

# Timing of TolA and TolQ recruitment at the septum depends on the functionality of the Tol-Pal system

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1	Timing of ToIA and ToIQ recruitment at the septum depends on the
2	functionality of the Tol-Pal system
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- 39 Abstract.
- 40

Efficient cell division of Gram-negative bacteria requires the presence of the Tol-41 42 Pal system to coordinate outer membrane (OM) invagination with inner membrane invagination (IM) and peptidoglycan (PG) remodeling. The Tol-Pal system is a trans-43 envelope complex that connects the three layers of the cell envelope through an 44 energy-dependent process. It is composed of the three IM proteins, ToIA, ToIQ and 45 ToIR, the periplasmic protein ToIB and the OM lipoprotein Pal. The proteins of the Tol-46 47 Pal system are dynamically recruited to the cell septum during cell division. ToIA, the central hub of the Tol-Pal system, has three domains: a transmembrane helix (TolA<sub>1</sub>), 48 49 a long second helical periplasmic domain (TolA<sub>2</sub>) and a C-terminal globular domain 50 (TolA<sub>3</sub>). The TolQR complex uses the PMF to energize TolA, allowing its cyclic 51 interaction via TolA<sub>3</sub> with the OM TolB-Pal complex. Here, we confirm that TolA<sub>2</sub> is sufficient to address ToIA to the site of constriction, whereas ToIA<sub>1</sub> is recruited by ToIQ. 52 53 Analysis of the protein localization as function of the bacterial cell age revealed that ToIA and ToIQ localize earlier at midcell in the absence of the other ToI-Pal proteins. 54 55 These data suggest that ToIA and ToIQ are delayed from their septal recruitment by 56 the multiple interactions of ToIA with ToIB-Pal in the cell envelope providing a new 57 example of temporal regulation of proteins recruitment at the septum. 58

*Keywords:* molecular motor, Tol-Pal system, septum, OM invagination, Cell division

61 *Abbreviations used:* OM, outer membrane; IM, inner membrane; PG, peptidoglycan;

62 pmf, proton-motive force; sfGFP, super-folder green fluorescent protein.

- 64 Introduction.
- 65

Cell division in Gram-negative bacteria is orchestrated by a large protein complex 66 called the divisome [1]. This macrostructure is composed of 12 essential proteins and 67 additional non-essential proteins which extend from the cytoplasm to the three layers 68 of the cell envelope. Once the divisome is completely assembled, the constriction 69 70 process begins. Despite major advances in understanding IM invagination and PG 71 remodelling during cell division, little is known about the coordinated constriction of the 72 OM. Since the OM is not energised and the periplasm is deprived of ATP, it has been 73 suspected for a long time that OM invagination might be achieved passively by simple 74 tethering of OM lipoproteins to the PG [2].

75 Recently, significant findings indicated that the Tol-Pal system plays a major role 76 in the OM constriction and provides the energy required for this process [3,4]. The Tol-77 Pal system is a highly conserved trans-envelope complex among Gram-negative 78 bacteria. The core-components are composed by the three IM proteins ToIA, ToIQ and 79 ToIR, the periplasmic protein ToIB and the OM lipoprotein Pal [5]. All the ToI-Pal 80 proteins are recruited to the divisome [3,6]. TolB and Pal form an OM complex. Pal 81 interacts both with ToIB and the PG layer, but these two interactions are mutually exclusive [7,8]. ToIA, ToIQ and ToIR form an inner membrane complex. The ToIQR 82 subcomplex is part of the emerging family of molecular stators that converts chemical 83 energy derived from the proton motive force (pmf) to mechanical movements. TolQR 84 85 are homologous to ExbBD that energize TonB involved in nutrients uptake, to MotAB 86 that power the flagellar rotation and to AglQRS implied in the gliding motility of Myxococcus xanthus [9-13]. The TolQR stator uses the PMF to energize TolA, allowing 87 its cyclic interaction with the outer membrane TolB-Pal complex and the release of Pal 88 to bind the PG. This cycle of exclusive ToIA, ToIB and Pal interactions, the ability of 89 Pal to bind noncovalently the PG, and the proposed contractile activity of the Tol-Pal 90 91 complex regulated by the energy state of the TolQR stator would assure sufficient contacts between the three layers of cell envelope for efficient invagination at the 92 93 septum [3,4].

TolA is the central hub of the Tol-Pal complex. This elongated protein is divided into three domains. One transmembrane helix (TMH), termed TolA<sub>1</sub>, anchors the protein to the IM. Both cross-linking experiments and isolation of suppressive mutations revealed that TolA<sub>1</sub> interacts with TolQ through its TMH [14-16]. A predicted 98 long helical second domain, termed TolA<sub>2</sub>, is thought to stretch across the periplasm. 99 Finally, TolA C-terminal domain, referred as TolA<sub>3</sub>, interacts with the N-terminal 100 domain of TolB but also with the Pal OM lipoprotein in a pmf dependent manner [17-101 20]. Using a fluorescent chimeric protein composed of the sfGFP fused to the 102 cytoplasmic N-terminus of TolA (GFP-TolA), previous microscopy studies showed that 103 TolA is dynamically recruited to the cell septum during cell division independently of 104 the other Tol-Pal proteins [3,6].

In this study, we aimed to identify the domain of ToIA responsible for its septal 105 106 localization during cell division. We engineered three truncated versions of ToIA fused 107 to a fluorescent marker. GFP-ToIA1 and GFP-ToIA1-2 were expressed from their 108 endogenous chromosomal loci whereas GFP-TolA<sub>3</sub> was expressed from a plasmid. In 109 the first part of this paper we checked that the chimeric proteins were correctly 110 expressed and addressed to their intended location, that is GFP-ToIA<sub>1</sub> and GFP-ToIA<sub>1</sub>-<sub>2</sub> in the IM and GFP-ToIA<sub>3</sub> in the periplasm. Then, using a fluorescence microscopy 111 112 approach we studied the intracellular localization of the GFP-ToIA derivatives and we compared their timing of localization as function of the progression of the division cycle. 113 114 Our results shed light on the dynamic of the Tol proteins during cell division.

115 **Results.** 

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#### 117 Expression and functionality of truncated forms of TolA fused to sfGFP.

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To determine the region of ToIA involved in its recruitment to the septum during 119 cell division, we engineered strains producing sfGFP fused to truncated forms of ToIA. 120 Thus, sfGFP was fused to the N-terminus of ToIA, of ToIA<sub>1</sub> (residues 1 to 64) and of 121 122 TolA<sub>1-2</sub> (residues 1 to 290) (Fig. 1). These constructs, designated as GFP-TolA, GFP-123 TolA<sub>1</sub> and GFP-TolA<sub>1-2</sub>, were introduced at their respective native locus on the 124 chromosome. Additionally, the periplasmic TolA<sub>3</sub> domain was fused at its N-terminus 125 to GFP preceded by a Tat sequence signal in the pBad/HisC vector (Fig. 1). Tat-GFP 126 has been previously described to be efficiently exported across the IM by the Twin-127 Arginine Transport system [3,21]. We first checked that all the TolA derivative fusion proteins were correctly expressed. As shown in Fig. 2a, the fusion proteins were 128 129 detected at their expected size with antiserum directed against TolA or against GFP. GFP-ToIA1 was not detected with antiserum directed against ToIA presumably 130 131 because TolA<sub>1</sub> was too small to contain epitopes. Then we checked the functionality 132 of the different fusion proteins. It has been well established that mutations in the tol-133 pal genes lead to increased sensitivity of cells to detergents such as SDS and bile salts, engender Escherichia coli tolerance towards group A colicins and filamentous 134 bacteriophages and form cell filaments in medium of low osmolarity [22-24]. Here we 135 show that strains producing GFP-ToIA<sub>1</sub>, GFP-ToIA<sub>1-2</sub> and GFP-ToIA<sub>3</sub> are tolerant to 136 137 colicin A, are hypersensitive to the addition of SDS in the culture medium and form cell filamentsn when grown in LB medium lacking added NaCl (Table 1 and S1). As 138 139 expected, all the truncated forms of ToIA that lack critical functional domains are not functional. In contrast, we confirm that GFP-ToIA is fully functional for the three tested 140 141 criteria as previously shown [3] (Table 1 and S1).

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#### 143 Intracellular location of the fluorescent fusion proteins.

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As the different truncated proteins were non-functional, we investigated their intracellular location by fractionation experiments. Cells producing the various TolA variants were converted into cytoplasmic (C), periplasmic (P) and membrane (M) fractions, and the different fusion proteins were detected with antiserum directed

against either ToIA or GFP. As shown in Fig. 2b, GFP-ToIA, GFP-ToIA1 and GFP-149 TolA<sub>1-2</sub> were recovered in the membrane fraction whereas GFP-TolA<sub>3</sub> was detected in 150 the periplasmic fraction. Finally, we performed membrane extraction treatments in 151 152 order to determine the nature of protein association with the membrane. The Fig. 2c shows that the transmembrane segment of GFP-ToIA, GFP-ToIA<sub>1</sub> and GFP-ToIA<sub>1-2</sub> is 153 154 correctly inserted into the membrane since all these fusion proteins were only solubilized in the presence of Triton X-100. Based on these results, we concluded that 155 156 the various fluorescent fusion proteins were correctly produced and addressed to their 157 intended location.

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#### 159

- **TolA**<sub>2</sub> is responsible for the TolA recruitment at the septum.
- 160

The intracellular localization of the various GFP-TolA derivatives were analysed by a live fluorescent microscopy approach. Previous studies clearly showed that TolA was dynamically recruited to the cell septum during cell division [3,6]. Here, we demonstrated that all the constructs possessing the transmembrane segment of TolA were recruited to the septum during cell division (Fig. 3) whereas sfGFP-TolA<sub>3</sub> remained spread in the cell envelope (Fig. 3). These results indicate that TolA<sub>1</sub> segment is responsible for the septal location of TolA during cell division.

In a previous paper, we showed that ToIA recruitment to the septum did not require 168 the presence of the other Tol-Pal proteins [3]. However, multiple interactions between 169 170 ToIA and the stator ToIQR, involving their TMHs, have been described. De facto, we wondered if GFP-ToIA<sub>1</sub> contrary to GFP-ToIA could be recruited to the cell septum by 171 the TolQR complex. During the time course of our manuscript reviewing, Hale et al., 172 173 2022 showed that ToIA1 required the presence of ToIQ for its septal recruitment, unlike ToIA2. Here, we analysed the localization of GFP-ToIA1 and GFP-ToIA1-2 in a tolQR 174 deletion mutant background and confirmed that TolA1-2 does not required TolQR to be 175 176 dynamically recruited to the septum unlike TolA<sub>1</sub> which required TolQR for its septal recruitment (Fig. 3). Accordingly, production of TolQR from plasmid pBadTolQR 177 178 restored accumulation of ToIA1 in a *toIQR* deletion mutant background.

179

#### **TolA1 and TolA1-2 localize earlier at the septum than TolA.**

It has been previously described that cell length correlates with cell cycle 182 progression and that appearance of fluorescence at midcell provides a quantitative 183 metric for temporal comparison [25,26]. A large-scale demograph analysis of cells 184 185 producing the various GFP-ToIA derivatives surprisingly demonstrates a temporal pattern of midcell recruitment during cell cycle. Indeed, mapping of the cell 186 187 fluorescence profiles according to their length clearly indicate that GFP-ToIA localizes later at the septum than GFP-ToIA1 or GFP-ToIA1-2, whereas GFP-ToIA3 never 188 189 localizes at midcell (Fig. 4a). To confirm these results, cells producing the various GFP-190 ToIA derivatives were separated in 10 age classes as function of their length, and for 191 each age class the average distribution of fluorescence along the normalized cell 192 length was measured (Fig. 4b). We showed that the fluorescence intensity of GFP-193 ToIA at midcell started to increase at about 30-40% of the division cycle, as previously 194 described [27]. Interestingly, the fluorescence intensity of GFP-ToIA<sub>1</sub> and GFP-ToIA<sub>1</sub>-195 <sub>2</sub> started to increase at only 10-20% of the division cycle, confirming that GFP-TolA<sub>1</sub> 196 and GFP-ToIA<sub>1-2</sub> are recruited earlier at the septum than GFP-ToIA (Fig. 4b).

- 197

#### 198 ToIA activity in the ToI-Pal complex delays its recruitment to the septum. 199

200 Clearly, ToIA deprived of the ToIA<sub>3</sub> domain is recruited earlier than full length ToIA to the septum. We know that TolA interacts via TolA<sub>3</sub> with the OM TolB-Pal complex in 201 a PMF-linked ToIQR stator dependent manner. We speculate that ToIA1 and ToIA1-2 202 203 are recruited earlier at midcell during the cell cycle because they don't interact with the 204 TolB-Pal complex in the OM all around the cell before accumulating at the septum. To 205 test this hypothesis, we compared the timing of GFP-ToIA midcell localization in WT, 206  $\Delta tolB$ , or  $\Delta tolQR$  genetic backgrounds. As revealed by the demograph analysis 207 presented in Fig. 5a, the absence of ToIB or the absence of the ToIQ-ToIR stator 208 significantly accelerates TolA recruitment at midcell. Moreover, the fluorescence 209 intensity of GFP-ToIA at the septum in these two mutant backgrounds started to increase at only 10-20% of the division cycle, as described above for ToIA1 and ToIA1-210 211 <sub>2</sub> (Fig. 5b). All of these results indicate that preventing the ToIA-ToIB interaction accelerates the recruitment of ToIA to the constriction site. As mentioned above, ToIA1 212 213 unlike ToIA1-2 required ToIQ for its early septal recruitment. This suggest that ToIQ 214 should be recruited faster at the septum in the absence of ToIA or ToIB. To test this 215 hypothesis, we compared the timing of TolQ-mCherry midcell localization in WT,  $\Delta tolA$ ,

216	or $\Delta tolB$ genetic backgrounds. In Fig 6a we show that TolQ is efficiently recruited at
217	the septum in the different genetic backgrounds. However, the fluorescence intensity
218	of TolQ-mCherry at the septum started to increase at 30-40% of the division cycle in
219	in the WT strain and at only 10-20% of the division cycle in the two mutant backgrounds
220	(Fig 6b)
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229 Discussion.

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It has been established that the Tol-Pal system is involved in the late stages of cell 231 232 division, contributing to the coordinated invagination of all three cell envelope layers. Its role would be dual: first, concentrating Pal at the septum in order to bind the PG 233 234 and to invaginate the OM; secondly, participating to the efficient processing of septal 235 PG to complete the cell division cycle [3,4,28]. A mechanistic model has been 236 proposed to explain the role of the Tol-Pal system in the OM invagination. In dividing 237 cells, all the Tol-Pal proteins are recruited to the site of constriction [3,6]. TolA, 238 energized by the TolQR stator, captures the diffusing TolB-Pal complex in the OM and 239 dissociates Pal from TolB. Accumulated Pal at the septum is now free to bind septal 240 PG, thus linking the OM and the PG layers and promoting OM invagination [4].

241 The mechanism by which the Tol-Pal proteins are attracted to the septum is currently unknown. However, both ToIA and ToIQ have been shown to be recruited at 242 243 midcell independently of each other and of the other Tol-Pal proteins [3,6]. FtsN was 244 proposed to be the candidate for such recruitment but its role remains controversial 245 and questionable [6, 29, 30]. To gain new information about the mechanism of TolA 246 recruitment at the septum, we aimed to identify the region of TolA responsible for its midcell localization. The constructs GFP-ToIA, GFP-ToIA<sub>1</sub> and GFP-ToIA<sub>1-2</sub> were 247 introduced at their respective native locus on the chromosome. Based on western-blot 248 analysis, we check, that GFP-ToIA and GFP-ToIA<sub>1-2</sub> were expressed at the same level 249 250 as ToIA, thus eliminating any risk of misinterpretation of subsequent microscopy 251 studies. We verified that all the fluorescent fusion proteins containing the 252 transmembrane segment of ToIA were correctly addressed and inserted into the inner 253 membrane although some of them were not functionals (Fig. 2b, c). Finally, we show 254 that all the fusion proteins containing the transmembrane segment of TolA are dynamically recruited to the divisome. However, in accordance with recent work by 255 256 Hale et al., 2022, we confirm that TolQ is responsible for TolA<sub>1</sub> recruitment to the septum but not TolA<sub>1-2</sub>. This result also confirms the importance of the transmembrane 257 258 segment of ToIA in its interaction with ToIQ (Germon et al., 1998). In summary, ToIA 259 is dynamically recruited to the septum by TolA<sub>1</sub> via its interaction with TolQ and by 260 TolA<sub>2</sub> via its interaction with an unknown partner. In contrast to the results published 261 by Hale et al., 2022, we do not observe recruitment of ToIA3 to the septum. Our 262 discrepancy in observations is certainly due to the fact that in our experiments ToIA3

is not fused to a transmembrane segment and a periplasmic domain, but directly
addressed to the periplasm. Similar behaviour has been previously described for FtsN.
Indeed, the short cytoplasmic tail of FtsN has been described as containing the
information necessary for its initial septal targeting, although it must be fused to a TMH
[31].

In the second part of this study we quantitatively compared the cellular distribution 268 269 of the various fluorescent TolA derivatives as a function of cell age. The components 270 of the *Escherichia coli* divisome are incorporated in two temporally distinct stages [25]. 271 During the early stage, the essential components FtsZ, FtsA, ZipA, and FtsEX are 272 recruited at the future division site and form the Z-ring. This stage starts temporally at 273 10-20% of the cell division cycle. After a temporal delay of about 20% of the cell division 274 cycle, the second stage of divisome assembly occurs. During this stage the other 275 essential proteins, including the final FtsN, are recruited and lead to the maturation of 276 the divisome [26]. Here, we confirm that ToIA and ToIQ are recruited in the late stage 277 of cell division (Fig.5-6). Indeed, ToIA and ToIQ appeared at midcell after 30-40% of 278 the cell cycle in our experiments, as previously reported [27]. Surprisingly, we observed 279 that TolA variants deleted from the periplasmic TolA<sub>3</sub> domain are recruited earlier in 280 the cell division process, suggesting that TolA's partner is an early division protein. 281 Since TolA<sub>1</sub> is recruited early and requires the presence of TolQ this suggests that TolQ's partner may also be an early division protein. As shown in (Fig. 4), GFP-TolA<sub>1</sub> 282 and GFP-ToIA<sub>1-2</sub> appeared at midcell after only 10-20% of the cell cycle. Difference of 283 284 the cellular distribution of the various ToIA derivatives as a function of cell age is 285 unambiguous since these fluorescent ToIA variants were expressed at a similar level, 286 correctly addressed and inserted in the IM. Our results suggest that the topological cue 287 involved in ToIA and ToIQ recruitment at midcell is present in the early stage of the cell division but not yet accessible. Interestingly, a similar behaviour has been previously 288 289 described for FtsN, an essential protein that interacts with FtsA in the divisome [31]. 290 Indeed, FtsN recruitment to the Z-ring is belated, suggesting that it is somehow sequestered from the assembly divisome complex by an unknown mechanism. In fact, 291 292 many studies have suggested that divisome assembling is more complicated that 293 sequential binary interactions [33,34].

In the last part of this study, we investigated the mechanism regulating the temporal recruitment of ToIA and ToIQ during cell division. We showed that in the absence of ToIB, or in the absence of the ToIQR stator, ToIA appears at midcell after

297 only 10-20% of the cell cycle. The same result is observed with the recruitment of TolQ 298 in the absence of ToIA or ToIB. This suggests that preventing ToIA and ToIQ from 299 interacting with their partners throughout the cell accelerates their recruitment to the 300 septum. It should be noted that the estimated number of Pal molecules per cell is largely in excess compared to the estimated number of ToIA. Indeed, previous studies 301 302 estimated between 30,000 and 60,000 copies of Pal, 6,000 copies of TolB and 303 between 400 to 800 copies of ToIA [5,35,36]. Based on the high affinity of ToIB for Pal 304 (Kd = 30 nM) and the hypothesized low affinity of Pal for the PG (Kd =  $\mu$ M), it has been 305 proposed that all TolB molecules will be in complex with Pal [4]. It is reasonable to 306 postulate that the IM complex ToIQ-R-A continually scans OM for an accessible ToIB-307 Pal complex as previously suggested [4]. Thus, each contact, even transient, restricts 308 the mobility of the IM complex TolQ-R-A limited by the PG mesh until TolA succeeds 309 in dissociating the TolB-Pal complex anchored in the OM. Conversely, in the absence 310 of their Tol's partners; TolA and TolQ never forms a trans-envelope complex 311 susceptible of transiently restricting their mobility, which results in an earlier 312 recruitment at the septum.

313 Our results provide new information on ToIA and ToIQ recruitment at midcell and 314 raise new perspectives. We show that in artificial conditions ToIA and ToIQ may be 315 recruited in the early stage and not in the late stage of the cell division process, providing a new example for an alternative non-sequential assembly of the divisome 316 and suggesting that what attracts ToIA and ToIQ to the septum is present in the early 317 318 stage. Finally, we propose that the mechanism that sequesters and delays ToIA and 319 TolQ from the assembling divisome complex is the repeated interactions of TolA with 320 the TolB-Pal complexes in the OM that they encounter throughout their journey to the 321 septum. This suggests that the Tol-Pal complex operates not only at the septum but 322 anywhere in the envelope.

323

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- *Conflict of interest*—The authors declare that they have no conflicts of interest with
- the contents of this article.

### 336 Material and Methods

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Bacterial strains, plasmids, and growth

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Strains and plasmids used for this study are listed in Table S1. *E. coli* strains were grown aerobically at 37°C in Lysogeny Broth medium (Bactotryptone 10 g/l, Bacto yeast extract 5 g/l, NaCl 10 g/l) supplemented with antibiotics if necessary (ampicillin 100 µg/ml, chloramphenicol 30 µg/ml).

344

#### 345 Plasmid construction

346

PCRs were performed in a Biometra thermocycler, using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). Primers were synthesized by Sigma. Plasmids pToIA, pGFP-ToIA and pTat-GFP-ToIA<sub>3</sub> were constructed by a double PCR technique [37], allowing amplification of the *toIA*, *sfGFP-toIA* and *toIA*<sub>3</sub> sequences, flanked by extensions annealing to the target vector pBad/HisC or pTat-GFP respectively. The product of the first PCR was then used as primers for a second PCR with the target vector as a template.

354

### 355 Cloning and expression of *E. coli* Tol-Pal proteins

356

357 The E. coli W3110 strains carrying the chromosomal gene deletions of interest were 358 obtained using a modified protocol from Datsenko & Wanner (2000) [38] as described previously [39]. Briefly, for each gene portion deletion, a kanamycin cassette was 359 amplified from the vector pKD4 using a pair of oligonucleotides carrying 50-nucleotide 360 361 extensions homologous to regions that are adjacent to the target DNA region. After electroporation of the PCR product into E.coli W3110 cells carrying the pKOBEG 362 363 vector, kanamycin-resistant clones were selected and confirmed by colony-PCR. The kanamycin cassette was then excised using vector pCP20 and gene deletions were 364 verified by PCR. 365

366

### 367 Western Blots and protein production

Protein productions from gene expression at chromosomal loci were checked by western blot experiments on over-night cultures. Heat treated samples ( $A_{600} = 0.3$ ) were loaded on 12,5% SDS-PAGE and analyzed by western-blot immunodetection using specific antibodies. When necessary, gene expression from a plamsid was induced with L-arabinose 0,02% for 30 min.

374

#### 375 Fluorescence microscopy

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377 Overnight cultures of E. coli W3110 wild-type or derivative strains were diluted to  $A_{600} \sim 0.05$  in LB medium and grown at 30°C with agitation to reach an  $A_{600} \sim 0.8$ . Cells 378 were spotted on LB agar pads prepared with Frame-Seal<sup>TM</sup> slide chambers (Bio-Rad) 379 380 and covered with a coverslip, and incubated for 20 to 30 min at room temperature 381 before microscopy acquisition. Fluorescence microscopy recordings were performed 382 with a Nikon Eclipse Ti microscope equipped with a 100 X NA 1.3 Ph3 objective, an 383 Orcaflash 4.0 LT digital camera (Hamamatsu) and a perfect focus system to maintain 384 focus. Fluorescence images were acquired with an exposure time of 30 ms for phase 385 contrast, and 100 ms for GFP.

386

#### 387 Statistical analysis

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First, noise and background were reduced using the 'Subtract Background' plugin from Fiji. Cells and fluorescent foci were automatically detected with MicrobeJ plugin for ImageJ (<u>http://www.microbej.com</u>). To avoid false positive, each event was manually controlled in the original raw data. Demograph or Heatmap representations were made with the 'Demograph' or 'Heatmap' options from MicrobeJ. Data for the average distribution profiles for specific age ranges, were output using the MicrobeJ XStatProfile Plot function with the parameters: Stat: Mean, Y axis: MEDIAL.intensity.

396

#### 397 Cell fractionation.

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Fractionation experiments were performed as previously described [40]. Cells were grown in LB medium with or without antibiotics. Cells (20 ml) with or without induction with 0,02 % arabinose for 30 min, were pelleted by centrifugation, and suspended in 0.2 ml spheroplast buffer (0.2 M Tris-HCl buffer, pH 8.0, containing 0.5 M sucrose and 403 0.5 mM EDTA). A 10 µl freshly prepared solution of lysozyme (2 mg/ml in water) was 404 added, followed by the addition of 0.4 ml of spheroplast buffer in 0.4 ml of water. The 405 cells were gently mixed and then incubated for 20 min at 4°C. The spheroplasts were 406 pelleted by centrifugation at 2,000 x g for 10 min. The supernatants corresponded to the periplasm. The pellet was resuspended in 1 ml of spheroplast buffer containing 2 407 408 mM phenylmethylsulfonyl fluoride (PMSF) and the spheroplasts were lysed by three 409 cycles of freezing and thawing. This suspension was then centrifuged at 100,000 x g 410 for 30 min. The supernatant corresponded to the cytoplasmic fraction. The pellet was 411 washed with 1 ml of spheroplast buffer, suspended in 1 ml of the same buffer, and 412 saved as the membrane fraction. Membrane fractions were also incubated overnight 413 at 4°C with either 0.7 M NaCl, 4 M urea or 1% (v/v) Triton X-100. These suspensions 414 were centrifuged at 150,000 x g for 30 min and the pellets were suspended in gel 415 electrophoresis buffer. The supernatants were incubated for 1 h at 4°C with 10% (v/v) 416 trichloroacetic acid (TCA) and centrifuged at 18,000 x g for 30 min. The pellets were 417 washed with acetone and suspended in gel electrophoresis buffer. Total, periplasmic, 418 membrane, and cytoplasmic fractions were analysed by western blotting with the 419 indicated antibodies. Protein markers of different compartments (Ef-Tu for cytoplasm, 420 TolB for periplasm, Pal for membrane) were detected in the corresponding fractions.

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#### 423 Phenotypic studies

424

425 Phenotypic studies were performed in order to visualize effects of the fluorescent 426 marker chromosomal insertion and gene deletions onto the tol-pal operon. Three 427 assays were achieved to check the OM integrity, the Tol-Pal complex assembly and 428 the cell division process. Colicin tolerance assay: Colicin A and D lethal activities were 429 checked by the presence of halos on a cell lawn of the strain to be tested, as previously 430 described [41]. SDS sensitivity assay: Sensitivity to Sodium Dodecyl Sulphate (SDS) was determined by measuring the level of survival of the strain after 4 hours of 431 432 incubation at 37°C in liquid LB medium containing various concentration of SDS (from 433 0.25% to 2%). Cell division assay: Cells were grown for 4 hours at 37°C in LB 434 (Bactotryptone 10 g/l, Bacto yeast extract 5 g/l, NaCl 10 g/l) or LB-NaCl (Bactotryptone 10 g/l, Bacto yeast extract 5 g/l) liquid medium. Then cells were fixed on fresh Poly-L-435

- 436 Lysine (0,1% w/v in  $H_2O$ ) coated microscope slides and were observed with an optical
- 437 microscope (Eclipse 50i, Nikon, France).

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#### 566 Figures legends

567

**Fig. 1.** Representation of the different TolA fluorescent hybrid proteins. (a) Schematic representation of the components of the Tol-Pal complex in the *Escherichia coli* envelope. (b) Location of the fusion and truncation sites in the TolA fluorescent hybrid proteins (strains are referenced in Table S1).

572

573 Fig. 2. Expression and cellular location of the TolA derivatives. (a) Western blot 574 analysis was used to check native and chimeric TolA protein productions. GFP-TolA, 575 GFP-TolA<sub>1-2</sub> and GFP-TolA<sub>1</sub> were introduced at their native locus on the chromosome 576 and were expressed from their own promotor. Plasmid encoded GFP-TolA<sub>3</sub> was 577 introduced in W3110 \(\Delta to IA\) strain and expression was induced with L-arabinose 0,02% 578 for 30 min. The antibodies used are indicated. GFP-ToIA1 was not detected with 579 antiserum directed against ToIA, presumably because ToIA<sub>1</sub> did not contain epitopes. 580 Alternatively, GFP-TolA<sub>1</sub> was detected with antiserum directed against GFP. (b) 581 Intracellular location of the TolA derivatives. Cells (T) producing the various TolA 582 derivatives were separated into cytoplasmic (C), periplasmic (P) and membrane (M) 583 fractions as described in Materials and Methods. We observed two different forms of GFP-TolA<sub>3</sub>, with an apparent molecular weight of approximately 45 and 50 kDa. These 584 bands probably correspond respectively to the mature protein after cleavage by the 585 leader peptidase and to its precursor form. Fractions of 0.3 unit ( $A_{600}$ ) were tested for 586 587 the presence of the various ToIA derivatives by immunoblotting with the indicated 588 antibodies (p = precursor form; m = mature form). The same fractions were tested for 589 the presence of ToIB, Pal and EF-Tu, markers of the periplasm, cytoplasm and 590 membrane fractions, respectively. (c) GFP-ToIA, GFP-ToIA<sub>1-2</sub> and GFP-ToIA<sub>1</sub> were 591 correctly inserted in the IM. Membrane fractions containing the indicated ToIA 592 derivatives were subjected to various treatments; overnight at 4°C with 0.7 M NaCl, 593 4M Urea or 1% Triton X-100, and were centrifuged for 30 min at 18,000 g. The various ToIA derivatives were detected in the resulting pellets (P) or supernatants (S) by 594 595 immunoblotting with the indicated antibodies.

596

**Fig. 3.** Localization of the TolA derivatives to cell septum during cell division. Fluorescence microscopy images of W3110 *E. coli* cells expressing the chromosomal fluorescent protein GFP-TolA, GFP-TolA<sub>1</sub> and GFP-TolA<sub>1-2</sub> or the plasmid encoded 600 GFP-TolA<sub>3</sub> and fluorescence microscopy images of  $\Delta tolQR$  strain cells expressing the 601 chromosomal fluorescent protein GFP-ToIA1 and GFP-ToIA1-2. GFP-ToIA3 is observed in the periplasm whereas GFP-ToIA, GFP-ToIA<sub>1</sub> and GFP-ToIA<sub>1-2</sub> localize at 602 603 constriction sites during cell division. GFP-TolA<sub>1-2</sub> localizes at constriction sites in the absence of ToIQR unlike GFP-ToIA<sub>1</sub>. However, in a  $\Delta toIQR$  mutant carrying the 604 plasmid pBadTolQR encoding TolQ and TolR, GFP-TolA1 localization at the septum 605 is restored. Heatmap distribution in cells of the fluorescent proteins tested is 606 607 represented on the right of each panel where n represents the number of cells for each 608 study. Scale Bar: 2 µm. In all these experiments, E. coli W3110 derivative strains were 609 grown in LB medium at 30°C.

610

611 Fig. 4. Localization of the various ToIA derivatives as function of cell cycle progression. 612 (a) Fluorescent profiles (demographs) of E. coli W3110 cells expressing the 613 chromosomal fluorescent protein GFP-ToIA, GFP-ToIA<sub>1</sub> and GFP-ToIA<sub>1-2</sub> or the 614 plasmid encoded GFP-ToIA<sub>3</sub> where n represents the number of cells for a triplicate study. The signal intensity in each cell is represented by a color code, from dark blue 615 616 (low intensity) to yellow (high intensity). On the y-axis, cells are sorted for length, 617 ascending from top (the shortest cell) to down (the longest cell) and indicates cell cycle 618 progression. Each horizontal line corresponds to a single cell. The longest cells 619 correspond to cells in the last stage of cell division. In all these experiments, strains were grown LB medium at 30°C. (b) Average distribution of indicated ToIA derivatives 620 621 for different cell-cycle progression age groups. Profiles of the 10 age classes for each 622 protein have been offset equally to facilitate comparison and minimize overlap. 623 fluorescence intensity profiles were normalized by length, then averaged and 624 normalized to the peak local brightness for each age group. Each age group contains 625 around 100-200 cells. \*Age class when increased fluorescent signal at mid-cell is first 626 observed.

627

**Fig. 5.** Localization of GFP-ToIA as function of cell cycle progression in different genetic backgrounds. (a) Fluorescent profiles (demographs) of *E. coli* W3110 cells expressing the chromosomal fluorescent protein GFP-ToIA, in WT,  $\Delta toIB$  or  $\Delta toIQR$ backgrounds where n represents the number of cells for a triplicate study. Same legend as figure 4a. (b) Average distribution of GFP-ToIA for different cell-cycle progression age groups in WT, *∆tolB or ∆tolQR* backgrounds. Same legend as figure
4b.

635

**Fig. 6.** Localization of TolQ-mCherry as function of cell cycle progression in different genetic backgrounds. (a) Fluorescent profiles (demographs) of *E. coli* W3110 cells expressing the chromosomal fluorescent protein TolQ-mCherry, in WT,  $\Delta tolB$  or  $\Delta tolA$ backgrounds where n represents the number of cells for a triplicate study. Same legend as figure 4a. (b) Average distribution of TolQ-mCherry for different cell-cycle progression age groups in WT,  $\Delta tolB$  or  $\Delta tolA$  backgrounds. Same legend as figure 4b.

**Table 1.** Phenotypic analysis of *tol-pal* chromosomal fusions and deletions strains.

644 W3110 cells expressing different chromosomal fusions and deletions of the tolA gene 645 were tested for level of import of colicin A (Tol dependent) and colicin D (Ton 646 dependent), susceptibility to SDS and cell division +/- NaCI. SDS sensitivity was tested 647 in liquid LB medium containing various concentrations of SDS (R: resistant, S: 648 sensitive). Colicin A and D sensitivity was tested on bacterial lawns by a spot dilution 649 assay. The number indicated represents the maximal 10-fold dilution of the colicin 650 stock (1 mg/ml) still able to kill the strain tested (0: resistant, 4: sensitive to 10<sup>4</sup> dilution). Cell division was observed during exponential phase after growth in LB or LB - NaCl 651 652 (N = Normal cell division, C = Chain formation).

## **Table 1:** *E. coli* strains and phenotypes.

654 655 656 657	Strains	OM stability SDS	Cell chaining	Colicin A	uptake D
658					
659	W3110	R	Ν	4	4
660	∆TolA	S	С	0	4
661	GFP-TolA	R	Ν	4	4
662	GFP-TolA <sub>1-2</sub>	S	С	0	4
663	GFP-ToIA1	S	С	0	4
664 665	∆TolA: pTat-GFP-TolA <sub>3</sub>	S	С	0	4

#### Supplemental information

Fig. S1. Functionality of the various TolA derviatives. (a) Phase contrast imaging of 668 WT and derivative mutant strains. Phase contrast imaging of WT and modified strains 669 670 showing cell division after growth at 37°C in LB - NaCl (top panel) or LB (bottom panel). tolA gene deletions lead to cell septation defect in hypo-osmolarity conditions. In 671 672 contrast the strains encoding tolA or gfp-tolA present a wild-type phenotype. Scale Bar: 2.5 µm. (b) Sensitivity to colicins of WT and derivative mutant strains. Sensitivity of WT 673 and modified strains to colicin A (Tol dependent) and colicin D (Ton dependent) lethal 674 activities was tested using dilutions from 10<sup>-1</sup> to 10<sup>-4</sup> of colicins spotted on freshly 675 seeded lawns. Zones of clearance indicate colicins activity against the strain. Colicin 676 677 D uses the TonB system to enter and to kill cells and its lethal activity is not affected by a tol-pal deletion. (c) Sensitivity to SDS of WT and derivative mutant strains. 678 679 Sensitivity to SDS of the indicated strains was tested in liquid LB medium containing various concentrations of SDS: 0.25% to 2%. The percentage of surviving cells in liquid 680 681 LB medium was estimated after 4 hours at 37°C from the turbidity ratio of the SDStreated and the control sample. Error bars indicate the error on triplicate samples for 682

683 each condition.

# 686

#### 687 **Table S1:** *E. coli* strains and plasmids.

3 9 0	Strains or plasmids	Genotype	Source
1	Strains		
	W3110	F'λ <sup>'</sup> IN(rrnD-rrnE)1 rph-1	[1]
	∆TolA	W3110 ∆tolA::frt	[2]
	GFP-TolA	W3110 sfGFP-toIA::frt	[2]
	GFP-TolA:∆TolQR	W3110 sfGFP-tolA::frt, <i>∆tolQR::kan</i>	[2]
	GFP-TolA:∆TolB	W3110 sfGFP-tolA::frt, ∆tolB::kan	[2]
	GFP-ToIA <sub>1-2</sub>	W3110 sfGFP-toIA <sub>1-2</sub> ::frt	This study
	GFP-ToIA1	W3110 sfGFP-tolA₁::frt	This study
	GFP-TolA₁:∆TolQR	W3110 sfGFP-tolA₁::frt, ∆tolQR::kan	This study
	GFP-TolA₁-2:∆TolQR	W3110 sfGFP-tolA₁-₂::frt, ∆tolQR::kan	This study
	TolQ-mCherry	W3110 tolQ-mCherry::frt	[2]
	TolQ-mCherry:∆TolB	W3110 tolQ-mCherry::frt, ∆tolB::kan	[2]
	TolQ-mCherry:∆TolA	W3110 tolQ-mCherry:: frt, ∆tolA::kan	[2]
		-	
	Plasmids		
	pCP20	amp flp recombinase	[3]
	pKOBEG	$cm$ , $\lambda red$ integrase	[4]
	pTat-GFP	amp, pBAD24 tat-gfp	[5]
	pTat-GFP-TolA₃	amp, pBAD24 tat-gfp-tolA <sub>3</sub>	This study
	pBad/HisC	amp	Invitrogen
	pBadTolQR	amp, pBAD/HisC toIQR	[2]

713 714

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Figure 1.



GFPToIA<sub>1</sub>

GFP-TolA

GFP-TolA<sub>1-2</sub>

GFP-TolA<sub>3</sub>

GFP-TolA<sub>1</sub>

Controls

Figure 2.

 $\alpha$ -GFP



Figure 3.



## Figure 4.



# Figure 5.



## Figure 6.





Fig:S1