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PII: S0923-2508(23)00050-5

DOI: https://doi.org/10.1016/j.resmic.2023.104075

Reference: RESMIC 104075

To appear in: *Research in Microbiology*

Received Date: 28 September 2022

Revised Date: 25 April 2023

Accepted Date: 26 April 2023

Please cite this article as: Y. Li, J. Santos-Moreno, O. Francetic, The periplasmic coiled coil formed by the assembly platform proteins PulL and PulM is critical for function of the *Klebsiella* type II secretion system, *Research in Microbiologoy*, https://doi.org/10.1016/j.resmic.2023.104075.

The periplasmic coiled coil formed by the assembly platform proteins PulL and PulM is critical for function of the *Klebsiella* type II secretion system

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ABSTRACT

Bacteria use type II secretion systems (T2SS) to secrete to their surface folded proteins that confer diverse functions, from nutrient acquisition to virulence. In the *Klebsiella* species, T2SS-mediated secretion of pullulanase (PulA) requires assembly of a dynamic filament called the endopilus. The inner membrane assembly platform (AP) subcomplex is essential for endopilus assembly and PulA secretion. AP components PulL and PulM interact with each other through their C-terminal globular domains and transmembrane segments. Here, we investigated the roles of their periplasmic helices, predicted to form a coiled coil, in assembly and function of the PulL–PulM complex. PulL and PulM variants lacking these periplasmic helices were defective for interaction in the bacterial two-hybrid (BACTH) assay. Their functions in PulA secretion and assembly of PulG subunits into endopilus filaments were strongly reduced. Interestingly, deleting the cytoplasmic peptide of PulM nearly abolished the function of variant PulMΔN and its interaction with PulG, but not with PulL, in the BACTH assay. Nevertheless, PulL was specifically proteolyzed in the presence of the PulMΔN variant, suggesting that PulM N-terminal peptide stabilizes PulL in the cytoplasm. We discuss the implications of these results for the T2S endopilus and type IV pilus assembly mechanisms.

Keywords:

Type II secretion system, Endopilus; type IV pili; assembly platform; pullulanase; *Klebsiella*

1. Introduction

Bacteria have an astonishing capacity to adapt to diverse environments, growth conditions and nutrient sources. Understanding the molecular basis of this adaptation is fundamentally important and might lead to applications in biotechnology and bioremediation. Among the numerous nanomachines that contribute to bacterial adaptation to diverse environments, type II secretion system (T2SS) plays a prominent role [1].

T2SS is typically composed of 15 proteins forming a megadalton complex that spans the envelope of Gram-negative bacteria. Recent reviews summarize our current knowledge of T2SS architecture and function [2, 3]. In the outer membrane, 15 subunits of the GspD protein form the secretin channel that allows the passage of large folded proteins or their complexes to the cell surface [4, 5, 6]. According to the current models, protein secretion is driven by the polymerization of periplasmic filaments called endopili (formerly pseudopili, [7]) in the inner membrane. The core of the endopilus is a helical homopolymer composed of the major subunit GspG [8, 9]. The minor pilins GspH, I, J and K form a complex at the endopilus tip [10, 11] and initiate endopilus assembly [12]. The minor pilins are essential for protein secretion and have also been implicated in substrate recognition [13]. Endopili are thought to be continuously polymerized by the inner membrane assembly platform (AP) subcomplex composed of GspF, GspL and GspM proteins [14]. GspL forms a stable complex with the cytoplasmic ATPase GspE [15, 16] and is itself stabilized by GspM [17, 18]. During endopilus elongation, GspM promotes targeting of the GspG and GspH pilins to the AP [19, 20]. In the cytoplasm, cycles of ATP binding and hydrolysis promote conformational changes of the hexameric GspE ATPase complex [21, 22]. These

changes are proposed to induce rotational movements of the central AP component GspF that orchestrates endopilus assembly [23].

Deciphering the structural basis of endopilus assembly is important to understand how T2SS functions at the molecular level. Structure-function analyses have provided molecular insight into the majority of individual T2SS components. Extending this knowledge towards larger subcomplexes will bring clues on the connectivity and cooperation between the different nanomachine components. So far, cryo-electron microscopy (cryo-EM) has been essential to gain insight into the secretin channel structure [4, 6], a large mostly pentadecameric complex with a periplasmic gate. Combining cryo-EM, nuclear magnetic resonance (NMR), modeling and mass spectrometry provided a detailed view of the pilus core [24, 25] and predicted its connection to the tip complex [26].

Recent studies of the *Klebsiella oxytoca* T2SS that secretes pullulanase (PulA) [27] combined X-ray crystallography and NMR to obtain information on the structure of a complex formed between C-terminal domains (CTDs) of PulL (GspL) and PulM (GspM) [28]. Mutations at the interface of these CTDs affect the stability of the two proteins and their function in protein secretion. Cys crosslinking characterized the hydrophobic interface at the membrane level, and led to a model of the PulL – PulM complex (lacking the cytoplasmic domain of PulL) (Fig. 1A). This model predicts additional interactions *via* periplasmic helices of the two partners. Here we investigated the importance of this interface for the formation and function of the PulL – PulM complex. In addition, we studied the role of the short cytoplasmic region of PulM in the stability and function of the AP complex.

2. Materials and methods

100 *2.1. Bacterial strains and culture conditions*

101 *Escherichia coli* strain DH5αF'*lacI*^{Q} was used for cloning and transformation
102 experiments. Functional assays were performed in *E. coli* PAP7460 [29] and PAP5378
103 strains. PAP5378 was constructed by P1 transduction by introducing the *ompT::kan*
104 gene from the Keio collection strain BW2511 *ompT::kan* into strain PAP5299 [12]. The
105 *kan* cassette was then deleted thanks to the helper plasmid pCP20 as described [30]
106 to give strain PAP5299 *ΔompT*. The bacterial two-hybrid assays were performed in
107 strain DHT1 [31]. Bacteria were grown at 30°C or 37°C in Lysogeny Broth (LB) [32]
108 supplemented with antibiotics as required: ampicillin (Ap) at 100 μg.mL^{-1},
109 chloramphenicol (Cm) at 25 μg.mL^{-1}, Kanamycin (Km) at 25 μg.mL^{-1}. Solid LB media
110 contained 1.5% agar (Difco). Expression of genes under *lacZ* promoter control was
111 induced with 1 mM isopropyl-β-1-D-thiogalactopyranoside (IPTG). The *pul* gene
112 promoters were induced with 0.4% D-maltose. For secretion assays, media were
113 buffered with 0.1 volume of M63 salt solution [32].
114
115 *2.2. Plasmid construction and site-directed mutagenesis*
116 Plasmids used in this study are listed in Table 1. Site-directed mutagenesis was
117 performed using a modified QuickChange protocol, with fully overlapping
118 oligonucleotide primers listed in Table S1 (Eurofins). Template DNA was amplified with
119 high fidelity DNA polymerase Q5 (New England Biolabs) in two-steps: first, 6 cycles of
120 amplification were performed with forward and reverse primers alone. The reactions
121 were mixed and another 15 cycles of amplification were performed. The DNA was
122 treated with *Dpn*I enzyme (Fermentas) and transformed into DH5αF' ultracompetent
123 cells prepared as described [33]. Isolated colonies were inoculated into 5 mL of LB
124 with appropriate antibiotics and plasmids were purified using the alkaline lysis protocol

and Qiaquick miniprep kit (Qiagen). Plasmids were verified by sequencing (GATC, Eurofins). For the *pulL* bacterial two hybrid constructs, vectors pUT18C and pKT25 were digested with *Eco*RI and *Kpn*I and the purified vector fragments were ligated with the PCR amplified *pulL Δ270-302* digested the same enzymes. For the *pulM* cloning, the BACTH vectors and PCR fragments were digested with *Eco*RI and *Bam*HI. Deletion of *pulM* residues 2-15 in plasmid pCHAP8882 was performed by QuickChange mutagenesis using primers PulM delN5 and PulM delN3 (Table S1). The same approach was used to introduce PulM2-15 deletion in the BACTH constructs pCHAP8154 and pCHAP8155 with primers PulM delN For and PulM delN Rev2 (Table S1).

2.3. Protein electrophoresis and Western blot analysis

Total bacterial extracts were analyzed on denaturing sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the Tris-tricine gels [34]. Proteins were transferred on nitrocellulose membranes using the Fast Blot semi-dry transfer system and one-step transfer buffer (Thermo). Membranes were blocked with 5% skim milk in Tris-buffer saline containing 0.05% Tween-20 (TBST) for one hour and probed with primary antibodies diluted in 5% skim milk in TBST. Polyclonal anti-PulM and anti-PulL sera were used at 1:1000 dilution, anti-PulA and anti PulG sera were diluted at 1:2000 and the monoclonal anti-CyaA antibodies were used at 1:10000. After four 10-min washes in TBST, membranes were incubated 1 hour in the secondary goat anti-rabbit or anti-mouse antibodies coupled to horse-radish peroxidase (Amersham) (1:10000). Fluorescence signals were developed with ECL2 (Thermo) and recorded on Typhoon FLA9000 (GE, Cytiva). Chemiluminescence signals were developed with ECL (Thermo) and recorded on an Amersham 680 imager.

2.4. PulA secretion assays

Secretion of the non-acylated variant of PulA was assessed by cell fractionation in strain PAP7460 harboring plasmid pCHAP8251 complemented with pCHAP8258 derivatives carrying *pulL* variants or pCHAP8496 complemented with pCHAP1353 derivatives carrying *pulM* variants. Bacteria were cultured overnight at 30°C in LB containing Ap and Cm, then inoculated in inducing medium containing in addition 0.4 % D-maltose and 0.1 vol of M63 salts for another 4 hours. Cultures were normalized to OD_{600nm} of 1. Bacterial pellets were collected after 5-min centrifugation at 16000 x g in a table-top Eppendorf centrifuge at 4°C and resuspended in an equal volume of SDS sample buffer. Supernatants were subjected to another 10-min round of centrifugation and 0.1 mL was taken off top, then mixed with 0.1 mL of 2 x SDS sample buffer. Cell and supernatant fractions from the same amount of cultures were analyzed by SDS-PAGE on 10% Tris-glycine gels followed by Western blot with anti-PulA antibodies. PulA bands were quantified with ImageJ and the fraction of PulA in the supernatant was calculated. Data were plotted and analyzed with GraphPad Prism9 software.

2.5. PulG pilus assembly assays

To quantify the PulG pilus assembly, bacteria of strain PAP7460 harboring the *pul* operon on plasmid pCHAP8185 and derivatives were cultured for 48 h or 72 h at 30°C on LB plates containing 1.5 % agar (Difco), appropriate antibiotics and 0.2% D-maltose. Bacteria were resuspended in 1 mL of LB and the cell density was normalized to OD_{600nm} of 1. The suspensions were vigorously vortexed for 1 min to detach surface pili. Bacterial pellets were then collected by 5-min centrifugation at 4°C and 16000xg and resuspended in SDS sample buffer at the concentration of 10 $OD_{600nm}.mL^{-1}$. The

pili-containing supernatants were cleared from remaining bacteria by a 10-min centrifugation at 16000xg and 0.7 mL was mixed with tri-chloro-acetic acid at a final concentration of 10%. After a 30-min precipitation on ice, the samples were centrifuged for 30 min at 4°C and 16000 x g. Pellets were washed twice with acetone and air dried, then resuspended in 70 μL of SDS sample buffer. Equivalent volumes were analyzed by SDS-PAGE on 10 % Tris-tricine gels followed by Western blot with anti-PulG antibodies. PulG bands were quantified using ImageJ and the results were analyzed and plotted with Prism 9 software (GraphPad).

2.6. Bacterial two-hybrid assays

Bacterial two-hybrid (BACTH) assay was used to study interactions between proteins fused to T18 and T25 fragments of the CyaA catalytic domain [35]. Plasmids were co-transformed into *E. coli* DHT1 and grown on LB Ap Km plates for 48-60 hours at 30°C. Independent single co-transformants were picked at random, inoculated into 1 mL of LB Ap Km and grown overnight at 30°C. These precultures were used to inoculate 1-mL cultures of LB Ap Km containing 1 mM IPTG, which were incubated 4-6 hours at 30°C with shaking. These cultures were used to measure beta-galactosidase activity as described [32]. The data were plotted and analyzed using the Prism 9 software (GraphPad).

3. Results

3.1. The periplasmic helices of PulL and PulM are important for T2SS function

PulL and PulM share a similar architecture of their transmembrane and periplasmic domains (Fig. 1A). Their hydrophobic alpha-helical transmembrane segments extend into polar periplasmic helices, followed by the flexible linkers connecting them to the ferredoxin-like C-terminal domains (CTDs). The two periplasmic helices are predicted to form a coiled coil [28] (Fig. 1A). To test their functional importance, we generated PulL and PulM variants lacking these periplasmic helices. In PulL, we removed the region comprising residues 270 to 302 (Fig. 1A) to yield variant PulL$^{\Delta CC}$, encoded by plasmid pMS1349 (Table 1). We first tested the ability of PulL$^{\Delta CC}$ to promote secretion of the nonacylated variant of pullulanase (PulA), the substrate of the *K. oxytoca* T2SS. Whereas the native PulL (PulL^{WT}) promoted efficient PulA secretion, the majority of the PulA pool accumulated in the cell fraction in the presence of PulL$^{\Delta CC}$ (Fig. 1B,C). No extracellular PulA was detected in the negative control lacking PulL. We used antibodies directed against PulL^{CTD} to assess PulL stability in these strains (Fig. 1D). Quantification of PulL$^{\Delta CC}$ signals showed that protein levels were reduced by about 30% compared to the native PulL (Fig. 1E). The *pulL$^{\Delta CC}$* mutation did not affect PulM levels (Fig. 1F,G).

To study the role of the periplasmic helix of PulM, we removed the region comprising residues 37 to 68 (Fig. 1A) resulting in variant PulM$^{\Delta CC}$ encoded by plasmid pMS1350 (Table 1). This deletion significantly reduced PulA secretion efficiency (Fig. 2A,B), whereas PulM $^{\Delta CC}$ stability was reduced by 10% on average compared to PulM (Fig. 2C,D). While PulL levels were reduced in *pulM* mutant, as expected, PulL stability was comparable in *pulM^{WT}* and in the *pulM $^{\Delta CC}$* mutant (Fig. 2E,F).

220

221 *3.2. Pilus assembly defects in* pulL$^{\Delta CC}$ *and* pulM$^{\Delta CC}$ *mutants*

222 The AP components PulL and PulM directly participate in endopilus assembly,
223 which is thought to drive PulA secretion. We therefore asked whether the periplasmic
224 helices of PulL and PulM are required for PulG pilus assembly. Under conditions where
225 bacteria are cultured on solid media, *E. coli* harboring a moderate copy-number
226 plasmid encoding a complete set of *pul* genes produces surface pili composed of PulG,
227 the major pilin subunit [36]. We tested the ability of PulL$^{\Delta CC}$ and PulM$^{\Delta CC}$ to restore this
228 function in mutants lacking plasmid-encoded *pulL* (pCHAP8251) or *pulM*
229 (pCHAP8496). After 48 h of growth in the presence of maltose to induce the *pul* gene
230 expression, bacteria complemented with wild type *pulL* and *pulM* genes produced pili
231 that could be sheared from their surface and separated from the cell-associated PulG
232 pool (Fig. 3). In the presence of PulL$^{\Delta CC}$ and PulM$^{\Delta CC}$ variants, the PulG pilus assembly
233 was nearly abolished (Fig. S1). Only upon extending the growth period to 72 h, we
234 could detect some PulG pili on the surface of the coiled coil deletion mutants. Piliation
235 levels were strongly and significantly reduced by the deletions of periplasmic helices
236 (Fig. 3).

237

238 *3.3. The periplasmic coiled coil is required for assembly of the PulL–PulM*
239 *complex*

240 To determine whether the deletion of periplasmic helices affects the PulL–PulM
241 interaction, we used the bacterial two-hybrid (BACTH) assay based on the
242 reconstitution of CyaA adenylyl cyclase signaling pathway [35]. Full-length PulL, PulM
243 and their variants were fused to the C-termini of T18 and T25 CyaA fragments (Table
244 1). The plasmid constructs were co-transformed in *E. coli cya* mutant strain DHT1 to
245 evaluate the reconstitution of adenylyl cyclase activity. By measuring the cAMP-

246 dependent beta-galactosidase activity, we compared interactions of full-length PulL
247 and PulM constructs described previously [28] with constructs harboring the PulL$^{\Delta CC}$
248 and PulM$^{\Delta CC}$ variants.

249 Bacteria containing plasmids encoding T18-PulM and T25-PulL showed high
250 beta-galactosidase activity, comparable to that of the yeast leucin zipper positive
251 control, indicating efficient heterodimer formation as observed previously [28] (Fig. 4A).
252 In strains harboring T18-PulM$^{\Delta CC}$ and T25-PulL, the mean activity was significantly
253 reduced. Deleting the PulL helix in T25-PulL$^{\Delta CC}$ had an even stronger effect and nearly
254 abolished the interaction with T18-PulM. Combining the two deletions in T18-PulM$^{\Delta CC}$
255 and T25-PulL$^{\Delta CC}$ slightly increased the mean activity, presumably by placing the
256 respective CTDs at the same level relative to the membrane to facilitate their contacts.
257 However, this increase was weak and not statistically significant in this experimental
258 context (Fig. 4A).

259 Strains harboring the T18-PulL and T25-PulM hybrids showed weaker β-
260 galactosidase activity overall (Fig. 4B), probably due to the generally lower levels of
261 PulL relative to PulM [28]. In this context, beta-galactosidase activity was reduced to
262 background levels upon removal of the periplasmic helix residues in T18-PulL$^{\Delta CC}$ or
263 T25-PulM$^{\Delta CC}$ and combining the two deletions did not measurably improve the
264 interaction (Fig. 4B). This lack of activity was not due to instability of BACTH constructs,
265 which was verified by Western blot using monoclonal anti-CyaA antibodies directed
266 against T18 fragment (Fig. S2). Overall, the BACTH data suggest that the periplasmic
267 coiled coil - forming regions play a major role in the PulL-PulM assembly.

268

269 *3.4. Periplasmic helix of PulM is not required for binding to PulG*

PulM interacts with the pilins PulG and PulH and has been characterized as a targeting factor for these subunits during pilus elongation [19, 20]. Since the deletion of the PulM periplasmic helix affected PulG pilus assembly, we asked whether this was due to an effect on PulM interaction with PulG. The mature portion of PulG fused to the C-terminus of T18 or T25 CyaA fragments was used to probe the interactions with PulM hybrids in the BACTH assay. Although deleting the PulM periplasmic helix reduced the interaction of the T18-PulM – T25-PulG pair, the PulM$^{\Delta CC}$ variant still interacted with PulG strongly and significantly relative to the negative control. In addition, there was no difference in the interaction when T18-PulG was combined with T25-PulM or T25-PulM$^{\Delta CC}$ (Fig. 5A). The β-galactosidase activities of the bacteria co-expressing the *pulG* and *pulL* BACTH constructs were at the level of the negative control, indicating no interaction as shown in previous studies [19]. Like the PulL^{WT}, the PulL$^{\Delta CC}$ variant did not interact with PulG (Fig. 5B). Based on these data, we concluded that it is unlikely that the piliation defect caused by PulM$^{\Delta CC}$ is due to a defect in PulG binding.

3.5. The role of the cytoplasmic PulM peptide

In support of the above conlclusion, the PulM-PulG interaction is affected by mutations neat the N-terminus of mature PulG [20]. This suggests that the corresponding region of PulM, near the cytoplasmic membrane interface is involved in PulG binding. The cytoplasmic region of PulM is a 16-residue peptide, presumably functioning as a positively charged membrane anchor. We hypothesized that this region interacts with the polar N-terminal residues of PulG, as their substitutions in variants PulG^{T2A} and PulG^{E5A} strongly reduce interactions with PulM [19, 20]. To test this hypothesis, we deleted the cytoplasmic peptide of PulM, and studied interactions

of this variant, designated PulMΔN, with PulL and PulG. In the BACTH assay, the PulMΔN variant showed a strong and significant defect in binding to PulG, confirming our predictions (Fig. 6A). This defect was comparable to that of PulM binding to the $PulG^{E5A}$ variant, characterized previously [20]. At the same time, deleting the PulM cytoplasmic peptide did not significantly affect its interactions with PulL in this assay.

We tested the ability of PulMΔN to promote PulA secretion and found that nearly all PulA remained cell-bound in the secretion assay, similar to the negative control lacking PulM (Fig. 6B,C). This phenotype is probably caused by the defective interaction of PulMΔN with PulG. Although the levels of PulMΔN variant were reduced compared to $PulM^{WT}$, the protein was still well produced and migrated on SDS-PAGE according to its expected Mw (~16.5 kDa) (Fig. 6D). Interestingly, this variant seemed to form some SDS-resistant dimers that were not detectable with $PulM^{WT}$. We also tested the levels of PulL in these strains. Surprisingly, although the deletion of PulM N-terminal peptide did not affect its interaction with PulL in the BACTH assay, we found that PulL was strongly destabilized in the presence of PulMΔN, resulting in a proteolytic product of a size similar to that of PulM (Fig. 6E). Antibodies directed against the $PulL^{CTD}$ detected this product, suggesting that it comprises the inner membrane and periplasmic regions of PulL. As it appears to be generated by a specific cleavage that removes the PulL cytoplasmic domain, we designated this protein PulLΔN. These results suggest that the PulM cytoplasmic peptide not only ensures PulG targeting to the assembly site through direct interaction, but also protects PulL from specific proteolysis.

4. Discussion

Structural information on the $PulL_{CTD}$–$PulM_{CTD}$ heterodimer, together with the BACTH and cysteine crosslinking data, allowed Dazzoni and collaborators to propose a structural model of membrane-embedded PulL–PulM complex (Fig. 1A) [28]. Sequence-based predictions [37] and biochemical data [28] suggest that periplasmic helices of PulL and PulM interact together to form a coiled coil. Our mutational, interaction and functional studies described here support this model and show that this coiled coil plays a major role in PulL–PulM assembly. Deleting residues 270-302 of PulL abolished interaction with PulM in the BACTH assay, and removal of PulM residues 37-68 reduced or abolished PulL binding, depending on the plasmid construct and expression levels. In structural models of PulL and PulM, these deletions would shorten their periplasmic helices by 4.8 nm. This might preclude the interaction of ferredoxin-like CTDs in the deletion variant with the native, full-length partner. Consistent with this model, the BACTH interaction signal was somewhat improved when the two deletions were combined in T18-$PulM^{\Delta CC}$ and T25-$PulL^{\Delta CC}$. However, this interaction remained weak overall and was not restored to original levels, suggesting that deleted regions play more than just an alignment role. The binding of $PulL_{CTD}$ to $PulM_{CTD}$ is weak and dynamic in solution [28]. Our results show that this interaction is also weak even in the context of $PulL^{\Delta CC}$ or $PulM^{\Delta CC}$ variants, where the two CTDs are anchored in the membrane *via* transmembrane segments followed by their flexible linkers. This suggests that the main role of the coiled coil is to stimulate PulM binding to PulL.

When isolated and in solution, $PulL_{CTD}$ and $PulM_{CTD}$ can form different types of parallel and anti-parallel homodimers, as shown by X-ray crystallography [28]. Similar behavior was found for the CTDs of EpsM, the PulM homologue from *V. cholerae* [38] and for XcpL, the PulL homologue from *P. aeruginosa* [39]. Likewise, homo-

oligomerization was observed in the ferredoxin-like domains of T4P assembly proteins PilN and PilO, considered as orthologues of PulL transmembrane and periplasmic regions and PulM, respectively. The CTD of PilN from *Thermus thermophilus* [40] and soluble periplasmic regions of *P. aeruginosa* PilO and PilN formed homodimers in crystals [41]. This is in contrast to their poor oligomerization *in vivo* when they are anchored in the bacterial membrane *via* their transmembrane segments, a constraint which reduces the degrees of freedom for CTDs binding.

In a yeast two-hybrid study of protein-protein interactions in the *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) T2SS, GspL periplasmic region interacted with the $GspM^{CTD}$ [14] and both periplasmic regions were found to form homo and heterodimers [42]. The recent BACTH data indicate that full-length PulL and PulM preferentially form heterodimers, and that their isolated CTDs interact in a parallel orientation compatible with their membrane insertion [28]. Similarly, full-length *P. aeruginosa* PilN and PilO form heterodimers, with no homodimers observed in the BACTH assay [43]. The long periplasmic helices thus seem to provide a compatible interacting surface to promote efficient formation of the heterodimer coiled coil. Studies in other systems also support the role precise and dynamic interactions between AP components. Mutations in the coiled coil regions of PilN and PilO in the *P. aeruginosa* T4P assembly system cause defects in pilus retraction [44]. In the *Dickeya dadanti* T2SS, the periplasmic regions of OutL and OutM comprise the major interacting interfaces revealed by yeast two-hybrid, BACTH and copurification, although the results of these studies suggested a more prominent role for their CTDs relative to the coiled coil regions [14, 18].

In the Pul T2SS, deletions of the PulL and PulM coiled coil regions strongly affected their function. Although variant $PulM^{\Delta CC}$ was more stable and retained the capacity to interact with PulL in the BACTH assay, it was as defective as $PulL^{\Delta CC}$ for both PulA

secretion and PulG pilus assembly, suggesting an impaired structure and function of the PulL–PulM complex. The coiled coil might provide structural rigidity to the complex compared to the individual proteins. Shortening of PulL and PulM helices by 4.8 nm could also result in defective interactions with other T2SS components or cellular structures, such as the peptidoglycan layer. In PulL$^{\Delta CC}$ and PulM$^{\Delta CC}$ variants, the globular ferredoxin-like domains (CTDs) would be placed proximal to the membrane, connected *via* the flexible linkers to the transmembrane helices. This might affect the overall architecture of the T2SS and interactions with its substrates.

While we showed here that PulM$^{\Delta CC}$ retained its ability to bind PulG, the next step in endopilus assembly, possibly involving binding to PulL, might be impaired. Like in other pilins, the TM segment of PulG is alpha-helical in its membrane-embedded state, but undergoes stretching upon incorporation into the endopilus [25]. This results in the loss of secondary structure in the region between Gly14 and Pro22 of PulG, which adopts an extended coil conformation [25]. The force that causes this stretching is likely to be generated during PulG extraction from the membrane, a key step in endopilus assembly which involves PulG binding to PulM [20]. The PulL–PulM coiled coil might provide an anchoring point during this step. The rigidity or the length of PulL–PulM dimer might be essential to exert force on PulG and operate its stretching during membrane extraction. The PulE ATPase in complex with the PulL cytoplasmic domain might be directly involved in this process. A defect in membrane extraction could be the cause of the major piliation defect observed in *pulL*$^{\Delta CC}$ and *pulM*$^{\Delta CC}$ mutants.

Our BACTH data suggest that the PulM cytoplasmic region in not essential for interaction with PulL, and that the periplasmic regions play a dominant role. The specific cleavage of PulL cytoplasmic domain in the *pulMΔN* mutant was therefore surprising. We hypothesize that the N-terminal peptide of PulM protects PulL from

395 proteolysis by binding to a disordered linker that connects $PulL_{cyto}$ domain with the
396 transmembrane and periplasmic regions. This linker is present in the PulL model
397 retrieved from the AF2 database [45] and predicted by the Alpha Fold 2 algorithm [46]
398 (Fig. S3).

399 Binding to the disordered PulL linker might require the free N-terminus of PulM, and
400 thus might occur in the native context, but not in the BACTH constructs, where PulM
401 N-terminus is fused to the T18 and T25 CyaA fragments. A similar interaction is
402 observed in T4P assembly systems, between the conserved N-terminal peptide of PilN
403 and PilM, which is an orthologue of the $PulL_{cyto}$ domain [47, 48]. This contact is highly
404 important for pilus assembly [49] and involves a conserved sequence ΦNLLP (where
405 Φ is a V, I or L) at the N-terminus of PilN homologues. Structural studies have shown
406 that this N-terminal peptide fits into a groove in PilM, allowing them to form a stable
407 PilM–PilN complex – a functional equivalent of PulL. The conserved Asn residue (N5
408 in PilN of *T. thermophilus*) plays a major role in PilM binding by forming a network of
409 hydrogen bonds with several residues in the PilM groove [47]. Functional data support
410 the crucial role of its equivalent Asn8 in the *Neisseria meningitidis* PilN, reflected by
411 the strong piliation defect of the $PilN^{N8A}$ variant [49]. Although not conserved in the
412 GspM homologues, the presence of a similar N-terminal sequence in PulM, MH**NLL**
413 (identical residues shown in bold) led us to test the effect of N3A substitution in PulM.
414 The $PulM^{N3A}$ variant showed a strong secretion defect and affected the stability of both
415 PulM and PulL [28]. Similar effects were observed in the PulMΔN variant described
416 here. The *pulMΔN* mutants show reduced PulM and PulL stability, generating a distinct
417 PulL fragment corresponding in size and likely in domain organization to the PilN
418 component of T4P assembly systems. Of note, a similar PulL degradation product is
419 also observed in the presence of native PulM [28], suggesting that the cytoplasmic

linker region of PulL is dynamic and protease sensitive, even in the presence of its interacting partner(s).

The above results argue that, although PulM is often considered as an orthologue of PilO in T4P systems, its role may be more similar to that of PilN. Importantly, direct interaction of PulM with the major pilin PulG parallels the binding of the PilN homologue HofN to the major pilin PpdD in the T4P assembly system of Enterohaemorrhagic *E. coli* [50].

The N-terminal regions of PulM and PilN might therefore play analogous roles in pilin targeting to the assembly site. The phenotype of the PulMΔN variant, strongly defective in PulG binding and affecting PulL stability, suggests a role of the cytoplasmic PulM peptide in passing the pilin subunits to PulL for incorporation into the growing pilus. PulM is also more abundant in the cell relative to PulL [28] consistent with its role as a shuttle between the large membrane pool of free PulG and the PulL protein stably bound to the ATPase PulE at the endopilus assembly site. Crosslinking studies of *V. cholerae* T2SS provided evidence for a transient complex of EpsL with EpsG [51], supporting at least a transient formation of G-M-L tripartite complex. Clearly, further studies are needed to identify their precise interactions within this complex and conformational changes of partners that promote this key step during endopilus assembly.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

This work was funded by Institut Pasteur, CNRS and ANR grant Synergy-T2SS ANR-19-CE11-0020-01. We are grateful to R. Ieva, R. Voulhoux, N. Izadi-Pruneyre and E. Bouveret for critical reading of the manuscript. We thank I. Guilvout and members of the BIM unit for helpful discussions. YL was a student of the Pasteur Paris University (PPU) international PhD program. JSM was funded by a fellowship from the Basque Government.

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592 **Table 1. Plasmids used in this study**
593

Name	Relevant characteristics	Source/Reference
pCHAP8185	*pulC-O, pulAB, pulS*, Ap^R, ColE1*ori*	[12]
pCHAP8496	pCHAP8185 *ΔpulM*	[20]
pCHAP8251	pCHAP8185 *ΔpulL*	[20]
pCHAP8258	*placZ-pulL*, Cm^R, p15A *ori*	[20]
pMS1349	pCHAP8258 *pulL*Δ270-302 (*pulL*$^{\Delta CC}$),	This study
pCHAP1353	*placZ-pulM*, Cm^R, p15A *ori*	[29]
pMS1350	pCHAP8258 *pulM*Δ37-68 (*pulM*$^{\Delta CC}$),	This study
	pulMΔ2-15 (*pulMΔN)* Cm^R, p15A *ori*	This study
pCHAP8882	*cyaT18* Ap^R, ColE1*ori*	This study
pUT18C	*cyaT25* Km^R, p15A ori	[35]
pKT25		[35]
pMS1222	*T18-pulL* Ap^R, ColE1*ori*	[28]
pMS1229	*T25-pulL*, Km^R, p15A ori	[28]
pCHAP8154	*T18-pulM,* Ap^R, ColE1*ori*	[19]
pCHAP8155	*T25-pulM,* Km^R, p15A ori	[19]
pMS1355	*T18-pulLΔ270-302*, Ap^R, ColE1*ori*	This study
pMS1356	*T25-pulLΔ270-302*, Km^R, p15A *ori*	This study
pMS1357	*T18-pulM*Δ37-68 Ap^R, ColE1*ori*	This study
pMS1358	*T25-pulM*Δ37-68 Km^R, p15A *ori*	This study
pCHAP7330	*T18-pulG,* Ap^R, ColE1*ori*	[23]
pCHAP7332	*T25-pulG,* Km^R, p15A *ori*	[23]
pMS1147	*T18-pulM*Δ2-15 Ap^R, ColE1*ori*	This study
pMS1148	*T25-pulM*Δ2-15 Km^R, p15A *ori*	This study

594

595 **Figure legends**

596

597 **Figure 1.** Deletion of the PulL periplasmic helix affects PulA secretion. **A**. Schematic
598 view of the PulL-PulM dimer in the inner membrane (IM). PulL is shown in green with
599 its periplasmic helix in lime; PulM is shown in magenta and its periplasmic helix in pale
600 pink. The numbers indicate positions of residues (shown as spheres) at the boundaries
601 of deleted segments in PulL$^{\Delta CC}$ (270-302) and PulM$^{\Delta CC}$ (37-68). **B**. Secretion of PulA
602 in strains producing native PulL (WT), no PulL (ΔL) and PulL$^{\Delta CC}$. PulA secretion assay
603 was performed (Materials and Methods) and 0.005 OD_{600nm} equivalents of cell- (CF)
604 and supernatant fractions (SN) were analyzed by Western blot with anti-PulA
605 antibodies. **C**. PulA secretion efficiency quantified from 3 independent assays as in
606 (**B**). The column height shows the mean values and dots represent values from

607 independent experiments. **D.** Concentrated cell fractions (from 0.05 OD_{600nm} of
608 bacteria) as in (**B**) analyzed with anti-PulL antibodies. **E**. Mean relative levels of PulL
609 quantified from concentrated cell fractions of strains from 3 independent experiments
610 (black dots) as in (**D**). Bar graphs indicate mean signal intensities of bands detected
611 with anti-PulL antibodies in strains producing $PulL^{WT}$ and $PulL^{\Delta CC}$. Mean levels of
612 $PulL^{\Delta CC}$ signal relative to $PulL^{WT}$ are indicated above the bar. **F**. Concentrated cell
613 fractions from 0.05 OD_{600nm} of bacteria as in (**D**), analyzed with anti-PulM antibodies.
614 **G**. Relative levels of PulM quantified from 3 independent experiments as in (**F**). Bar
615 heights indicate mean signal intensities and dots indicate individual values. Statistical
616 analysis was performed with Prism9 (GraphPad) using ordinary one-was ANOVA with
617 multiple comparisons. ****, $p<0.0001$, ns (non-significant difference). Nonspecific
618 bands cross-reacting with anti-PulL and anti-PulM antibodies are indicated by a star.
619
620 **Figure 2.** PulM periplasmic helix region is required for full T2SS function. **A**. Secretion
621 of PulA in strains producing native PulM (WT), no PulM (ΔM) and $PulM^{\Delta CC}$. PulA
622 secretion assay was performed and 0.005 OD_{600nm} equivalents of cell- (CF) and
623 supernatant fractions (SN) were analyzed by Western blot with anti-PulA antibodies.
624 **B**. PulA secretion efficiency quantified from 3 independent assays in the presence of
625 PulM (WT) or $PulM^{\Delta CC}$ and without PulM (ΔM). Bar heights show the mean values and
626 dots represent values from 4 independent experiments. **C**. Cell fractions from 0.05
627 $0D_{600nm}$ of bacteria as in (**A**) probed with anti-PulM antibodies. **D**. Signal intensities of
628 bands detected with anti-PulM antibodies from 3 independent sample as in (**C**) (black
629 dots). Bar heights indicate mean signal intensity and error bars show standard
630 deviations. Mean levels of $PulM^{\Delta CC}$ signal relative to $PulM^{WT}$ are indicated above the
631 bar. **E**. Concentrated cell fractions of samples as in (**C**) from 0.05 OD_{600nm} of bacteria,

analyzed with anti-PulL antibodies. **F**. Relative levels of PulL quantified from 3 independent experiments as in (**E**). Bar heights indicate mean signal intensities, dots indicate individual values and error bars show standard deviation. Statistical analysis was performed using ordinary one-was ANOVA with multiple comparisons. *, $p<0.05$; **, $p<0.01$, ns (non-significant difference).

Figure 3. Piliation defect of PulL and PulM lacking periplasmic helices. PulG pilus assembly on the surface of PAP7460 bacteria containing plasmid pCHAP8251 complemented with pCHAP8252 ($PulL^{WT}$), pMS1348 ($PulL^{\Delta CC}$) or vector pSU19 (ΔL), or PAP7460 containing plasmid pCHAP8496 complemented with pCHAP1353 ($PulM^{WT}$), pMS1350 ($PulM^{\Delta CC}$) or vector pSU18 (ΔM). Percentage of PulG present in the sheared fractions (SF) from bacteria cultured for 72 h at 30°C as described in Materials and Methods. Bar heights represent mean values from five independent piliation experiments (dots) and error bars indicate standard deviation. Statistical analysis was performed by one-way ANOVA with multiple comparisons. ****, $p<0.0001$, ns (non-significant difference).

Figure 4. Periplasmic helices are required for PulL – PulM interaction. **A**. Analysis of interactions between T18-PulM with T25-PulL and their derivatives. **B**. Analysis of interactions between T18-PulL with T25-PulM and their derivatives. Beta-galactosidase activity (Miller units) of DHT1 bacteria co-transformed with plasmids producing T18 and T25 or their hybrids as indicated: Z, yeast leucin zipper; M (PulM), L (PulL); MΔCC ($PulM^{\Delta CC}$) and LΔCC ($PulL^{\Delta CC}$). The bar graph heights indicate mean values and error bars show standard deviation. Black dots show β-gal. activities of individual cultures. Statistical analysis was performed with GraphPad Prism 9 using

One-way ANOVA test with multiple comparisons. ****, p<0.0001; ns (no significant difference).

Figure 5. PulM periplasmic α-helix is not essential for interaction with PulG. **A**. Interaction analysis of PulG with PulM and derivatives. **B**. Interaction analysis of PulG with PulL and derivatives. Beta galactosidase activity measured for indicated pairs of T18- and T25- hybrids. Z, yeast leucin zipper; M (PulM), L (PulL); MΔCC ($PulM^{\Delta CC}$), LΔCC ($PulL^{\Delta CC}$) and G (PulG). Bar graphs indicate mean values and error bars standard deviation. Black dots show activity values of independent cultures (n≥4). The data were plotted and analyzed with GraphPad Prism 9 software, using one-way ANOVA test with multiple comparisons. ****, p<0.0001, ns (non-significant difference).

Figure 6. PulM cytoplasmic peptide is essential for interaction with PulG and for the PulL stability. **A**. BACTH analysis of PulM and PulMΔN interactions with PulG and PulL Black dots show beta-galactoidase activity values of 4 independent cultures and bar graphs indicate mean activities. Statistical analysis was performed by one-way ANOVA with multiple comparisons. ****, p<0.0001, ns (non-significant difference). **B**. PulA secretion assay in strain PAP5378 containing plasmid pCHAP8496 *(ΔpulM*) complemented with $pulM^{WT}$ (on pCHAP1353), *pulMΔN* (on pCHAP8882) and empty vector pSU18 *(ΔpulM*). Secretion assay was performed as described in Materials and Methods. **C**. Quantification of secreted PulA fraction (%) from three independent assays as in (**B**). (**D**) PulM and (**E**) PulL levels in strain PAP7460 carrying pCHAP8496 *(ΔpulM*) complemented with $pulM^{WT}$ (on pCHAP1353), empty vector (pSU18) and *pulMΔN* (on pCHAP8882). The asterisk in (**D**) marks a nonspecific band cross-reacting with the anti-PulM antibody.

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