



28 **Abstract**

29 PapC ushers are outer-membrane proteins enabling assembly and secretion of P pili  
30 in uropathogenic *E. coli*. Their translocation domain is a large  $\beta$ -barrel occluded by a  
31 plug domain, which is displaced to allow the translocation of pilus subunits across the  
32 membrane. Previous studies suggested that this gating mechanism is controlled by a  
33  $\beta$ -hairpin and an  $\alpha$ -helix. To investigate the role of these elements in allosteric signal  
34 communication we developed a method combining evolutionary and molecular  
35 dynamics studies of the native translocation domain and mutants lacking the  $\beta$ -  
36 hairpin and/or  $\alpha$ -helix. Analysis of a hybrid residue interaction network suggests  
37 distinct regions (residue “communities”) within the translocation domain (especially  
38 around  $\beta$ 12- $\beta$ 14) linking these elements, thereby modulating PapC gating. Antibiotic  
39 sensitivity and electrophysiology experiments on a set of alanine-substitution mutants  
40 confirmed functional roles for four of these communities. This study illuminates the  
41 gating mechanism of PapC ushers and its importance in maintaining outer-  
42 membrane permeability.

## 43 Introduction

44 Gram-negative pathogens commonly express a vast variety of complex surface  
45 organelles that are involved in different cellular processes. One of these organelles,  
46 known as pili (or fimbriae), forms a class of virulence factors involved in host cell  
47 adhesion and recognition, invasion, cell mobility and biofilm formation. P pili from  
48 uropathogenic *Escherichia coli* are specifically required for the colonization of the  
49 human kidney epithelium, a critical event in the kidney infection process  
50 (pyelonephritis) (Roberts et al., 1994). P pili are assembled on the bacterial outer  
51 membrane (OM) via the chaperone/usher (CU) pathway (Thanassi et al., 1998),  
52 which is often used as a model system to elucidate the mechanism of pilus  
53 biogenesis (Waksman and Hultgren, 2009).

54 The biogenesis of pili via the CU pathway is a highly ordered process that comprises  
55 sequential steps. The chaperone protein (PapD) brings the pilins to the bacterial OM  
56 where they are assembled into a pilus at a transmembrane pore protein known as  
57 the usher (PapC). The usher (~800 residues) is composed of 5 domains (Figure 1A):  
58 a periplasmic N-terminal domain (NTD), an OM central translocation domain (TD)  
59 that comprises a translocation pore domain (TP), interrupted by a conserved Ig-like  
60 plug domain (PD), and two domains at the periplasmic C-terminal end (CTD1 and  
61 CTD2) (Capitani et al., 2006, Geibel et al., 2013, Ng et al., 2004, Thanassi et al.,  
62 2002, Phan et al., 2011). The structure of the apo TD (Figure 1B,C) consists of a 24-  
63 stranded kidney-shaped  $\beta$ -barrel where the PD is inserted into the loop connecting  
64 two  $\beta$ -strands ( $\beta 6$ - $\beta 7$ ), occluding the luminal volume of the pore (Remaut et al., 2008,  
65 Huang et al., 2009). In the activated form of another archetypal member of the usher  
66 family, FimD, the PD is located outside the pore lumen in the periplasm, next to the  
67 NTD (Phan et al., 2011, Geibel et al., 2013). In addition to the PD, there are two  
68 secondary structure elements that uniquely characterize the large  $\beta$ -barrel structures  
69 of the usher TD (Figure 1B). The first element is a  $\beta$ -hairpin that creates a large gap  
70 in the side of the  $\beta$ -barrel, a feature unprecedented in previously known OM  $\beta$ -barrel  
71 structures (Remaut et al., 2008). This element (located between strands  $\beta 5$  and  $\beta 6$  of  
72 the barrel, Figure 1C) folds into the barrel lumen and constrains the PD laterally  
73 inside the barrel pore. Mutants lacking the  $\beta$ -hairpin show an increased pore  
74 permeability suggesting that the  $\beta$ -hairpin has a role in maintaining the PD in a  
75 closed conformation (Volkan et al., 2013). The second element is an  $\alpha$ -helix (located  
76 on the loop between  $\beta 13$  and  $\beta 14$ , Figure 1B), which caps the  $\beta$ -hairpin from the  
77 extracellular side. Mutants lacking the  $\alpha$ -helix, or in which the interface between the  
78 helix and the PD is disrupted, present a remarkable increase in pore permeability,

79 comparable with that of the mutant lacking the PD, suggesting a role for the helix in  
80 maintaining the PD in a closed state (Mapingire et al., 2009, Volkan et al., 2013).

81 The mutant lacking both the  $\beta$ -hairpin and the  $\alpha$ -helix is defective for pilus biogenesis  
82 (Mapingire et al., 2009). It has been observed in other OMP  $\beta$ -barrels that such  
83 secondary structure elements (e.g., an  $\alpha$ -helix that protrudes inside the barrel or  
84 packs against the transmembrane strands) can use complex allosteric mechanisms  
85 to mediate their function (Naveed et al., 2009). These are often combinations of large  
86 conformational changes (“global motions”) dictated by the overall architecture  
87 (including movement of secondary structure elements) and smaller changes (“local  
88 motions”, such as the motion of recognition loops and side-chain fluctuations) (Liu  
89 and Bahar, 2012). Additionally, it has been shown that important residues in terms of  
90 evolution (highly-coevolved or conserved) could have a pivotal role in mediating such  
91 allosteric communications (Suel et al., 2003, Tang et al., 2007).

92 Here, to understand the allosteric mechanism leading to the plug displacement in  
93 PapC and the involvement of the  $\alpha$ -helix and  $\beta$ -hairpin, we used a hybrid  
94 computational approach and verified our results experimentally. By combining  
95 sequence conservation analysis, mutual information-based coevolution analysis, and  
96 all-atom molecular dynamics (AA-MD), we modelled the interaction network within  
97 the native PapC TD as well as within different mutants lacking the  $\alpha$ -helix,  $\beta$ -hairpin,  
98 and both. This unique computational approach allowed us to identify residues that  
99 are likely to be involved in the transmission of the allosteric signal between the  $\alpha$ -  
100 helix,  $\beta$ -hairpin elements and the plug. These residues were investigated by site-  
101 directed mutagenesis, functional studies and planar lipid bilayer electrophysiology.  
102 The results confirmed the involvement of 4 of the 5 distinct communities in  
103 modulation of the usher’s channel activity and gating, suggesting that they all  
104 participate in the allosteric mechanism controlling plug displacement.

105

## 106 **Results**

107

108 To investigate if the  $\beta$ -hairpin or  $\alpha$ -helix (or both) of the TD (residues 146-637 in the  
109 full length PapC) have a role in the allosteric communication leading to the  
110 displacement of the PD (residues 264-324) we performed four independent MD  
111 simulations, corresponding to the PapC TD model (sim1, Table 1) and three mutants  
112 (sim2-sim4, Table 1) embedded in a mixed lipid bilayer: sim2 where the region  
113 corresponding to the hairpin between  $\beta$ 5 and  $\beta$ 6 (residues 233-240) was deleted,

114 sim3 where the  $\alpha$ -helix between  $\beta$ 13 and  $\beta$ 14 (residues 447-460) was removed, and  
115 sim4 where both regions were removed. The last 50 ns of simulation were  
116 considered for analysis, where the averaged root-mean-square deviation of  $C_{\alpha}$  atoms  
117 ( $C_{\alpha}$ -RMSD) from the averaged structures stabilized around  $2.00\pm 0.09$  Å,  $1.80\pm 0.09$   
118 Å,  $1.86\pm 0.11$  Å, and  $2.03\pm 0.10$  Å, for the *native* (sim1), *hairpin mutant* (sim2), *helix*  
119 *mutant* (sim3), and *helix-hairpin mutant* (sim4), respectively (Table 1–figure  
120 supplement 1). This timescale, although limited for a full exploration of the structural  
121 changes induced by the mutations, was informative in revealing how local structural  
122 perturbations may affect allosteric changes leading to the plug displacement in PapC  
123 TD.

124

125 *Non-Covalent Interaction Network In the Native PapC Translocation Domain and Its*  
126 *Perturbation in the Absence of the  $\beta$ -Hairpin,  $\alpha$ -Helix or Both.*

127

128 The changes in the non-covalent interactions (hydrogen bonds and salt bridges)  
129 between all residue pairs were analysed within the native TD by calculating their *non-*  
130 *covalent interaction score* (NCI score) (see Methods). A non-covalent residue-  
131 residue interaction network (RIN) comprising 492 nodes (residues) and 1350 edges  
132 (interactions) was then constructed as a weighted undirected graph for the native TD  
133 (Figure 2) and the three mutant systems (Figure 2- figure supplement 1A-C), with the  
134 weight for each edge given by the corresponding NCI score (Table 2). All four RINs  
135 have properties typical of small-world networks (Haiyan and Jihua, 2009, Atilgan et  
136 al., 2004, Taylor, 2013), with significant higher clustering coefficient compared to a  
137 corresponding random network and a higher mean short path length (Table 2).  
138 Within the constructed non-covalent native RIN we identified 246 weak-to-strong  
139 interactions (connecting 362 nodes) with an NCI score of at least 0.3. Among these,  
140 231 nodes connected by 133 edges showed an NCI score greater than 0.6 (*i.e.*,  
141 strong interaction) of which 78 involve residues that are part of the barrel strands  
142 (58.6%).

143 Comparative analysis between the RINs of native and mutants systems revealed  
144 slight changes, suggesting a rearrangement in the interaction network. To better  
145 understand the mutation-induced changes in network components we calculated the  
146 difference in non-covalent interaction score ( $\Delta$ NCI score) between the native TD  
147 system and each of the mutant systems (The weakened interaction are shown in  
148 Figure 2- figure supplement 2A-C). This information was then added as a weighted  
149 undirected edge to the pre-existing native non-covalent RIN (the  $\Delta$ NCI edges are

150 shown in Figure 2- figure supplement 2D). Interestingly, 24% of the strong  
151 interactions in the native RIN were weakened relative to the RIN of the mutant  
152 lacking the  $\beta$ -hairpin, 22.6% relative to the mutant lacking the  $\alpha$ -helix and 23.3%  
153 relative to the mutant lacking both, suggesting that interactions between nodes that  
154 are not part of the deleted secondary structure elements were consistently weakened  
155 in the absence of these elements.

156

### 157 *Evolutionary analysis of PapC TD*

158

159 We first extracted evolutionary information from a multiple sequence alignment of the  
160 PapC TD family. The patterns of conservation in the TD using ConSurf (Ashkenazy et  
161 al., 2010) analysis suggested that the highly conserved residues (score 9) tend to be  
162 clustered in two specific regions of the usher (Figure 3A). The first cluster mapped  
163 onto the PD and the P-linkers (P-linker1 residues 248-263; P-linker2 residues 325-  
164 335) connecting it to the TP. The second cluster (which included the majority of the  
165 highly-conserved residues) mapped onto one side of the TP (strand  $\beta$ 1-14 and  $\beta$ 24).  
166 It includes residues: (i) near the periplasmic side of the  $\beta$ -barrel within  $\beta$ 1-4 strands  
167 and  $\beta$ 24 strand; (ii) on the extracellular side of the barrel (within  $\beta$ 5-10); (iii) in the  $\beta$ -  
168 hairpin region ( $\beta$ -hairpin and  $\beta$ 7-9); and (iv) in the area of  $\beta$ 10-14 capped by the  $\alpha$ -  
169 helix region, which comprises the  $\alpha$ -helix and its linkers: H-linker1 (residues 445-450)  
170 and H-linker2 (461-468, respectively). Surface representation of the TD reveals a  
171 continuous patch of conserved residues facing the lipid bilayer, including  $\beta$ 13, the  
172 extracellular half of  $\beta$ 14 and the periplasmic half of  $\beta$ 12 (Figure 3B). Intriguingly, this  
173 patch ( $\beta$ 13 conserved patch) reaches the full height of the pore from the  $\alpha$ -helix  
174 region to a functionally important loop located between  $\beta$ 12 and  $\beta$ 13 strands (Volkan  
175 et al., 2013, Farabella, 2013).

176 In addition to investigating conservation we performed an analysis to identify the  
177 coevolutionary relationships between residues in the structure. Using normalized  
178 mutual information (NMI) analysis (Martin et al., 2005) with a Z-score cut-off = 4 (see  
179 Methods) to detect the intra-molecular coevolved residues within PapC TD, a  
180 coevolutionary RIN containing 100 coevolved residues (nodes) and 357 connections  
181 (edges) was derived (Figure 3D). Mapping the network onto the PapC TD structure  
182 showed that many of the residues involved are also connected spatially and are  
183 clustered in the same regions where the highly-conserved residues were found (P-  
184 linkers, the PD, and the barrel wall capped by the  $\alpha$ -helix, in close proximity to the  $\beta$ -  
185 hairpin) (Figure 3C and D). The obtained coevolutionary RIN showed a significant

186 clustering coefficient compared to a corresponding random network (of 0.493 vs.  
187 0.187, respectively) and a comparable mean short path length (3.15 vs. 2.57,  
188 respectively) (Daily et al., 2008).

189

### 190 *Identifying Allosteric 'Hot Spots' From a Hybrid Residue Interaction Network*

191

192 We constructed one hybrid RIN in which the attributes for the nodes and edges are  
193 defined by the properties described above (non-covalent networks and evolutionary  
194 analysis, see Methods). Starting from the secondary structure elements (that  
195 uniquely characterise the barrel - the  $\alpha$ -helix and  $\beta$ -hairpin) in **this hybrid** RIN, we  
196 used a multi-step procedure to reconstruct a pathway of communication between  
197 them (Figure 4).

198 **This initial large sub-network is formed by 208 nodes (residues) connected by 456**  
199 **NCI edges (in the native RIN). Applying the dynamic filter (independently) on the**  
200 **edges, based on the difference in non-covalent interaction score between the native**  
201 **TD and each of the mutants ( $\Delta\text{NCI}>0$ ), revealed that in each case a large part of**  
202 **network have weakened interactions (hairpin mutant: 200 nodes, 438 NCI edges;**  
203 **helix mutant: 199 nodes, 437 NCI edges; helix-hairpin: 202 nodes, 443 NCI edges)**  
204 **(Figure 4- figure supplement 1A). The application of the evolutionary filter revealed**  
205 **that only a small part of the sub-network is made of evolutionary important residues**  
206 **(75 nodes connected by 104 native NCI edges). Combining the filters (Figure 4-**  
207 **figure supplement 1B) resulted in 69 nodes connected by 100 NCI edges (thus**  
208 **representing interacting residues in the native PapC network). The residues of this**  
209 **sub-network (14% of all residues in the TD) were considered 'hot spots' in the**  
210 **communication pathway of PapC TD ('hot spot' sub-network). Mapping the hot spot**  
211 **residues onto the structure revealed that they are located close together in a**  
212 **continuous area within PapC TD.**

213 We analysed the community structure of the hot spot sub-network using the edge-  
214 betweenness clustering algorithm (Girvan and Newman, 2002, Morris et al., 2010).  
215 This analysis shows that the sub-network has a modular structure, with a modularity  
216 index of 0.73 (maximum value of the modularity index is 1), which is typical of 3D-  
217 structure based RIN (Newman and Girvan, 2004, Sethi et al., 2009). Here, a total of  
218 11 communities containing two or more residues were identified, from which only five  
219 communities are composed by more than 5 residues. For further analysis, we chose  
220 to consider only these five largest communities, which are located: between  $\beta$ 7-9 and  
221 the P-linkers (C1); between the  $\beta$ -hairpin and the conserved region at the base of the

222  $\alpha$ -helix ( $\beta$ 12-14) (C2); between  $\beta$ 12-13 loop and the P-linker1 (C3); between the  $\beta$ -  
223 hairpin, P-linker2 and the PD ( $\beta$ E-F) (C4); and on the tip of the PD ( $\beta$ E-F loop and  
224  $\beta$ A-B loop) (C5) (Table 3 and Figure 5).

225 We selected a number of key residues – core hot-spot residues – from the  
226 communities that are linking different elements within each community for further  
227 experimental investigations (Figure 5). These were found in communities C1-C4: in  
228 C1, residues linking the P-linkers and the barrel wall that possibly help in maintaining  
229 the P-linkers in a closed configuration (P-linker1:D249, P-linker2:Y329, P-  
230 linker2:T331,  $\beta$ 7:R337, and  $\beta$ 8:S363); in C2, residues that bridge the base of the  $\alpha$ -  
231 helix (the extracellular end of the  $\beta$ 13 conserved patch) and  $\beta$ -hairpin ( $\beta$ -  
232 hairpin:R237,  $\beta$ 12:S420,  $\beta$ 13:R442,  $\beta$ 13:S444); in C3, residues on the interface  
233 between P-linker1 and  $\beta$ 12-13\_loop ( P-linker1:Y260,  $\beta$ 12- $\beta$ 13 loop:K427,  $\beta$ 13:T437,  
234  $\beta$ 13:F438); and in C4, residues that are part of the interface with P-linker1 and the  
235 periplasmic end of the  $\beta$ 13 conserved patch (P-linker2:A325, P-linker2:V327).

236

### 237 *Experimental Analysis of Residues In The Hot Spot Sub-Network*

238

239 To test experimentally if the key hot spot residues identified above (linking elements  
240 within each community) contribute to allosteric signalling within PapC, we  
241 constructed a set of single alanine substitution mutations (Table 4). Each of the  
242 mutants was present at a similar level in the OM compared to the wild-type PapC  
243 usher, and the mutations did not affect the ability of the usher to form a stable  $\beta$ -  
244 barrel in the OM (data not shown). [1F2] The functionality of the PapC substitution  
245 mutants was assessed by ability to assemble P pili on the bacterial surface. P pili  
246 bind to receptors on human red blood cells, and assembly of functional P pili was  
247 determined using a hemagglutination assay (HA). Seven (D249A, T331A, R442A,  
248 S444A, Y260A, K427A, T437A) of the 14 tested mutants exhibited greater than 2-fold  
249 defects in agglutination titers compared with wild-type PapC, with 4 of the mutants  
250 (D249A, R442A, Y260A, and K427A) exhibiting no agglutination activity (HA titer = 0)  
251 (Table 4). The defective mutants were in key residues from communities C1, C2 and  
252 C3, confirming roles for these communities in proper usher function.

253 We next used an antibiotic sensitivity assay to screen the PapC substitution mutants  
254 for effects on channel activity of the usher. The OM of Gram-negative bacteria has  
255 low permeability to detergents such as SDS and to antibiotics such as erythromycin  
256 and vancomycin, providing resistance to these molecules. In its resting state, the  
257 usher TP is gated closed by the PD, preserving integrity of the OM. Mutations that  
258 disrupt channel gating by the PD will result in opening of the large TD channel,

259 leading to increased sensitivity of the bacteria to antibiotics. Bacteria expressing 5 of  
260 the PapC substitution mutants (T331A, R237A, Y260A, F438A and V327A) exhibited  
261 increased sensitivity to one or more of the tested molecules (Table 4). Y329A<sup>[IF3]</sup>,  
262 R337A, S363A and S420A did not appear to permutate the allosteric signalling within  
263 PapC, showing the same antibiotic sensitivity phenotype and ability to assembly pili  
264 of the native PapC (Table 4). However, the hemagglutination assay and antibiotic  
265 sensitivity assay are screening tools, and as such, they lack in sensitivity to pick up  
266 smaller changes in the ability to assemble pili or channel activity of the usher.

267

#### 268 *Electrophysiological analysis of selected mutants*

269

270 In total, ten mutated PapC TDs were found to be affected either in their ability to  
271 trigger hemagglutination or in their permeability to SDS or antibiotics (T331A, D249A  
272 in C1; R237A, R442A, S444A in C2; Y260A, K427A, T437A, F438A in C3; and  
273 V327A in C4). We attempted to purify those mutants in view of examining their  
274 channel activity using planar lipid bilayer electrophysiology, which is a more sensitive  
275 assay. Unfortunately, only seven of these 10 mutants yielded protein stable enough  
276 (as wild-type) in detergent solutions to carry out the planned experiments (T331A in  
277 C1; R237A, S444A in C2; K427A, T437A, F438A in C3; and V327A in C4). During  
278 the OM extraction procedure, D249A, R442A, and Y260A were not stable enough  
279 due to the loss of the membrane bilayer environment and inability to maintain their  
280 native conformation in detergents. Insertion of PapC purified proteins (see Methods)  
281 was promoted in planar lipid bilayers by clamping the membrane potential to – 90  
282 mV. As soon as channel activity was observed, the potential was briefly returned to  
283 zero and the chamber stirring stopped to minimize further insertions. Ten-minute long  
284 recordings of channel activity at + and – 90 mV, and at + and – 50 mV were  
285 performed.

286 The typical electrophysiological signature of the wild-type PapC usher is  
287 characterized by prolonged dwell times at a low current level, representing the closed  
288 state of the usher, and brief transitions of various current amplitudes. These  
289 transitions represent short-lived openings of various conductance, ranging from 50 to  
290 600 pS (“transient-mixed” behaviour, TM) (Figure 6A). Although it is not possible to  
291 know exactly how many individual pores were inserted into the bilayer, the observed  
292 fluctuations of various sizes are taken to represent various conformational states of a  
293 single pore. As documented previously, the openings of the “transient-mixed”  
294 behaviour appear rather small and may be due to the jiggling of the plug within the  
295 TP and/or the thermally-induced mobility of various domains of the protein, such as

296 the NTD and CTDs or loops (Mapingire et al., 2009). Occasionally, and more so at  
297 higher membrane potential, very large and sustained openings (“large-open”  
298 behaviour, LO) are observed in wild-type PapC usher (Figure 6B). These openings  
299 have a conductance of ~ 3-4 nS, which is similar to the monomeric conductance of  
300 the mutant lacking the PD (Mapingire et al., 2009) and are interpreted as  
301 representing a full displacement of the PD from a single monomer. Prolonged  
302 opening of intermediate conductance (0.5-1 nS) can also be observed and may  
303 represent partial PD displacement.

304 Because the electrophysiological behaviour of wild-type PapC usher is quite variable,  
305 and in attempt to quantify the propensity at spontaneous PD displacement, we have  
306 counted the number of 10-min long recordings (sweeps) that show “large-open”  
307 behaviour, and we report the percent of such sweeps in various conditions. The  
308 frequency of observing these large openings in wild-type PapC usher is ~ 20% at ±  
309 50 mV, but increases to ~ 60% at ± 90 mV. The application of a larger  
310 transmembrane voltage is likely to disrupt the interactions between key residues  
311 involved in keeping the PD in place, leading to a more frequent spontaneous  
312 displacement of the latter.

313 Three of the seven analysed mutants, V327A, T331A and F438A, showed an  
314 increased propensity at displaying large openings, relative to the wild-type PapC  
315 usher, as illustrated for V327A and T331A (Figure 6C, D). This was particularly true  
316 at ±90 mV where the percent of sweeps with large openings reaches values of 75-  
317 90% (Figure 6F). The T331A mutant was consistently more prone to open than WT  
318 and any other mutants, which led to the occasional simultaneous opening of several  
319 monomers (Figure 6D). Two of the seven mutants R237A and T437A still opened  
320 occasionally to the 3 nS level, but the frequency of sweeps with such events was  
321 slightly diminished relative to the wild-type PapC usher at ±90 mV (Figure 6F)  
322 suggesting that these mutants are likely to be insignificantly different from the wild-  
323 type PapC usher.

324 The K427A and S444A mutants showed a decreased frequency (or complete  
325 absence) of large openings. The K427A mutant almost never showed “large-open”  
326 behaviour at ±50 and ±90 mV, indicating an extremely closed channel (Figure 6E,  
327 Figure 6E, F). The S444A mutant was even less prone to open, with 0% occurrence  
328 of PD displacement at ±50 mV in the 11 bilayers that we investigated (Figure 6F).  
329 However, increased activity with fast flickers and occasional more prolonged  
330 openings could be seen for both mutants if the membrane potential was switched to  
331 voltages in the ± 100-150 mV range, indicating that the channels are present in the  
332 bilayer, but require higher voltages for activation.

333

334 **Discussion**

335

336 PapC usher catalyses the translocation across the outer membrane of P-pili, and its  
337 gating mechanism is important for bacterial homeostasis and for catalysis of pilus  
338 assembly. The TD of PapC is formed by the largest  $\beta$ -barrel pore known to be  
339 formed by a single chain. The PD occludes the pore in an inactive state and  
340 maintains the permeability of the channel. As previously documented, the native  
341 PapC channel is highly dynamic and is characterized by spontaneous short-lived  
342 openings of various conductance levels (Mapingire et al., 2009). Two distinct  
343 structural elements, the  $\beta$ -hairpin and the  $\alpha$ -helix, play an important role in  
344 maintaining the PD in a closed conformation. In the absence of both elements the  
345 usher is defective for pilus biogenesis. Our analysis of the non-covalent interaction  
346 RIN in the native PapC TD shows that the interactions found between the TP and the  
347 PD are mostly weak, possibly to allow an easy release of the PD. This finding  
348 supports the idea that the highly dynamic behavior of the native PapC channel is  
349 originated from the 'jiggling' of the PD within the TP (Mapingire et al., 2009). On the  
350 other hand, we find that the interactions between the TP and the P-linkers, between  
351 the TP and the  $\beta$ -hairpin, and between the TP and the  $\alpha$ -helix region were mainly  
352 stable, supporting their role in maintaining the PD in a closed conformation.

353

354 Analysis of the mutation-induced perturbation of the non-covalent interaction RIN of  
355 the native PapC TD in absence of the  $\beta$ -hairpin,  $\alpha$ -helix or both, shows that the  
356 interactions between nodes that are not part of the deleted secondary structure  
357 elements are consistently weakened (Figure 2- figure supplement 1), suggesting the  
358 two elements are not independent. This feature can be interpreted as part of a  
359 complex allosteric process regulating the PD gating mechanism. It has been  
360 proposed that only a few residues play essential roles during allosteric  
361 processes and that perturbing the interactions between these residues can facilitate  
362 the population shift of the conformational ensembles (Tsai et al., 2009, del Sol et al.,  
363 2009). Additionally, it has been shown that residues with a pivotal role in mediating  
364 such allosteric communications are also important residues in terms of evolution  
365 (both highly-coevolved residues and conserved residues) (Suel et al., 2003, Tang et  
366 al., 2007, Ferguson et al., 2007). Remarkably, we show here that PapC is  
367 characterised by an uneven distribution of the evolutionary important residues,  
368 clustered in the P-linkers, the PD, and the barrel wall capped by the  $\alpha$ -helix, in close  
369 proximity to the  $\beta$ -hairpin. Another interesting finding is the presence of the ' $\beta$ 13

370 conserved patch' that reaches the full height of the pore from the  $\alpha$ -helix region to a  
371 loop located between  $\beta$ 12 and  $\beta$ 13 strands ( $\beta$ 12-13\_loop), which has been previously  
372 identified as important (Volkan et al., 2013, Farabella, 2013).

373

374 To detect the allosteric network we implement a new method that integrates dynamic  
375 and evolutionary information in a hybrid RIN and then apply network analysis. This  
376 approach allows us to explore a large part of the protein, resulting in the detection of  
377 only 14% of all residues in the TD as potential candidates. It has been shown that  
378 detecting "residue communities" in protein structure networks leads to the  
379 identification of key residues that are often part of a signal transduction pathway (del  
380 Sol et al., 2007, Bode et al., 2007). The interconnection within and between the  
381 communities is pivotal for the flow of allosteric signalling. Residues in the same  
382 community are densely interconnected and have multiple routes to communicate with  
383 each another. However, the interconnections between communities involve only a  
384 few edges, which form the bottleneck for the flow of the signal in the network (del Sol  
385 et al., 2007, Bode et al., 2007, Sethi et al., 2009). Here, all the identified communities  
386 (C1-C5, Figure 5) comprise residues from multiple elements (e.g.  $\beta$ -hairpin, P-  
387 linker1, P-linker2 and distinct part of the TP) of PapC TD except C5 that is composed  
388 only by PD residues. Mutation of key residues linking elements within each of the  
389 four communities C1-C4 showed an altered antibiotic sensitivity phenotype,  
390 confirming a role of these communities in the pore gating mechanism. Additionally,  
391 communities C1-C3 are required for proper usher function (as estimated by the  
392 hemmoglutatione assay, suggesting dependency of the pore gating function and  
393 pilus assembly (based on some of the mutations). For example, mutations in  $\beta$ 12-  
394 13\_loop:K427 in C3 and  $\beta$ 13:S444 in C2, which lead to a drastic decrease in the  
395 frequency of plug displacement (stabilizing the closed state, based on channel  
396 activity analysis) also show defective pilus assembly (possibly due to their deficiency  
397 in relocating the plug in a functional conformation).

398

399 Additionally, our study identifies two residues with opposite effects on plug  
400 displacement in the same community: C3:  $\beta$ 12-13\_loop:K427, which is located on  
401  $\beta$ 12-13\_loop, and  $\beta$ 13:F438, which is located at the periplasmic end of the  $\beta$ 13  
402 conserved patch (linking  $\beta$ 12-13\_loop with the  $\alpha$ -helix region). This observation  
403 suggests that  $\beta$ 12-13\_loop has a pivotal role in modulating plug displacement (acting  
404 as a 'latch') as proposed previously (Farabella, 2013), and supported by additional  
405 mutagenesis studies (Volkan et al., 2013). Intriguingly, at the extracellular end of the  
406  $\beta$ 13 conserved patch there is another mutation ( $\beta$ 13:S444) leading to a channel more

407 reluctant to open, suggesting a regulatory role for the  $\beta$ 13 conserved patch (Figure 7)  
408 in modulating the 'latch' ( $\beta$ 12-13\_loop).

409  
410 Interestingly, our community residues in some cases are found to have very different  
411 patterns of interactions in an alternative conformation. For example, the functionally  
412 important 'latch' ( $\beta$ 12-13\_loop) is shown to be in a different conformation in the open  
413 state (based on FimD:FimC:FimH structure (Phan et al., 2011)) as well as other  
414 residues in C3 and C4 communities that change their interaction pattern in the open  
415 state. As a result, some residues that are identified by our method, such as V327,  
416 could in principle be selected also by visual inspection for further experimental  
417 investigation. V327 (located on P-linker2 in C4 at interface with P-linker1 and the  
418 barrel wall) is found to have a different interaction pattern in the open state of the  
419 usher and is also shown to promote plug displacement. However, more importantly,  
420 the method is able to predict residues in regions that would not attract our attention  
421 at all but may contain important information in respect to the allosteric pathway. (This  
422 aspect can become even more significant in the absent of an alternative  
423 conformation). For example, we predict two such residues to be functionally  
424 important –T331 and S444. T331 is located on P-linker2 at the interface between P-  
425 linker2 and the barrel wall, which stays intact in the open conformation of the usher;  
426 S444 is located on  $\beta$ 13 at the base of the  $\alpha$ -helix, i.e. on the barrel itself, and it lacks  
427 any direct contact with the plug domain. However, both residues were shown to have  
428 an effect on plug displacement. Thus, the main strength of our method is that the  
429 knowledge of the structure of the protein in one conformation only and enough  
430 sequence information to extract evolutionary information are sufficient for the  
431 detection of functionally important residues that can be pivotal for transferring the  
432 regulatory information within the protein.

433

## 434 **Conclusion**

435 In this study, we provide a first deep insight into the allostery regulating the gating  
436 mechanism of the usher family. Using PapC TD as model system, we developed an  
437 integrative approach, combining computational modelling, sequence conservation  
438 analysis, mutual information-based coevolution analysis and information from AA-MD  
439 simulations, to study the potential involvement of particular secondary structure  
440 elements (the  $\alpha$ -helix and  $\beta$ -hairpin) in the allosteric communication. The construction  
441 of a hybrid interaction network and the use of network analysis allowed us to identify  
442 communities of residues within the TD that potentially mediate this process. Antibiotic  
443 sensitivity and electrophysiology experiments on a set of alanine-substitution mutants

444 confirmed that residues located in the P-linkers, the  $\beta$ -hairpin, and  $\beta$ 13 conserved  
445 patch (part of four communities) alter channel gating and that residues located in P-  
446 linker2,  $\beta$ 12- $\beta$ 13\_loop and  $\beta$ 13 conserved patch (both periplasmic end and  
447 extracellular end) are sensitive to plug displacement. Therefore, we suggest that the  
448  $\beta$ 13 conserved patch acts as a regulator of the 'latch' ( $\beta$ 12- $\beta$ 13\_loop) mediating  
449 channel opening. Furthermore, our study shows how the integration of different  
450 computational approaches based on evolution, structure and dynamics of proteins,  
451 into a hybrid network can unveil communication pathways within proteins. Such an  
452 integrative network modelling approach can guide the experimental investigation by  
453 pinpointing key candidates involved in the transmission of the allosteric signal.

454

## 455 **Methods**

### 456 *Systems Modelling*

457 We built four model systems based on the X-ray structure of the TD (residues 1-492)  
458 of PapC at 3.2 Å resolution (PDB ID: 2vqi (Remaut et al., 2008)). The starting model  
459 for the simulation of the native TD (sim1, native) was generated by adding the  
460 missing loops to the X-ray structure using the *dope\_loopmodel* method (Shen and  
461 Sali, 2006) in MODELLER-9v7 (Sali and Blundell, 1993). Additionally, three mutant  
462 model systems were constructed based on the native model: a mutant that lacking  
463 the  $\beta$ -hairpin ( $\Delta$ 233-240) (sim2, hairpin mutant); a mutant lacking the  $\alpha$ -helix ( $\Delta$ 447-  
464 460) (sim3, helix mutant) and a mutant lacking both ( $\Delta$ 233-240 and  $\Delta$ 447-460) (sim4,  
465 helix-hairpin mutant).

466 Each of the systems was oriented with respect to the membrane normal (the Z axis  
467 by definition) using the database (Lomize et al., 2006). For the native model (sim1) a  
468 mixed lipid bilayer (POPE/POPG 3:1) was generated around the protein using the  
469 replacement method (Jo et al., 2007). To obtain a mixed lipid bilayer that reproduces  
470 an estimated surface area per lipid of  $61.5 \pm 0.2 \text{ \AA}^2$  (Murzyn et al., 2005) we used the  
471 InflateGRO method (Kandt et al., 2007).

472

### 473 *Systems Set Up For MD Simulations*

474 All MD simulations were performed using Gromacs 4.0.5 (Van Der Spoel et al.,  
475 2005). TIP3P parameters were used for water molecules (Jorgensen et al., 1983),  
476 the OPLSA-AA force-field (Kaminski et al., 2001) was applied to the protein and ions,  
477 and the Berger force-field (Berger et al., 1997) to the lipids. All four systems were  
478 solvated in water and ions were added to neutralize the total charge (0.15 M NaCl),  
479 resulting in more than 75,000 atoms in total. Next, each system was energy-  
480 minimized using a steepest descent algorithm in the presence of different position

481 restraints on the protein and the lipid bilayer head-groups, which were gradually  
482 removed. Note that in the simulations of the mutant systems (sim2 to sim4), the  
483 protein models were embedded in the pre-equilibrated membrane obtained after 15  
484 ns of unrestrained equilibration of the native TD (sim1).

485

#### 486 *Equilibration Procedure and Production Run*

487 The assembled systems were equilibrated in a multistage process using periodic  
488 boundary conditions and a 2 fs time step. Short-range interactions were used with a  
489 cut-off of 1 nm. The PME algorithm (Darden et al., 1993) was used for long-range  
490 electrostatic interactions. All bonds were constrained using the LINCS algorithm  
491 (Hess et al., 1997, Hess, 2008). The first equilibration step was performed in the NVT  
492 ensemble, using a restraining force of 1000 kJ/(mol nm<sup>2</sup>) for 0.1 ns on the protein  
493 and lipids. The V-rescale thermostat (Bussi et al., 2007) was employed to couple the  
494 temperature of the system to 310K with a time constant of  $t_{\tau} = 0.1$  ps. All the  
495 following equilibration steps were performed in the NPT ensemble. During the next 3  
496 steps different parts of the system were restrained using a force constant of 1000  
497 kJ/(mol nm<sup>2</sup>): the protein and lipids, the protein atoms only, and the protein backbone  
498 atoms.

499 The resulting model of each system was then simulated without restraints. Constant  
500 temperature of 310K was maintained using the Nose-Hoover thermostat (Hoover,  
501 1985) (Nose, 1984.) with a time constant of  $t_{\tau} = 0.1$  ps. Using semi-isotropic coupling  
502 with a Parrinello-Rahman barostat (Parrinello, 1981), a constant pressure of 1 bar  
503 was applied with a coupling constant ( $t_{\text{P}}$ ) of 1 ps and a compressibility  $4.5 \times 10^{-5}$  bar<sup>-1</sup>.  
504 Each unrestrained simulation was performed for ~70-72 ns. The last 50 ns of  
505 simulation were used for analysis.

506

#### 507 *Non-covalent residue interaction network*

508 Hydrogen bonds were defined using a cut-off of 30° for the acceptor–donor–  
509 hydrogen angle and a cut-off of 3.5 Å for the hydrogen–acceptor distance. The  
510 definition of salt-bridges was based on a 4 Å distance cut-off between any oxygen  
511 atoms of acidic residues and nitrogen atoms of basic residues. The *non-covalent*  
512 *interaction score* (NCI score) of the identified bonds was defined as the percentage  
513 of simulation time during which a bond occurs between two amino acids normalised  
514 by the number of bonds. Using the normalized score, a non-covalent residue  
515 interaction network (RIN) was built for each of the simulated systems as a weighted  
516 undirected graph, in which each node represents a residue and each edge is

517 weighted by the normalized score. The difference in non-covalent interaction score  
518 ( $\Delta$ NCI score) between the native TD system and each of the mutant systems was  
519 then calculated and added as a weighted undirected edge to the pre-existing non-  
520 covalent RIN.

521

#### 522 *Sequence Conservation Analysis*

523 The sequence corresponding to the X-ray structures of PapC TD (PDB id: 2vqi;  
524 Uniprot id: P07110) was used as input to psiBlast resulting in a set of unique related  
525 sequences from the non-redundant NCBI dataset (Altschul et al., 1997). The E-value  
526 threshold was set as  $10^{-3}$  and sequences with id > 90% and < 30% sequence identity  
527 were excluded. The structure-based multiple sequence alignment was calculated  
528 using Expresso (3DCoffee) (Armougom et al., 2006). Finally, an evolutionary  
529 conservation score was calculated for each residue an empirical Bayesian inference  
530 method (Mayrose et al., 2004) as implemented in the ConSurf web server  
531 (Ashkenazy et al., 2010).

532

#### 533 *Sequence Coevolution Analysis*

534 To estimate the coevolution within the residues in the usher TD we used Normalized  
535 Mutual Information (NMI) (Martin et al., 2005) over all position pairs in the multiple  
536 sequence alignment obtained as described above. NMI calculations were performed  
537 using PyCogent (Knight et al., 2007, Caporaso et al., 2008) and a Z-score was  
538 calculated for each residue pair based on the standard deviation from the mean NMI  
539 values. Only residue pairs that had Z-score > 4 were identified as coevolved  
540 pairs(Martin et al., 2005, Gloor et al., 2005).

541 Next, a coevolutionary RIN was built as a weighted undirected graph where each  
542 node represents a residue (as in the non-covalent RIN) and an edge connecting two  
543 nodes is the NMI score. The network was visualized and analyzed in Cytoscape  
544 2.8.2 (Smoot et al., 2011) using NetworkAnalyzer plug-in (Assenov et al., 2008) for  
545 calculating degrees of connectivity and RINalyzer plug-in (Doncheva et al., 2011) for  
546 mapping the network on the PapC structure.

547

#### 548 *Hybrid RIN*

549 To store the entire information we combined the coevolutionary RIN, the non-  
550 covalent RIN, and the conservation analysis into one network. In this hybrid network  
551 each node represents each PapC TD residue and is associated with the

552 corresponding conservation score. Two nodes can have multiple edges, each  
553 weighted according to the information it carries (NMI score, NCI score, or  $\Delta$ NCI).

554

#### 555 *Allosteric 'hot spots' sub-network reconstruction*

556 Using the hybrid network and starting from the  $\alpha$ -helix region (residues 445-468) and  
557  $\beta$ -hairpin residues (residues 230-240) we generated a sub-network of first  
558 neighbours residues based only on the NCI score higher than 0.3 in the native RIN.

559 This sub-network was expanded by again adding only neighbouring residues  
560 connected by NCI score higher than 0.3. The procedure was repeated until no more

561 new residues could be added to the sub-network. A first set was generated by

562 filtering the sub-network based on the evolutionary information. The filtering was

563 done by selecting nodes with a conservation score of 9 (i.e., highly conserved) or

564 nodes that are connected by an NMI edge (i.e., coevolved significantly). A second

565 set was generated by filtering out the nodes that are not connected by a weakened

566 interaction ( $\Delta$ NCI $>$ 0) in each of the mutant systems. Intersecting the identified sets

567 resulted in a 'hot spots' sub-network. ~~TA first set was generated by filtering the sub-~~

568 ~~network based on the evolutionary information. The filtering was done by only leaving~~

569 ~~nodes with a conservation score of 9 (i.e., highly conserved) or nodes that are~~

570 ~~connected by an NMI edge (i.e., coevolved significantly). A second set was~~

571 ~~generated by filtering out the nodes that are not connected by a weakened~~

572 ~~interaction ( $\Delta$ NCI $>$ 0) in each of the mutant systems. Intersecting the identified sets~~

573 ~~resulted in a 'hot spots' sub-network.~~ The 'hot spots' sub-network was then

574 decomposed into communities using the edge-betweenness clustering algorithm

575 (GLay) as implemented in clusterMaker (Girvan and Newman, 2002, Morris et al.,

576 2010). Cytoscape 2.8.2 (Smoot et al., 2011), RINalyzer plug-in (Doncheva et al.,

577 2011), and Chimera (Pettersen et al., 2004) were used for mapping the network on

578 the PapC structure.

579

#### 580 *PapC substitution mutants*

581 The PapC alanine substitution mutants were derived from plasmid pDG2 using the

582 QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the primers listed in

583 Supplementary file 1. Plasmid pDG2 encodes wild-type *papC* with a C-terminal,

584 thrombin-cleavable His-tag (Li et al., 2004). All mutants were sequenced to verify the

585 intended mutation.

586

#### 587 *Expression and folding of the PapC substitution mutants in the outer membrane*

588 Each of the PapC mutants was compared with wild-type PapC for expression levels  
589 and ability to fold into a stable  $\beta$ -barrel in the OM. OM isolation, analysis of usher  
590 protein levels, and the heat-modifiable mobility assay for  $\beta$ -barrel stability were done  
591 as previously described (Henderson et al., 2011).

592

#### 593 *Hemagglutination assay*

594 HA assays were performed to test the ability of each of the PapC substitution  
595 mutants to assemble functional P pili on the bacterial surface. HA assays were  
596 performed by serial dilution in microtiter plates as previously described (Henderson  
597 et al., 2011). HA titers were determined visually as the highest fold dilution of  
598 bacteria still able to agglutinate human red blood cells. Each assay was performed in  
599 triplicate; each mutant was analyzed twice and the values averaged.

600

#### 601 *Top soft agar assay for antibiotic sensitivity*

602 Bacteria were grown in LB medium supplemented with 100  $\mu$ g/ml ampicillin (Amp) to  
603 an OD<sub>600</sub> of 0.6 and then induced for PapC expression with 0.1% arabinose for 1 h.  
604 Aliquots of 0.1 ml bacteria were added to 3 ml melted soft top agar (0.75% LB agar)  
605 cooled to 45°C and supplemented with 100  $\mu$ g/ml Amp and 0.1% arabinose. The  
606 bacteria and melted agar were mixed well and poured on top of 1.5% solid LB agar  
607 plates containing 100  $\mu$ g/ml Amp and 0.1% arabinose. Once the top agar solidified,  
608 sterile 6 mm filter discs were placed on top and 10  $\mu$ l of the following antibiotics were  
609 added: 75 mg/ml SDS, 2 mg/ml vancomycin, or 1.5 mg/ml erythromycin. The  
610 diameter of the growth inhibition zone around the antibiotics, including the filter disc,  
611 was measured after overnight growth at 37°C. Each PapC mutant strain was tested  
612 twice and the values averaged.

613

#### 614 *Electrophysiological analysis of selected PapC substitution mutants*

615 PapC mutants (R237A, F438A, V327A, K427A, T437A, T331A and S444A) were  
616 purified according to published protocols (Henderson and Thanassi, 2013) and  
617 investigated by planar lipid bilayer electrophysiology. Planar bilayers were made from  
618 a preparation of L- $\alpha$ -phosphatidylcholine Type II-S from Sigma (also known as  
619 asolectin) according to the Montal and Mueller technique (Montal and Mueller, 1972)  
620 following a published protocol (Mapingire et al., 2013). Protein aliquots were diluted  
621 1:1 in buffer T (1M KCl, 5 mM Hepes, pH 7.2) containing either 1 or 2%  
622 *N*-Octyl- oligo- oxyethylene (octyl-POE, Axxora). Eight micrograms of protein from  
623 the diluted sample was added to the *cis* side of a planar lipid bilayer chamber  
624 containing ~ 1.5 mL of buffer T. Gentle stirring was applied to promote spontaneous

625 insertions of the protein into the bilayer. Channel activity was monitored by  
626 measuring current under voltage clamp conditions using an Axopatch 1D amplifier  
627 with a CV4B headstage or an Axopatch 200B amplifier (Axon Instruments). The  
628 current was digitized (ITC-18, Instrutech), and stored on a PC computer using the  
629 Acquire software (Bruyton). Ten-minute long traces were sampled at 1.25 ms  
630 intervals and filtered at 500 Hz. Both chambers contained buffer T and Ag/AgCl  
631 electrodes with pellet. The *trans* side of the bilayer was set as ground. Insertions  
632 were typically performed at -90 mV. Data display and analysis were done with  
633 pCLAMP software (Axon Instruments).

634

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840 **Figure Legend**

841

842 **Figure 1. PapC usher organization and detail of its translocation domain.** A. A  
843 diagram of the domain organization of PapC usher. NTD (dark-blue) represents the  
844 N-terminal domain, CTD1 (light-violet) and CTD2 (dark-violet) represent the C-  
845 terminal domains; TD represents the translocation domain, comprising the TP  
846 (translocation pore, light-blue) and the PD (plug domain, magenta). B and C: Ribbon  
847 representation of the starting model of the native translocation domain (TD) of PapC  
848 with the labels 'N' and 'C' indicating the N and C termini of the translocation channel.  
849 The  $\beta$ -barrel, PD (including the P-linkers),  $\beta$ -hairpin, and  $\alpha$ -helix (including the H-  
850 linkers) are coloured blue, magenta, orange, and yellow, respectively. The outer  
851 membrane position is represented schematically with the labels 'E', 'M' and 'P'  
852 indicating the extracellular side, the membrane and the periplasmic side,  
853 respectively. Side view of the TD (B) is shown with the  $\alpha$ -helix,  $\beta$ -hairpin, H-linker1,  
854 H-linker2, P-linker1, P-linker2, and PD, labelled. Extracellular top view of the TD (C)  
855 is shown with the barrel  $\beta$  strands labelled  $\beta$ 1 through  $\beta$ 24 and with the PD strands  
856 labelled  $\beta$ A through  $\beta$ F. The figures were created with Chimera (Pettersen et al.,  
857 2004).

858

859 **Figure 2. Non-covalent interaction network (non-covalent RIN).** A. Ribbon  
860 representation of the starting model of the native translocation domain (TD) of PapC  
861 with the labels 'N' and 'C' indicating the N and C termini of the translocation channel.  
862 The  $\beta$ -barrel, PD, P-linker1, P-linker2,  $\beta$ -hairpin, and  $\alpha$ -helix (including the H-linkers)  
863 are coloured grey, magenta, light purple, dark purple, orange, and yellow,  
864 respectively. The  $\alpha$ -helix,  $\beta$ -hairpin, P-linker1, P-linker2, and PD are labelled. B.  
865 Protein structure network representation of the native translocation domain (TD) of  
866 PapC visualized (see Figure 2-figure supplement 1 for the RINs of the mutant TD)  
867 with Cytoscape 2.8.2 (Smoot et al., 2011) based on RINalyzer plug-in analysis  
868 (Doncheva et al., 2011). The nodes (representing residues) are coloured by  
869 structural element as in A. Edges (connecting two residues) are shown in blue, the  
870 edge width is proportional to its NCI score from lower to higher values.

871

872 **Figure 3. Evolutionary analysis of PapC TD.** A-B. Sequence conservation  
873 calculated with ConSurf (Ashkenazy et al., 2010) and mapped onto the initial model  
874 of the native PapC TD (sim1, t=0). Amino acid conservation scores are classified into  
875 9 levels. The colour scale for residue conservation goes from cyan (non-conserved:  
876 grade 1) to maroon (highly conserved: grade 9), unreliable positions are coloured

877 light yellow. A. Ribbon representation of the model with the highly conserved  
878 residues (grade9) shown as spheres and key elements labelled. B. Molecular surface  
879 of the model with  $\beta$ 12- $\beta$ 14 labelled. C-D Sequence co-evolution calculated with  
880 PyCogent (Knight et al., 2007, Caporaso et al., 2008). C The co-evolving residues  
881 are mapped onto the initial model of the native PapC TD (sim1, t=0). D. The co-  
882 evolution network as visualized with Cytoscape 2.8.2 Cytoscape 2.8.2 (Smoot et al.,  
883 2011) based on RINalyzer plug-in analysis (Doncheva et al., 2011). Edges  
884 (connecting two co-evolved residues) are shown in blue, and nodes (representing  
885 coevolved residues) are coloured by structural element. The PD, P-linker1, P-linker2,  
886  $\beta$ -hairpin and  $\alpha$ -helix are indicated schematically and coloured as in Figure 2. The  
887 node size is proportional to its degree of connectivity.

888

889 **Figure 4. Detection of allosteric hot spots.** A flowchart representing the protocol  
890 to identify allosteric hot-spots. First a sub-network of the protein hybrid RIN was  
891 generated starting from the  $\alpha$ -helix and  $\beta$ -hairpin. Then filters based on the  
892 evolutionary information and on the interactions analysis were applied (see Figure 4–  
893 figure supplement 1).

894

895 **Figure 5. PapC TD communities.** The hot-spot network communities are shown as  
896 surface by colors and indicated schematically (C1 to C5). The inset shows a close up  
897 of the identified core residues located in  $\beta$ 7,  $\beta$ 8, the P-linkers, the  $\beta$ -hairpin, the  
898 conserved region at the base of the  $\alpha$ -helix, in the junction between  $\beta$ 12- $\beta$ 13\_loop.  
899 The core residues are labelled in bold and numbered according to the X-ray structure  
900 of the apo PapC TD (PDB id: 2vqi).

901

902 **Figure 6. Kinetic signatures of channel activity in wildtype and mutant PapC**  
903 **ushers and frequency of PD displacement.** Fifty-second segments of recordings  
904 obtained in planar lipid bilayers were selected to illustrate the behaviour of the  
905 different proteins. (A) Recording from the wild-type PapC usher showing the  
906 characteristic “transient-mixed” behaviour. (B) Recording from the wild-type PapC  
907 usher showing an example of spontaneous large openings due to plug displacement.  
908 Note the large amount of current fluctuations during the openings, and the “transient-  
909 mixed” behaviour in between such events. Examples of similar large openings (C, D)  
910 are shown for the V327A, and T331A mutants, respectively. (E) A recording from the  
911 K427A shows that the channel barely displays any activity at this voltage. The  
912 voltage was +90 mV for all panels. The current level for the closed channels is  
913 marked as “C”, and openings are seen as upward deflections of the traces; current

914 levels corresponding to fully open monomeric or dimeric forms are denoted by “M”  
915 and “D”, respectively. Note that the traces are plotted as conductance, rather than  
916 current, vs. time and the scale bars are given in nS. (F) The percent of sweeps  
917 displaying “large-open” behaviour” (LO) indicative of PD displacement is shown for  
918 WT and each mutant at the indicated voltages. The number of individual bilayers  
919 investigated in each case is given above the bars.

920

921 **Figure 7. Residues involved in the allosteric signalling to control PapC gating.**

922 A schematic model summarizing the location of the detected hot spots involved in the  
923 gating mechanism. The  $\beta$ 12- $\beta$ 13\_loop (the ‘latch’) and the  $\beta$ 13 conserved patch are  
924 colored in dark grey and light gray respectively. The PD, P-linker1, P-linker2,  $\beta$ -  
925 hairpin and  $\alpha$ -helix are coloured as in Figure 2. Hot spot residues are colour-coded  
926 based on their communities (C1-C4, as in Figure 5). The different symbols indicate  
927 the mutant’s electrophysiological behavior (‘X’ where no data were available).  
928 Mutants that show a pilus assembly defect or an increased antibiotic sensitivity, or  
929 both, are represented by a triangle, pentagon and rectangle, respectively.

930 **Tables**

931 Table 1 Summary of the simulations.

Simulation	Model Systems	Length (ns)
Sim1	Native PapC TD	72
Sim2	Hairpin Mutant	70
Sim3	Helix Mutant	70
Sim4	Helix-Hairpin Mutant	70

932

933 Descriptions of the items are: Simulation, the name of the simulation; model systems,  
934 PapC TD model systems simulated; and Length, the length of the simulation.

935 Table 2. Summary of the residue-residue interaction networks (RINs) parameter.

<b>RIN</b>	<b>Full RIN</b>	<b>C</b>	<b>Cr</b>	<b>C/Cr</b>	<b>L</b>	<b>Lr</b>	<b>L/Lr</b>
Native PapC TD	1350 (492)	0.384	0.012	32.00	6.67	3.78	1.76
Hairpin Mutant	1196 (485)	0.368	0.011	33.45	7.20	3.90	1.84
Helix Mutant	1225 (476)	0.362	0.011	32.90	6.67	3.90	1.71
Helix-Hairpin Mutant	854 (466)	0.262	0.008	32.75	8.10	4.70	1.72

936 Descriptions of the items are: RIN residue-residue interaction networks of the  
 937 different model systems; Full RIN, number of edges in the RIN, in parenthesis the  
 938 number of node; C, average clustering coefficient; L, average shortest path length;  
 939 Cr, average clustering coefficient for the random networks with the same size; Lr,  
 940 average shortest path length for the random networks with the same size; C/Cr is the  
 941 average clustering coefficient ratio (as used in (Atilgan et al, 2004)); L/Lr is the  
 942 average shortest path length ratio (as used in (Atilgan et al, 2004)).  
 943

944 Table 3 Communities in the hot-spot sub-network

Community	Residues
C1	E247, D249, Y329, L330, T331, G334, Q335, R337, K339, E361, S363, W364, G365, L366, S371, L372
C2	R237, D402, S420, Y441, R442, F443, S444, K468, E469, M470, E475, W496
C3	Y260, Y425, S426, K427, T437, F438, A439
C4	S233, R303, G304, L306, V308, F320, T324, A325, V327
C5	E269, E312, N314, G315, R316, K318

945

946 Descriptions of the items are: Community, the name of the community; Residues,

947 residues that are part of the community.

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Table 4 Analysis of PapC substitution mutants

PapC	Community	HA Titer	Antibiotic Sensitivity		
			SDS	Erythromycin	Vancomycin
WT		64	15	6	6
D249A	C1	0	15	6	6
Y329A	C1	32	15	6	6
T331A	C1	24	15	15	10
R337A	C1	64	15	6	6
S363A	C1	64	15	6	6
R237A	C2	64	16	6	15
S420A	C2	32	15	6	6
R442A	C2	0	15	6	6
S444A	C2	24	14	6	6
Y260A	C3	0	15	14	6
K427A	C3	0	14	6	6
T437A	C3	24	14	6	6
F438A	C3	32	14	12	6
V327A	C4	64	20	14	16

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Descriptions of the items are: PapC, the PapC construct tested; Community, the name of the community to which the mutated residue belongs; HA (hemagglutination assay) Titer, the maximum fold dilution of bacteria able to agglutinate human red blood cells; Antibiotic Sensitivity, the diameter of zone of inhibition (mm) around filter disc impregnated with SDS (750 µg), erythromycin (15 µg), or vancomycin (20 µg). The antibiotic sensitivity measurement includes the filter disc (6 mm diameter).

958 **Figure Supplement Titles and Legends**

959

960 **Table 1–figure supplement 1.** C $\alpha$ -RMSD values for each system throughout the  
961 simulations. C $\alpha$ -RMSD from the starting structure (t=0) for the native TD (A), the  
962 hairpin mutant (B), helix mutant (C), and helix-hairpin mutant (D).

963

964 **Figure 2–figure supplement 1. Non-covalent interaction network (non-covalent  
965 RIN).** Protein structure network representation of the hairpin mutant (A), helix mutant  
966 (B), and helix-hairpin mutant (C) translocation domain (TD) of PapC visualized as  
967 before.

968

969 **Figure 2–figure supplement 2. Non-covalent interaction network (non-covalent  
970 RIN).** RINs showing the difference in non-covalent interaction score ( $\Delta$ NCl score)  
971 between the native TD system and the hairpin mutant (A), helix mutant (B), and  
972 helix-hairpin mutant (C). The combined RIN (D) is created by merging the three  
973 RINs. The nodes (representing residues) are coloured as in figure 2A. Edges  
974 (connecting two residues) are shown in blue, note that in this RIN the edge width is  
975 proportional to its  $\Delta$ NCl score from lower to higher values.

976

977 **Figure 4–figure supplement 1. Contribution of each filter in the detection of  
978 allosteric hot spots.** Venn diagrams illustrating the contribution of each filters in the  
979 set definition. (A) The dynamic filter raises from the intersection of the difference in  
980 non-covalent interaction score between the native TD and each of the mutants. (B)  
981 The relative combination of the set from dynamic filter and the set from the  
982 evolutionary filter to the final hot spots sub-network.

983

984 **Additional File**

985

986 **Supplementary file 1.** Primers used in this study to generate PapC substitution  
987 mutations