl ,
600 5 mM Na_2SO_4 , 2 mM MgSO_4)⁷⁶ containing chloramphenicol (25 $\mu\text{g ml}^{-1}$) and carbenicillin
601 (100 $\mu\text{g ml}^{-1}$). After 24 hrs incubation at 37°C, cells were harvested, and resuspended in MBP-
602 lysis buffer (100 mM Tris-HCl pH 7.0, 200 mM NaCl) supplemented with EDTA-free protease
603 inhibitor cocktail (Roche) and lysed using sonication. The lysate was cleared by centrifugation at
604 20,000 *g*, 4°C for 30 minutes and loaded onto a 5 ml HighTrap MBP column (GE Healthcare)
605 using an Äkta-Pure system (GE Healthcare). The column was washed with lysis buffer and
606 protein eluted with 10 column volumes MBP-elution buffer (100 mM Tris-HCl pH 7.0, 200 mM
607 NaCl, 10 mM maltose). The elution fractions containing MalE-TfcP/MalE-TfcP^{C91M} were pooled
608 and diluted four fold in 100 mM Tris-HCl pH 7.0. The pooled and diluted samples were loaded
609 onto a HighTrap SP ion exchange column. The column was washed with IEX-wash buffer
610 (100 mM Tris-HCl pH 7.0) and protein eluted in a linear gradient with IEX-elution buffer (100 mM
611 Tris-HCl pH 7.0, 2 M NaCl). Samples were concentrated using an Amicon Ultra filter with
612 10 kDa cutoff and loaded on a HiLoad 16/600 Superdex 200 pg (GE Healthcare) size exclusion

613 chromatography column equilibrated with SEC-buffer (50 mM Tris-HCl pH 7.6, 50 mM NaCl).
614 Protein was either used fresh or snap-frozen in buffer containing SEC-buffer with 10% glycerol.

615 **UV-Vis spectroscopy.** UV-Vis measurements of purified (oxidised) and reduced MalE-
616 TfcP/MalE-TfcP^{C91M} was conducted on a Tecan M200Pro platereader or a Shimadzu 1900
617 spectrophotometer. Protein was diluted to an absorbance of ~0.7. After measurement of the
618 oxidised spectrum, protein was reduced by adding a few crystals of sodium-dithionite,
619 equilibrated for 15 min and the reduced spectrum recorded.

620 **Redox titrations.** Redox titrations were carried out in a Coy anaerobic tent (3% H₂, <5 ppm O₂).
621 MalE-TfcP in HEPES buffer, pH 7.0, was mixed with 20 μM (final concentration) of the following
622 redox mediators: Phenosafranin, safranin T, neutral red, benzyl viologen, and methyl viologen.
623 The solution potential was measured with an InLab redox micro combination electrode (Mettler
624 Toledo) under anaerobic conditions. Correction to redox potentials vs. H₂/H⁺ was done by
625 addition of 207 mV to the reading of the potentiometer. Stirring was done using a 8 mm teflon
626 coated stirrer bar. For redox titration using visible spectroscopy (using a Shimadzu 1900
627 spectrophotometer), automated addition of 15 μl buffered 0.2 mM sodium-dithionite solution was
628 done using a remotely controlled peristaltic pump (Pharmacia P1) for 60 sec followed by 2 min
629 equilibration and 2 min recording of the spectra in the 600-460 nm range. The normalised
630 absorbance increase at 550 nm (corrected by the absorbance for titration of mediators only)
631 was fitted to the Nernst equation for n=1 at 298 K. For the EPR titration, manual addition of
632 aliquots of buffered sodium-dithionite was used. After stabilisation of the solution potential,
633 300 μl samples were withdrawn, transferred to EPR tubes, which were capped with rubber
634 tubing and an acrylic glass stick. Samples were shock-frozen and stored in liquid nitrogen until
635 the EPR measurements.

636 **EPR spectroscopy.** EPR spectra were recorded with an X-band EPR spectrometer (Bruker
637 Elexsys E580) in a 4122HQE-W1/1017 resonator. The temperature of the samples in Ilmasil PN
638 quartz tubes (4.7±0.2 mm outer diameter, 0.45±0.05 mm wall thickness) was maintained at 12 K
639 with an ESR900 continuous flow helium cryostat (Oxford Instruments). The modulation
640 frequency was 100 kHz and the modulation amplitude 1.5 mT. Spectra were averages for four
641 90 sec scans. For the titration, the normalised amplitude of the derivative-shaped feature of the
642 low spin EPR signal of the ferric state at g=2.26 was used for a fit to the Nernst equation (n=1,
643 T=298 K).

644 **Operon mapping.** Total RNA was isolated from exponentially growing *M. xanthus* cultures
645 using the Monarch Total RNA Miniprep Kit (NEB). 10^9 cells were harvested and resuspended in
646 200 μ l lysis-buffer (100 mM Tris-HCl pH 7.6, 1 mg ml⁻¹ lysozyme). After incubation at 25°C for
647 5 min cells were lysed and RNA purified according to manufacturer's protocol with the exception
648 that the on-column DNase treatment was omitted. RNA was eluted in RNase-free water and
649 subsequently treated with Turbo DNase and purified using the Monarch RNA Cleanup Kit
650 (50 μ g) (NEB) and eluted in RNase-free water. 1 μ g of RNA was used for cDNA synthesis using
651 the LunaScript RT SuperMix Kit (NEB) with and without reverse transcriptase (RT). cDNA was
652 diluted 1:5 with water and 1 μ l of diluted cDNA used for PCR reactions.

653 **qRT-PCR.** For qRT-PCR RNA was isolated and cDNA synthesised as described for operon
654 mapping. qPCRs were carried out using the Luna Universal qPCR MasterMix (NEB) with the
655 primers listed in Supplementary Table 3 and measured on an Applied Biosystems 7500 Real-
656 Time PCR system. Relative gene expression levels were calculated using the comparative C_T
657 method⁷⁷. *Mxan_3298 (tuf2)*, which encodes elongation factor Tu, and *mxan_3303 (rpsS)*,
658 which encodes the small ribosomal subunit protein S19, were used as internal controls. All
659 experiments were done with three biological replicates and two technical replicates.

660 **Statistics and reproducibility.** Data shown for operon mapping, T4aP-dependent motility,
661 T4aP shearing assays, immuno-blot experiments, UV-Vis spectroscopy and fluorescence
662 microscopy were obtained in at least two biological replicates with similar results. For targeted
663 proteomics and LFQ-analysis of the pili proteome, four biological replicates were analysed.
664 qRT-PCR analysis were conducted with three biological replicates each with two technical
665 replicates. Redox titrations and EPR-spectroscopy were done in a single experiment.

666 **Data availability.** Source data are provided with this paper. The authors declare that all data
667 supporting this study are available within the article, its Supplementary Information file, and the
668 Source Data file.

669

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857
858 **Authors' contributions.** MH: Designed and conceived the study and performed most of the
859 experiments. ATL: Conceived the study, supervised research and provided strains and
860 plasmids. TG: Performed targeted and label-free mass spectrometry-based quantitative
861 proteomics. NW: Helped with the *in vitro* analyses of MalE-TfcP. EH: Generated the PilY1.1
862 antibodies. AJP: Conceived and supervised the *in vitro* analyses of MalE-TfcP. LSA: Conceived
863 the study, supervised research and provided funding. MH, ATL, TG, AJP and LSA: Analysed
864 and interpreted data and wrote the manuscript.

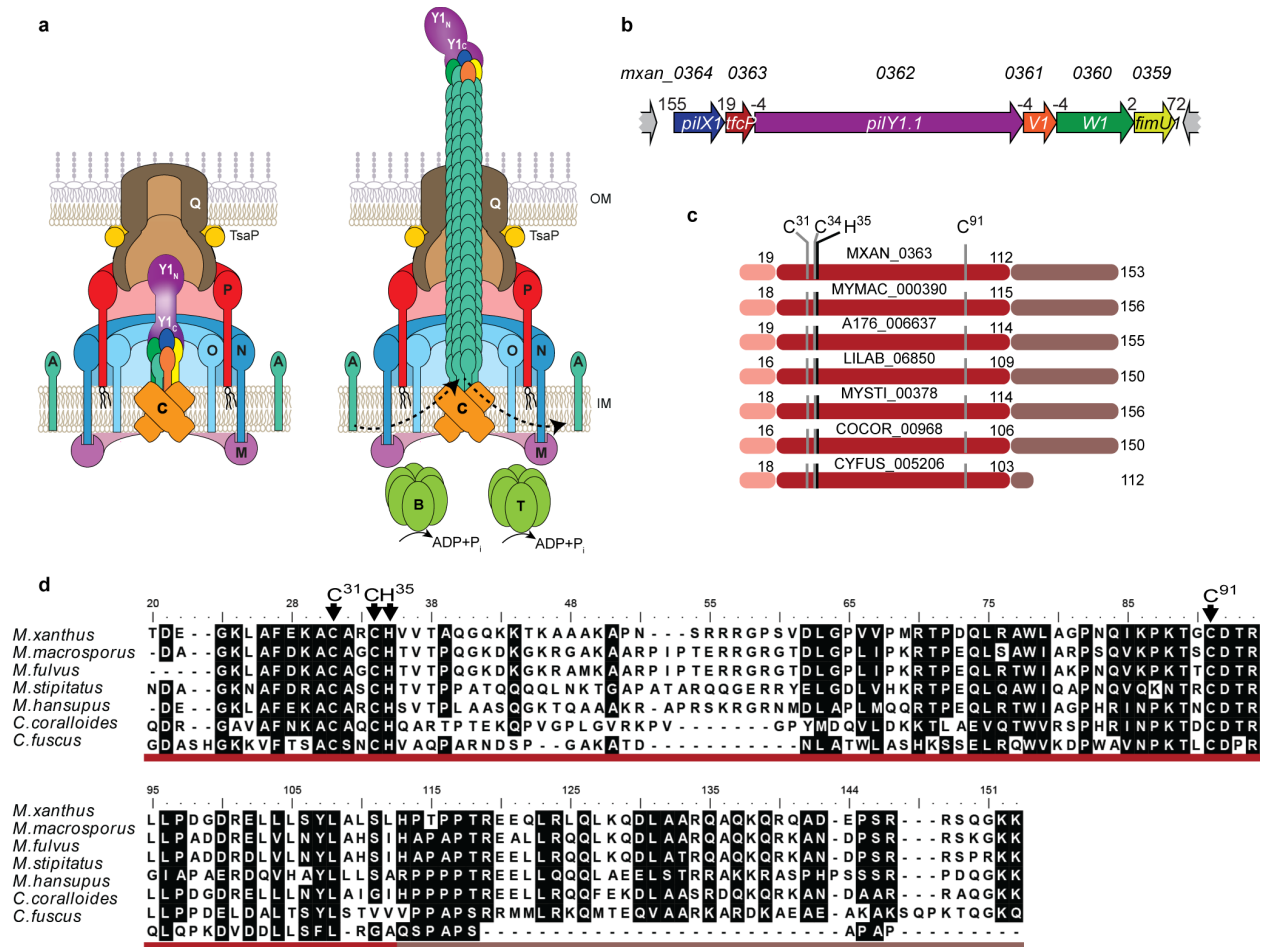
865 All authors approved the final manuscript.

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867 **Competing interests.** The authors declare no competing interests.

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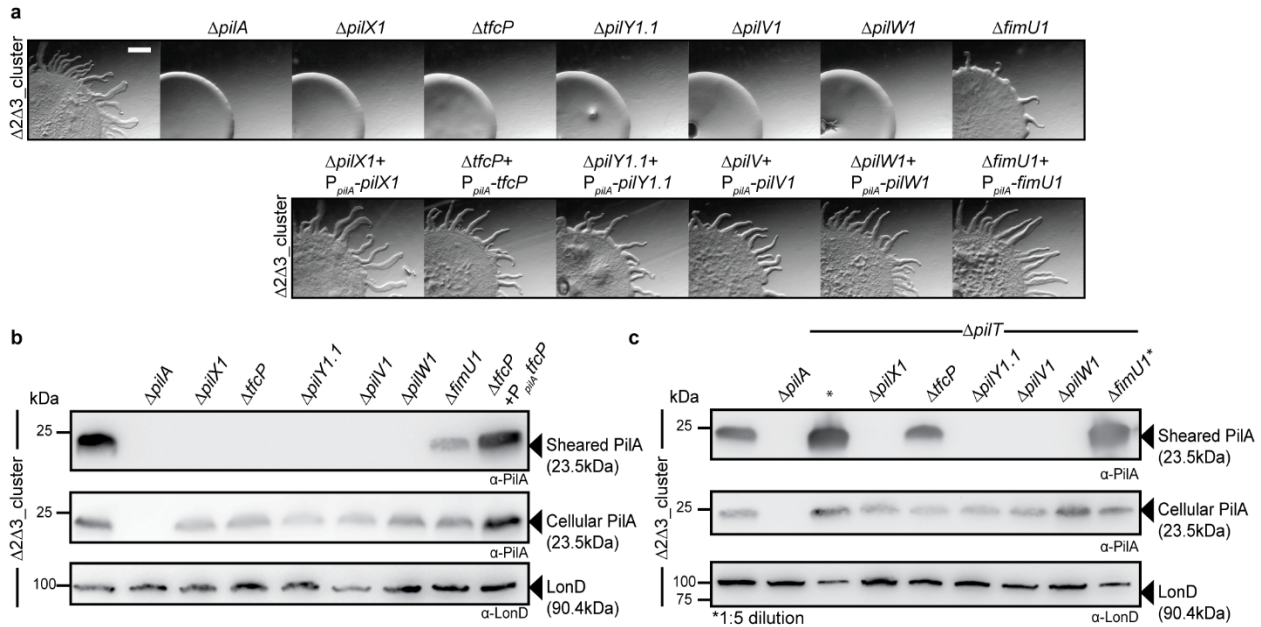
872 Figure 1. TfcP is a non-canonical cytochrome c

873 **a** Architectural model of non-piliated and pilated T4aPM. PilB and PilT associate with PilC in a
874 mutually exclusive manner during extension and retraction, respectively. Bent arrows,
875 incorporation and removal of the major pilin PilA from the pilus base during extension and
876 retraction, respectively. Proteins labelled with single letters have the Pil prefix. Y1_N and Y1_C
877 indicate the N- and C-terminal domains of PilY1, respectively. The colour code for the four minor
878 pilins is as in **b**. **b** Genetic organisation of cluster_1 encoding minor pilins, PilY1.1 and TfcP.

879 Locus tags are included above and gene names within genes. Distances between start and stop
880 codons are shown above. **c** Domain architecture of TfcP and homologs. Pink: Type I signal
881 peptide, red: Cytochrome c domain, and brown: C-terminal extension. The cytochrome c
882 signature motif CxxCH and the distal Cys⁹¹ residue are indicated. Numbering of amino acids is
883 according to the unprocessed, full-length protein.

884 **d** Sequence alignment of TfcP and homologs. Residues are highlighted based on >80%
885 similarity. Domains are indicated using the color code from **c**. The cytochrome c signature motif
886 CxxCH and the distal Cys⁹¹ residue are indicated. Numbering of amino acids is according to the
887 unprocessed, full-length protein.

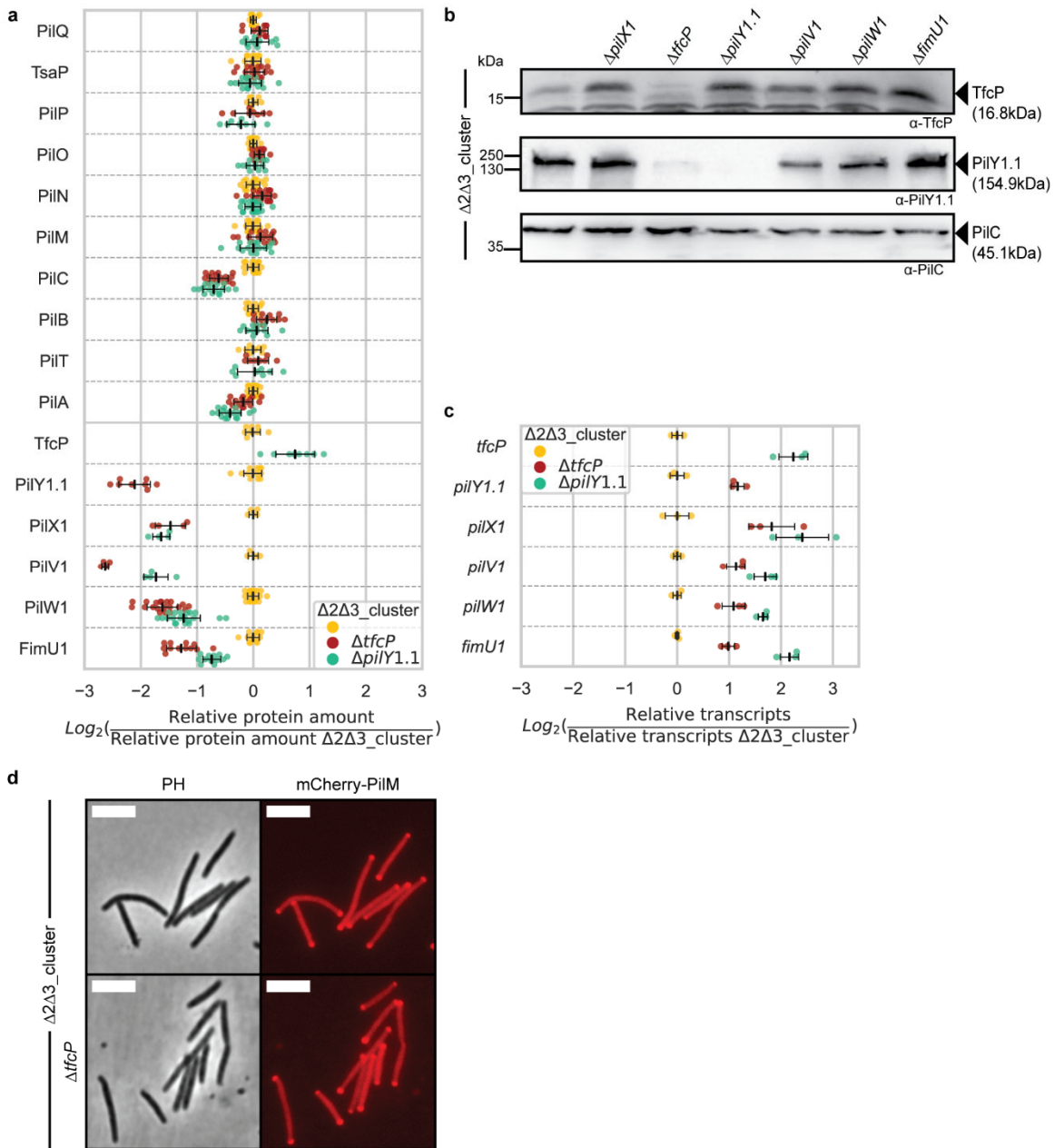
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891 **Figure 2. TfcP, minor pilins and PilY1.1 of cluster_1 are important for T4aPdM and T4aP**
892 **formation**

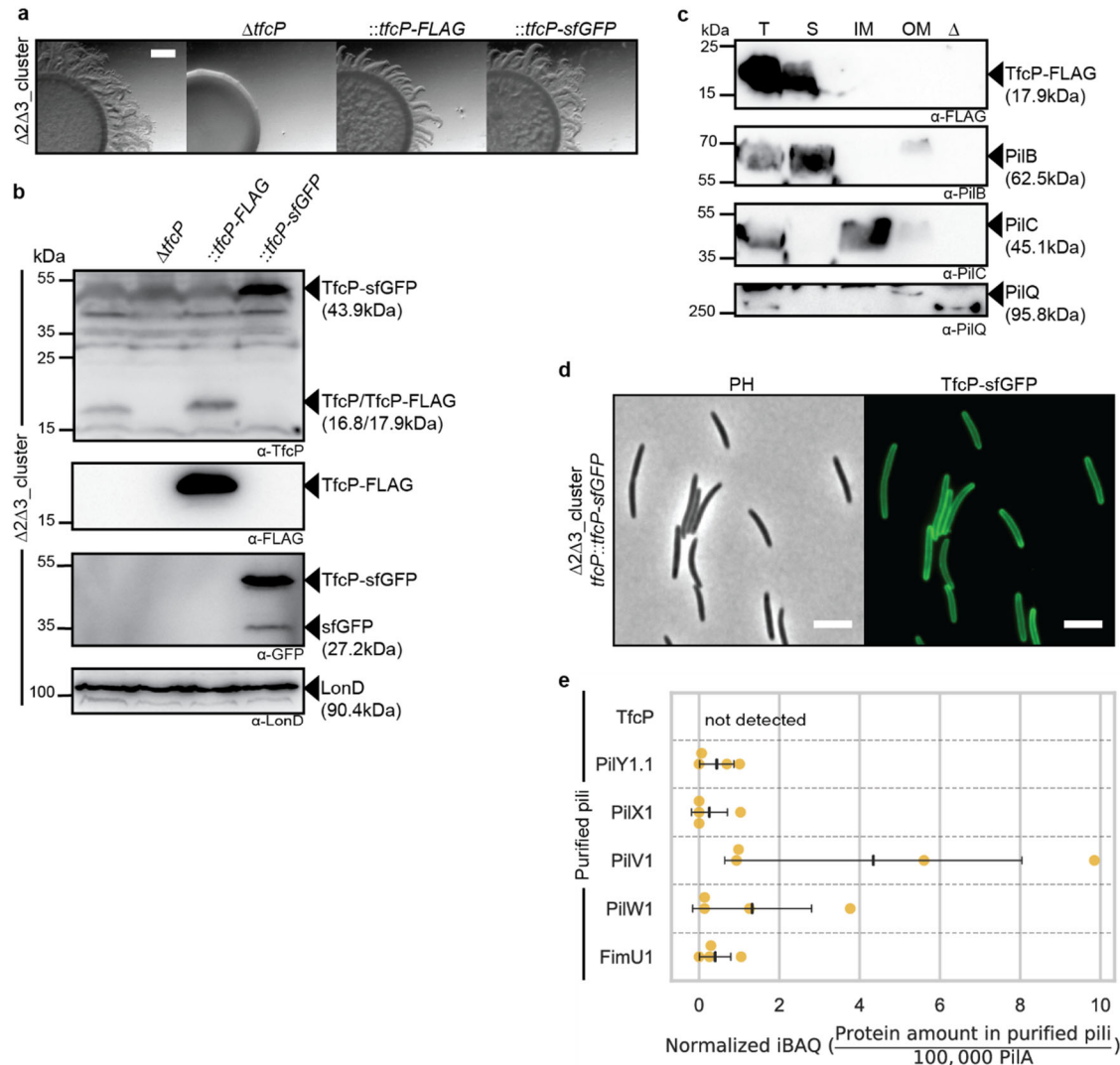
893 **a** Assay for T4aPdM. WT $_{\Delta 2\Delta 3}$ and strains with deletions of individual cluster_1 genes, and the
894 corresponding complementation strains were spotted on 0.5% agar supplemented with 0.5%
895 CTT and imaged after 24 hrs. Scale bar, 1 mm. **b** Shearing assay for T4aP formation. T4aP
896 sheared off from ~15 mg cells grown on 1.5% agar supplemented with 1.0% CTT were
897 separated by SDS-PAGE and probed with α -PilA antibodies (top panel). Middle panel, 40 μ g of
898 protein from total cell extracts separated by SDS-PAGE and probed with α -PilA antibodies and,
899 after stripping, with α -LonD antibodies as a loading control (lower panel). **c** Shearing assay for
900 T4aP formation in retraction deficient strains. T4aP-formation was assayed as in **b**. In lanes
901 labeled with *, five-fold less protein was loaded.
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Figure 3. TfcP is important for stability of PilY1.1 and minor pilins of cluster_1.
a Accumulation of proteins of the T4aPM and cluster_1. Cells were grown in 1.0% CTT suspension culture. Relative protein amounts were determined using targeted proteomics with one to five heavy labelled reference peptides for each protein spiked into the trypsin-digested cell lysates (Methods). To calculate relative protein amounts, the light-to-heavy intensity ratio of the endogenous (light) and reference (heavy) peptide was calculated. For every strain, four biological replicates were analysed. Individual data points represent the log_2 ratio of the relative amount of one peptide in one biological replicate to the mean relative amount of the same peptide in the WT $_{\Delta 2\Delta 3}$ strain (calculated from the four biological replicates). Center marker and error bars in black: Mean and standard deviation (STDEV) of all values for one protein. **b**

915 Immuno-blot analysis of TfcP and PilY1.1 accumulation. Cells were grown in 1.0% CTT
916 suspension culture. Total cell extracts from the same number of cells were separated by SDS-
917 PAGE and analysed by immuno-blotting. PilC was used as loading control. **c** qRT-PCR analysis
918 of transcript levels of cluster_1 genes. Total RNA was isolated from cells grown in 1.0% CTT
919 suspension culture. Individual data points represent three biological replicates with each two
920 technical replicates, and in which the ratio of the relative transcript level in a mutant over the
921 transcript level in the WT_{Δ2Δ3} strain is plotted. Center marker and error bars: Mean and STDEV.
922 **d** Localisation of mCherry-PilM in the *ΔtfcP* strain. Cells were grown in 1.0% CTT suspension
923 culture, placed on 1.0% agarose supplemented with TPM, and immediately imaged by phase
924 contrast (PH) and fluorescence microscopy. Scale bar, 5 μm.
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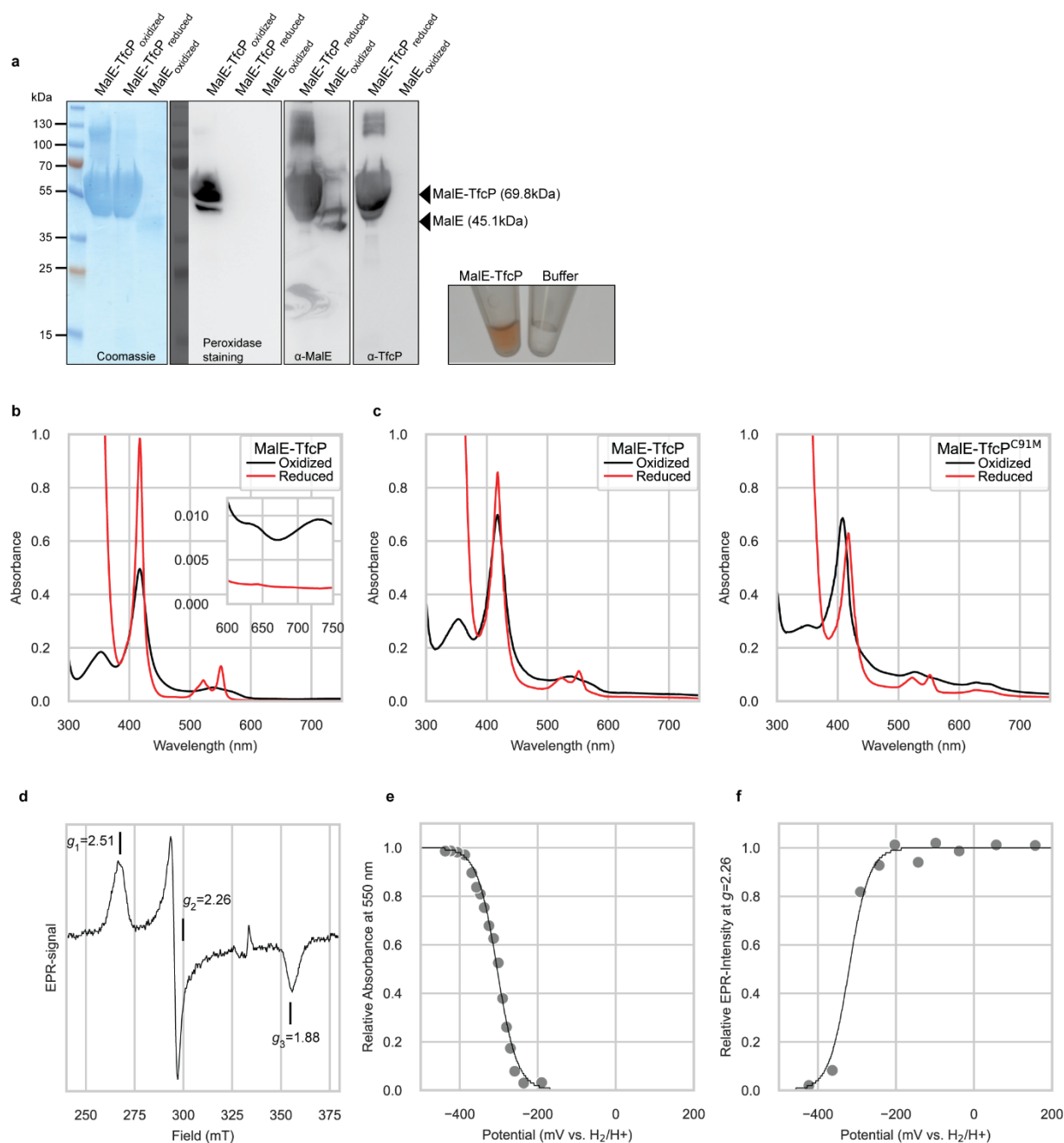
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Figure 4. TfcP is a periplasmic protein.

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a Assay for T4aPdM. Strains were assayed as in Fig. 2a. Scale bar, 1 mm. **b** Immuno-blot analysis of TfcP-FLAG and TfcP-sfGFP accumulation. Cells were grown in 1.0% CTT suspension culture and analysed as in Fig. 3b. **c** Subcellular localisation of TfcP-FLAG. Cells were grown in 1.0% CTT suspension culture and fractionated into fractions enriched for soluble proteins (S), IM proteins (IM) and OM proteins (OM). T indicates total cell extract. In the lane marked Δ , total cell extract of the $\Delta pilBTCMNOPQ$ mutant was used as negative control. Protein from the same number of cells was loaded per lane, and analysed by immuno-blotting. PilB, PilC and PilQ serve as controls for the fractionation and localise to the cytoplasm, IM and OM, respectively^{12,36}. **d** Determination of TfcP-sfGFP localisation. Cells were grown in 1.0% CTT suspension culture, and analysed as in Fig. 3d. Scale bar, 5 μ m. **e** Label-free quantification (LFQ) of cluster_1 proteins in purified pili. Pili were isolated from cells grown on 1.5% agar supplemented with 1.0% CTT. Normalised iBAQ (intensity based absolute quantification) values (Methods) were determined in four biological replicates for WT $_{\Delta 2\Delta 3}\Delta pilT$ and the negative control WT $_{\Delta 2\Delta 3}\Delta pilT\Delta pilB$. iBAQ values of WT $_{\Delta 2\Delta 3}\Delta pilT$ were background corrected by subtraction of the

942 mean iBAQ value of the four replicates of the negative control and rescaled to the iBAQ value of
943 100,000 PilA molecules in the same sample. Center marker and error bars: Mean and STDEV.
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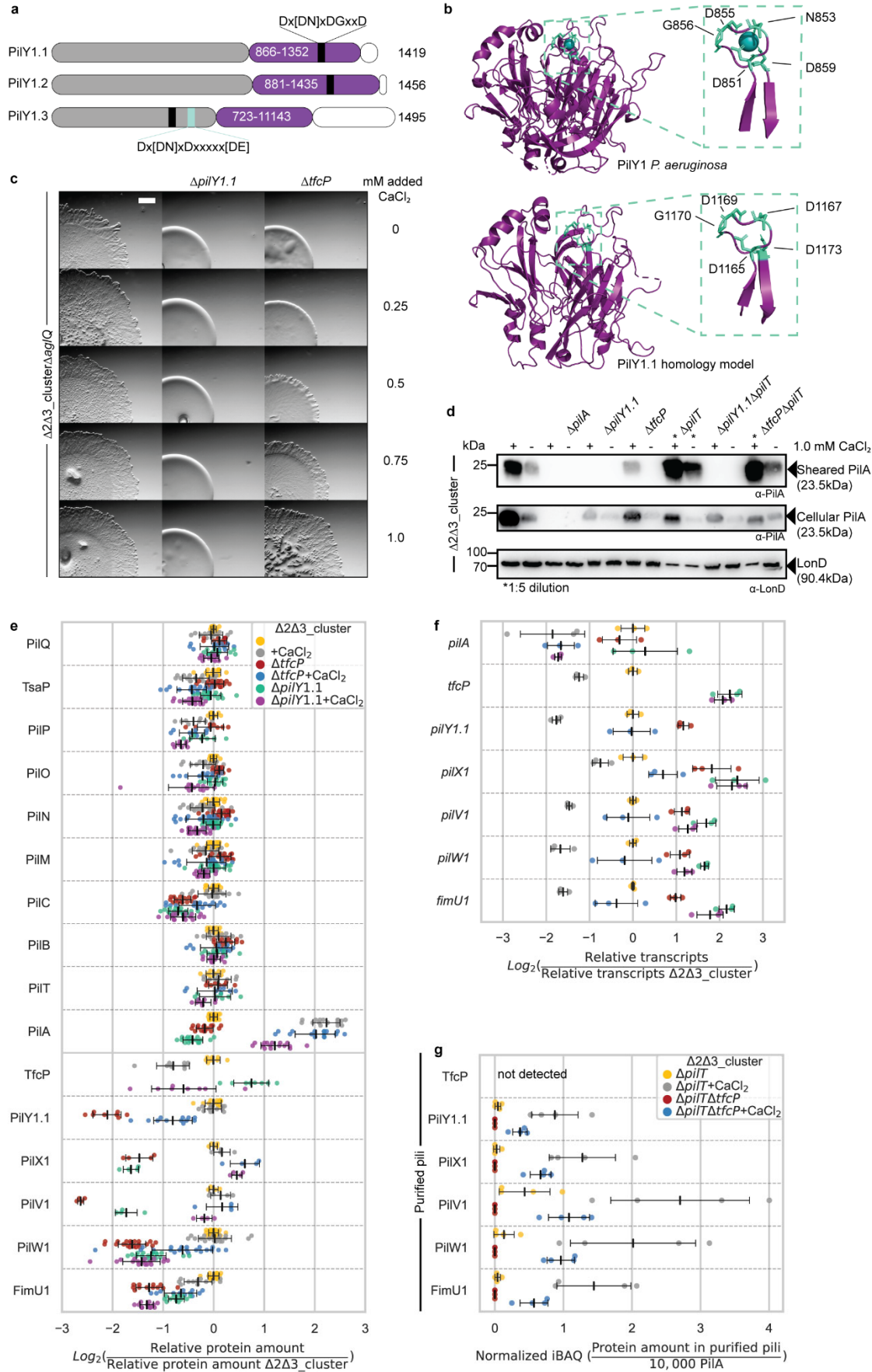


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Figure 5. TfcP is a redox active, heme-binding protein.

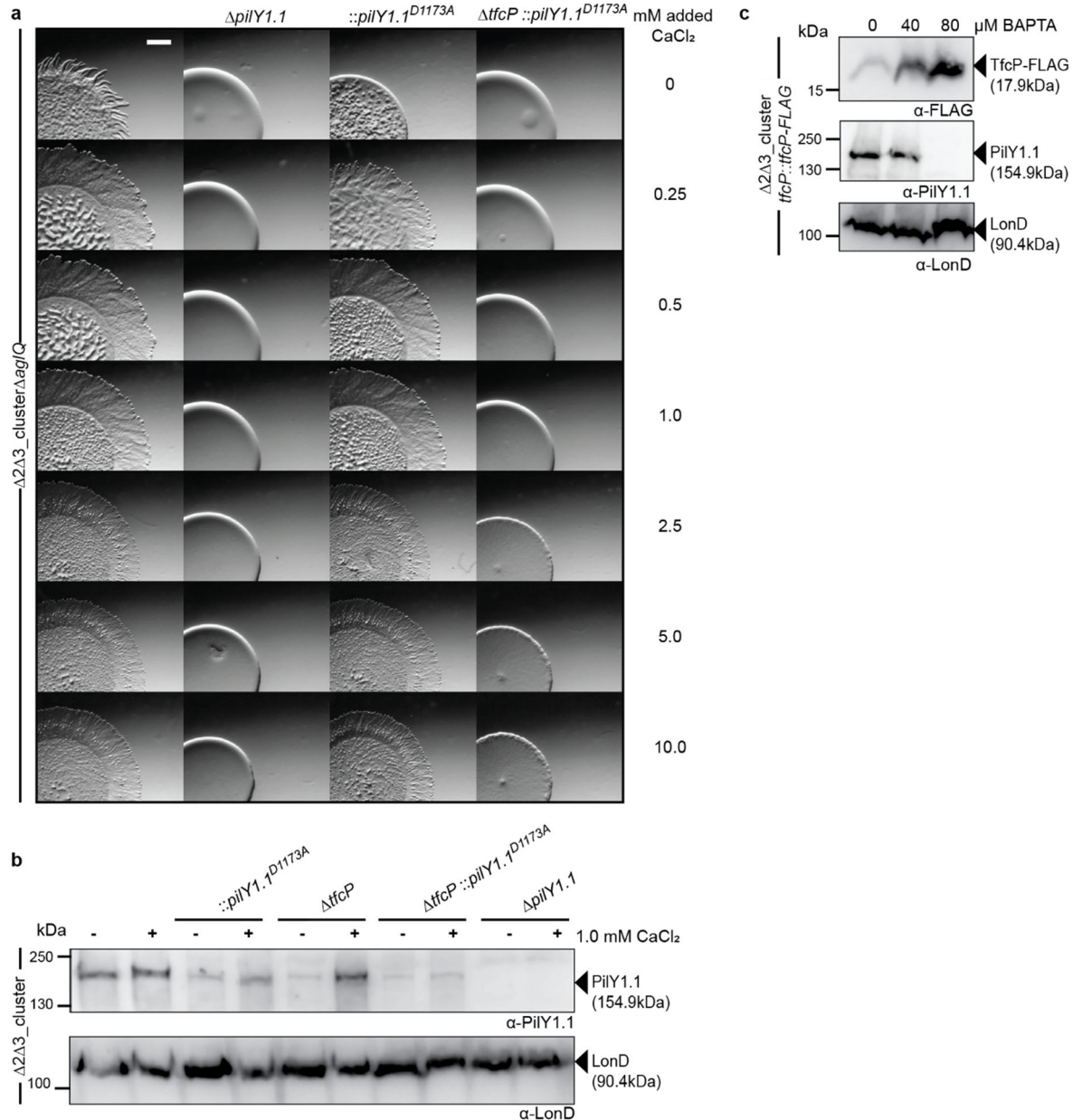
a TfcP heme-binding assay. Panels from left-to-right, MalE-TfcP in oxidised (as purified) and reduced state (after addition of DTT) stained with Coomassie Blue, analysed for heme-binding by peroxidase staining using a luminescent horse radish peroxidase (HRP) substrate and MalE as negative control, detected by immuno-blotting with α -MalE and α -TfcP as indicated, and image of purified MalE-TfcP in buffer. **b** UV-Vis spectra of purified MalE-TfcP in the oxidised and reduced (after addition of sodium-dithionite) state. Inset shows the absorbance in the 600-750 nm region. Experiment was done using a Shimadzu 1900 spectrophotometer. **c** UV-Vis spectra of purified MalE-TfcP variants in the oxidised and reduced state. Experiments were done using a Tecan200Pro plater reader and, therefore, the spectrum of WT TfcP is included

956 again. **d** EPR spectrum of MalE-TfcP. Spectra were recorded in the oxidised state at 12 K,
957 0.32 mW microwave power, 1.5 mT modulation amplitude (9.3523 GHz). **e** Redox titration of
958 MalE-TfcP. The 550 nm absorbance at 23°C is plotted versus the solution potential and fitted to
959 the Nernst equation. **f** Redox titration of MalE-TfcP following the EPR-intensity at $g=2.26$ of
960 samples poised at indicated solution redox potentials.
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963 **Figure 6. Added CaCl₂ compensates for lack of TfcP.**

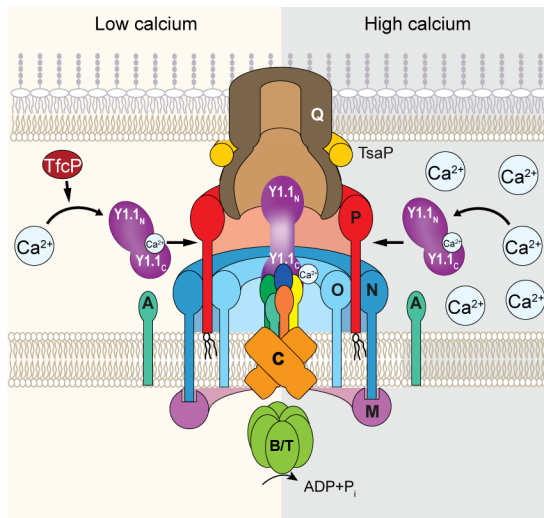
964 **a** Domain architecture of PilY1 proteins of *M. xanthus*. Purple, C-terminal PilY1 domain; grey, N-
965 terminal domains; white; C-terminal sequences. EF hand-like calcium binding motif is in black
966 together with the consensus sequence; light blue box indicates the second calcium binding motif
967 in PilY1.3 together with the consensus sequence²⁵. **b** Comparison of PilY1 structure of *P.*
968 *aeruginosa* (PDB: 3HX6)²⁷ and a homology model of PilY1.1; inset, zoom of calcium binding
969 motif. **c** Assay for T4aPdM. Cells were grown in 1.0% CTT suspension culture and plated on
970 0.5% agar supplemented with 0.5% CTT without or with added CaCl₂ and imaged after 24 hrs.
971 Final concentrations of added CaCl₂ are indicated. Scale bar, 1 mm. **d** Shearing assay for T4aP
972 formation. T4aP sheared off from ~15 mg cells grown on 1.5% agar supplemented with 1.0%
973 CTT and 1.0 mM CaCl₂ as indicated, and analysed as in Fig. 2b. **e** Accumulation of proteins of
974 the T4aPM. Cells were grown in 1.0% CTT suspension culture without or with 1.0 mM added
975 calcium as indicated. Proteins were quantified as in Fig. 3a. Data for samples without added
976 CaCl₂ are the same as in Fig. 3a and included for comparison. **f** qRT-PCR analysis of transcript
977 levels of cluster_1 genes and *pilA*. Total RNA was isolated from cells grown in 1.0% CTT
978 suspension culture without or with added calcium as indicated. Transcripts were quantified as in
979 Fig. 3c. Colour code is as in **e**. Data for samples without added CaCl₂ are the same as in Fig. 3e
980 and included for comparison. **g** LFQ proteomics of cluster_1 proteins in purified pili. Pili were
981 isolated from cells grown on 1.5% agar supplemented with 1.0% CTT without or with added
982 CaCl₂ as indicated. Normalised iBAQ values were calculated as in Fig. 4e and background
983 corrected by subtraction of the mean iBAQ value of the four replicates of the relevant negative
984 control, and rescaled to 10,000 PilA molecules in the same sample. Data for WT_{Δ2Δ3} without
985 added CaCl₂ are the same as in Fig. 4e and included for comparison.
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Figure 7. Calcium binding by PilY1.1 is essential for TfcP function.

a Assay for T4aPdM. Cells were grown in 1.0% CTT suspension culture and plated on 0.5% agar supplemented with 0.5% CTT and imaged after 24 hrs. The final concentration of added CaCl₂ is indicated. Scale bar, 1 mm. **b** Accumulation of PilY1.1 variants. Cells were grown in 1.0% CTT suspension culture without or with 1.0 mM CaCl₂, total cell extract isolated and analysed by immuno-blot as in Fig. 3b. **c** Accumulation of TfcP-FLAG and PilY1.1 in presence of BAPTA. Cells were grown in 1.0% CTT in suspension, exposed to indicated concentrations of BAPTA for 16 hrs, total cell extract isolated, and analysed by immuno-blot as in Fig. 3b.



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1000 **Figure 8. Model of TfcP function at low and high calcium concentrations.** For simplicity,

1001 PiiB and PiiT are not shown separately. Y1.1_N and Y1.1_C indicate the N- and C-terminal

1002 domains of PiiY1, respectively. The colour code for the four minor pilins is as in Fig. 1b.

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