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**Tol energy-driven localization of Pal and anchoring to the peptidoglycan promote outer membrane constriction**

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Running title: **Energy state of Tol-Pal and cell division**

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**Abstract**

During cell division, Gram-negative bacteria must coordinate inner membrane (IM) invagination, peptidoglycan (PG) synthesis and cleavage and outer membrane (OM) constriction. The OM constriction remains largely enigmatic and the nature of this process, passive or active, is under debate. The proton-motive force (PMF) dependent Tol-Pal system performs a network of interactions within these three compartments. Here we confirm that the trans-envelope Tol-Pal complex accumulates at constriction site in *Escherichia coli*. We show that the IM complex composed of TolA, TolQ and TolR recruits the OM complex TolB-Pal to the septum, in an energy-dependent process. Pal recruitment then allows its binding to PG and subsequently, OM constriction. Our results provide evidence that the constriction of the OM is an energized process.

**Keywords:** molecular motor, Tol-Pal system, septum, OM invagination, membrane vesicles

**Abbreviations used:** OM, outer membrane; IM, inner membrane; PG, peptidoglycan; PMF, proton-motive force; sfGFP, super-folder green fluorescent protein; OmpA, outer-membrane protein A; Lpp, Braun's lipoprotein

## 1 Introduction

2  
3 Gram-negative bacteria evolved a complex bacterial envelope consisting of two  
4 membranes, the inner (IM) and outer (OM) membranes, confining a periplasmic  
5 space in which a thin layer of PG resides. This three-layer structure acts as a  
6 physical and selective barrier against noxious compounds and contributes to  
7 mechanical robustness of the cell [1]. Cell division is the last event of the bacterial  
8 cell cycle and requires a coordinated constriction of these three layers to maintain the  
9 cell envelope integrity.

10 In *E. coli*, cell division is a highly coordinated dynamic process involving more than  
11 30 different proteins that form a large complex called the divisome. Assembly of the  
12 divisome at midcell is initiated by association of three proteins: a cytoplasmic protein  
13 FtsZ, a bitopic IM protein ZipA and an associated IM protein FtsA, which form a  
14 dynamic ring structure commonly referred as the proto-ring or the Z-ring. After a  
15 temporary delay, seven additional essential proteins, FtsK, FtsQ, FtsL, FtsB, FtsW,  
16 FtsI and FtsN are recruited to the proto-ring to complete the division machinery which  
17 ultimately extends from the cytoplasm to the three layers of the cellular envelope.  
18 Once the full divisome is assembled, the synthesis of septal PG starts driven by  
19 treadmilling of FtsZ filament bundles, which is coordinated with IM constriction and  
20 the constitution of the septum (for reviews see [2-5]). How Gram-negative bacteria  
21 coordinate OM invagination with IM invagination and septal synthesis remains largely  
22 unknown.

23 The Tol-Pal system is a trans-envelope complex highly conserved among gram-  
24 negative bacteria. The core of this system is composed of five proteins organized in  
25 two sub-complexes. The IM complex is composed of three proteins: the proton  
26 channel-forming TolQ, TolR and associated TolA anchored in the IM, while the OM  
27 complex is composed of TolB, a periplasmic protein, and Pal a lipoprotein anchored  
28 in the inner leaflet of the OM and able to bind the PG layer (Fig. 1a). *In vivo*, the two  
29 sub-complexes are transiently connected by TolA. TolA has a long predicted helical  
30 second domain (TolAII) that is thought to stretch across the periplasm where its C-  
31 terminal domain interacts with TolB N-terminal domain and potentially also with Pal in  
32 a PMF-dependent manner [6]. Thus, the Tol-Pal complex uses the PMF to form a link  
33 between the IM and OM. In *E. coli*, two additional proteins are encoded in the *tol-pal*

1 cluster (Fig. 1b): the cytoplasmic thioesterase YbgC and the periplasmic protein  
2 CpoB [7].  
3

4 Previous results suggest that the Tol-Pal complex could play a key role in the OM  
5 constriction. This complex transiently links the three layers of the cell envelope [6, 8-  
6 12], accumulates at the septum during cell division [13] and CpoB encoded by the  
7 last gene of the *tol-Pal* cluster, has been described to regulate activity of a major PG  
8 synthase (PBP1B) in response to the Tol energy state [7]. These results suggest that  
9 OM constriction could be an active process dependent of the Tol-Pal system. Despite  
10 this potential essential role, the cellular localization of the Tol-Pal complex in *E. coli*  
11 has been analyzed under multicopy production of Tol-Pal fluorescent derivatives [13]  
12 while the role of the energy state of the Tol-Pal complex in its localization has not  
13 been documented.

14 The PMF, TolQ and TolR proteins and the trans-membrane helix of TolA regulate  
15 the conformational change of the periplasmic region of TolA, and probably its ability  
16 to interact with Pal and perhaps TolB [6]. The TolQ-R complex is part of an emerging  
17 molecular motor family which is able to convert chemical energy derived from the  
18 PMF to mechanical movements [14]. This molecular motor family includes several  
19 multiprotein complexes that share sequence and conformational similarities. The  
20 MotA/MotB proteins drive the bacterial flagellum, the ExbB/ExbD proteins are  
21 involved in iron uptake and the recently characterized AglSQR proteins drive *M.*  
22 *xanthus* gliding motility; all work as motors as they convert chemical energy (PMF)  
23 into mechanical movement in order to energize a TolA-like partner [15-17].  
24 Paradoxically and in contrast to other Tol-like motors, the exact function of the Tol-  
25 Pal system remains enigmatic, essentially because of the pleiotropic phenotypes  
26 exhibited by the *tol* mutants [18-24], and in particular the exact contribution of Tol-Pal  
27 to cell division remains obscure [21,25].

28 In this study, we revisited the question of the Tol-Pal proteins localization. In order  
29 to monitor the intracellular localization and dynamic of each Tol-Pal protein during the  
30 cell cycle, we initiated a systematic fluorescence microscopy approach in *E. coli*. To  
31 further gain insight on how this machinery operates, we engineered chimeric proteins  
32 expressed from their endogenous chromosomal loci, fused to fluorescent markers  
33 such as sfGFP or mCherry. Only the core proteins of the Tol-Pal system that are  
34 conserved among Gram-negative bacteria were studied. In the first part of this paper

1 we showed that all the chimeric proteins were functional and stably produced. We  
2 confirmed that the Tol-Pal proteins are dynamically recruited to the constriction sites  
3 during cell division. In addition, we showed that TolR requires either TolA or TolQ for  
4 its localization whereas TolA and TolQ localize independently of any of the other Tol-  
5 Pal proteins. Strikingly, TolQ, TolR, TolA, and TolB are absolutely required for Pal  
6 association with division sites, an active recruitment process that requires the PMF.  
7 Finally, the PG binding domain of Pal is not required for its septal localization, but  
8 absolutely require for its function and for proper OM constriction during cell division.

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## 1 Results

### 3 Chromosomal-encoded fusion of the Tol-Pal components to fluorescent 4 domains results in functional fluorescent proteins.

6 To further gain information on the cellular localization of Tol-Pal components, their  
7 recruitment and dynamic behavior, we engineered strains producing super-folder  
8 GFP (sfGFP) or mCherry fused to the N or C terminus of TolQ, TolR, TolA, TolB, and  
9 Pal. All the constructs were introduced at their respective native locus on the  
10 chromosome. sfGFP was fused to the cytoplasmic N-terminus of TolA and TolR.  
11 Indeed, the C-terminal domain of TolA and TolR has been described to be involved in  
12 many interactions with the others proteins of the complex and the proximity of a  
13 fluorescent marker might inhibit their function (for a review see [26]). As mCherry-  
14 protein fusions have been described to be effectively transported to the periplasm of  
15 *E. coli* for protein localization experiments [27], this fluorescent marker was fused to  
16 the C-terminus of TolB, Pal and also to the cytoplasmic C-terminus of TolQ. The  
17 distal N-terminal part of TolB contains the TolA binding site and is therefore not  
18 suitable to fuse a fluorescent marker [28].

19 We first checked that all the fluorescent Tol-Pal fusions proteins were functional. It  
20 has been previously described that Tol-Pal mutants are hypersensitive to detergents  
21 [29], form cell chains in medium of low osmolarity [25] and are resistant to colicins  
22 [30]. Here we showed that all the strains producing a fluorescent Tol-Pal fusion  
23 protein displayed a wild-type phenotype (Table 1 and Fig. 2 and S1-3). These strains  
24 were able to grow as efficiently as the wild-type strain in presence of SDS (Fig. 2a  
25 and S1), did not form cell chains in LB medium lacking NaCl (Fig. 2b and S2), and  
26 were sensitive to different colicin concentrations (Fig. 2c and S3), in contrast to the  
27 strain W3110 deleted of the *tolA* gene (Table 1 and Figs. 2a-c, and S1-3). These  
28 results clearly showed that all the fluorescent Tol-Pal fusion proteins remained  
29 functional.

30 The *tol-pal* genes are transcribed as two distinct operons, *ybgC tolQRA* and *tolB*  
31 *pal cpoB* [31] (Fig. 1b). As shown in figure 2d by immunoblotting analysis with the  
32 indicated antibodies, insertion of sfGFP or mCherry coding sequence in these  
33 operons did not modify the level of expression of the resulting chimeric fluorescent  
34 proteins, as well as the level of expression of the others proteins of the Tol-Pal

1 complex. Nevertheless, the strain producing TolB-mCherry was associated with a  
2 decrease in Pal abundance. This decrease does not interfere with Tol-Pal function  
3 because the strain producing TolB-mCherry displayed a wild-type phenotype,  
4 absence of filamentation, growth on detergent and sensitivity to colicin A similar to  
5 the W3110 strain (Table 1 and Figs. S1-3). We therefore used that strain throughout  
6 this study.

### 7 8 **Tol-Pal proteins are recruited to cell constriction site.**

9  
10 The intracellular localization of each of the Tol-Pal proteins fused to a fluorescent  
11 reporter was analyzed by a live fluorescence microscopy approach. Time-lapse  
12 microscopy recordings showed that all tested fluorescent Tol-Pal proteins first  
13 appeared diffuse in the cell and were dynamically recruited to future division sites  
14 during cell division until they dispersed after daughter cell separation (Fig. 3a, left  
15 panel). Notably, Pal-mCherry remained longer than the other Tol proteins at the new  
16 cell poles (Fig. 3a, right panel). These results were confirmed by Kymograph analysis  
17 (Fig. 3a, right panel) and a large-scale demograph analysis of cells (Fig. 3b). While  
18 the demograph analysis (Fig. 3b) may suggest that there is a temporal pattern in the  
19 mid-cell recruitment of the Tol proteins during cell cycle, we believe that our data  
20 cannot be used to confirm or infirm this hypothesis. Indeed, the number of Pal  
21 molecules per cell has been estimated between 30,000 to 40,000 copies, whereas  
22 the number of TolA molecules per cell has been estimated between 400 to 800  
23 copies [26]. These differences of abundance make it easier, for example, to detect  
24 Pal at the septum. The precise timing interpretation of the Tol-Pal protein localisation  
25 would require a more stringent analysis of cell fluorescence, specifically the analysis  
26 of dual-labelled strains. Nevertheless, based on the recordings presented on Fig 3,  
27 our results confirm unambiguously that the Tol-Pal proteins are recruited to the  
28 septum during cell division.

### 29 30 **TolR requires TolA or TolQ for its localization at the cell constriction site.**

31  
32 To determine the role of each Tol-Pal proteins in the assembly of the core-  
33 complex and their recruitment to the septum, we analyzed the intracellular  
34 localization of the different chimeric fluorescent proteins in various deletion mutant



1 backgrounds. Each of these deletion mutants, *tolA*, *tolB*, *tolQ*, *tolR*, *tolQR* and *pal*,  
2 present a classical *tol* phenotype (Table 1 and Figs. S1-3). Western blot analysis  
3 confirmed deletion of each *tol-pal* gene and showed that *tolB* and *tolQ* deletions led  
4 to a reduced production of Pal and of TolR, respectively. The level of production of  
5 the others proteins were not affected (Fig. S4) First, we analyzed the behavior of the  
6 three IM proteins of the Tol-Pal complex, TolA, TolQ and TolR, during cell division.  
7 Using plasmid encoded fluorescent fusions, Gerding and co-workers previously  
8 reported that GFP-TolA and TolQ-GFP, but not GFP-TolR localize at the septum in a  
9 TolQ-R-A-B-Pal mutant background [13]. Here, we deciphered further the Tol  
10 recruitment process, showing that sfGFP-TolA, TolQ-mCherry and sfGFP-TolR  
11 accumulated at the constriction sites in all single deletion mutants tested. These data  
12 demonstrate that assembly of a functional molecular motor is not required for  
13 recruitment of the Tol-Pal proteins to the septum (Figs 4a-c). It has been previously  
14 described by crosslinking experiments that TolR can interact with TolQ in absence of  
15 TolA and with TolA in absence of TolQ [9,32]. Thus, although TolA and TolQ do not  
16 require any Tol-Pal protein for their septal location, and based on previous  
17 observations [13], it is likely that TolR requires either TolA or TolQ for its recruitment  
18 to the septum (Fig. 4b).

19  
20 **Pal requires the TolQ, TolR, TolA and TolB proteins and a functional TolQ-TolR**  
21 **molecular motor for its localization at the cell constriction site.**

22  
23 The Tol-Pal OM complex is composed of the OM anchored lipoprotein Pal and the  
24 soluble periplasmic TolB protein. TolB-mCherry also localized to the division septum  
25 (Fig. 5a). In the various *tol-pal* deletion mutants, TolB-mCherry was largely  
26 sequestered in apparent membrane vesicles that formed at constriction sites (Fig.  
27 5b). This could be expected because *tol-pal* mutants have been previously reported  
28 to show pleiotropic phenotypes, including pronounced local expansions of their  
29 periplasmic space at sites of constriction and abundant vesicle formations [13,33,34].  
30 To test whether periplasmic proteins could indeed be detected in vesicles, we also  
31 imaged a GFP protein fused to a Tat signal peptide (Tat-GFP), a periplasmic control  
32 protein, in WT and *tol-pal* mutant cells (Fig. 5a, 5b). In a wild-type background, Tat-  
33 GFP was localized all around the cell envelope suggesting that it is indeed  
34 homogeneously distributed in the periplasmic space. However, we observed

1 numerous highly fluorescent vesicles emerging from the constriction sites or located  
2 at the pole of the *tol-pal* mutant cells (Fig. 5b).

3 The intracellular localization of Pal in the different mutant backgrounds revealed  
4 that Pal-mCherry was not recruited at the constriction sites during cell division but  
5 remained spread in the OM (Fig. 5c). This result demonstrates that recruitment of  
6 Pal-mCherry to division sites requires all the Tol proteins. TolA interacts with Pal in a  
7 PMF-dependent manner (directly or via TolB, see below), and this interaction is  
8 regulated by the molecular motor TolQR. It has been previously described that a  
9 single D23A mutation in the transmembrane segment of TolR deactivates the TolQR  
10 motor without affecting TolQR-TolA complex assembly [35]. When we  
11 complemented, a *tolQR* strain with a plasmid encoding TolQR, Pal-mCherry was  
12 recruited to the septum (Fig. 5c). However, introduction of a plasmid encoding  
13 TolQR<sub>D23A</sub> failed to restore septal localization and Pal-mCherry was dispersed along  
14 the periphery of the cell (Fig. 5c). These results show that a functional TolQR motor  
15 is absolutely required to maintain Pal at the septum.

### 17 **Key role of Pal at the septum.**

18  
19 Previous studies demonstrated that Pal interacts both with TolB and the PG, and  
20 that these two interactions are mutually exclusive [10,11]. To analyze the role of Pal  
21 at the septum we first constructed a plasmid encoding the fluorescent hybrid protein  
22 Pal-sfGFP under the control of an arabinose inducible promoter and verified that this  
23 construction was correctly expressed, functional (Fig. 6a-c and Table 1) and localized  
24 at the constriction sites during cell division (Fig. 6e, WT panel). Then, we constructed  
25 two mutated version of Pal-sfGFP (Fig. 6d). A Pal (E102K) variant that prevents Pal-  
26 TolB interaction but does not affect PG-binding and a Pal (R104C) variant that  
27 interacts with TolB but does not bind PG [36]. The two mutated Pal-sfGFP proteins  
28 (Pal<sub>102</sub>-sfGFP and Pal<sub>104</sub>-sfGFP) were correctly expressed (Fig. 6a) but as previously  
29 shown were not able to complement a  $\Delta pal$  strain [36] (Fig. 6b, c and Table 1). Thus,  
30  $\Delta pal$  strain producing either Pal<sub>102</sub>-sfGFP or Pal<sub>104</sub>-sfGFP fusion protein showed a  
31 high sensitivity to detergents (Fig. 6c) and showed a chaining phenotype under low  
32 osmolarity growth conditions indicative of a division and cell separation defect (Fig.  
33 6b). The Pal<sub>102</sub>-sfGFP did not localize at the sites of constriction during cell division,  
34 indicating that interaction with TolB is essential for Pal recruitment (Fig. 6e). In

1 contrast, Pal<sub>104</sub>-sfGFP localized at the septum normally, indicating that once at the  
2 septum, Pal must interact with the PG to perform its function. Finally, in a *ΔtolB-pal*  
3 strain, Pal<sub>104</sub>-sfGFP showed no septal localization during cell division (Fig. 6e).  
4 These results clearly indicate that TolB is absolutely necessary for functional Pal  
5 localization and that Pal binding to PG is absolutely necessary to the function of the  
6 Tol-Pal system in cell division.

7

## 1 Discussion.

2  
3 Cell division in Gram-negative bacteria is orchestrated by the divisome and  
4 requires the coordinated constriction of the three cell envelope layers. Despite major  
5 progress understanding IM invagination, little is known about OM coordinated  
6 constriction. It has been suspected for a long time that OM invagination during cell  
7 division might be achieved passively, by simple tethering of OM lipoproteins to the  
8 PG. However recent evidence suggested a potential role of the Tol-Pal complex in  
9 cell division and particularly in OM constriction. The Tol-Pal proteins connect the  
10 three layers of the cell envelope [26], and they have been described to localize at the  
11 septum during cell division [13]. Interestingly, although Pal is a PG-binding OM  
12 lipoprotein, it is not covalently attached to the PG. Pal is engaged in many  
13 interactions with different partners, localized in the OM, the periplasm and the IM,  
14 and some of these interactions are exclusive. Moreover, interaction between TolA  
15 and Pal requires the PMF and a functional TolQR molecular motor [14]. These data  
16 led the hypothesis that Pal might facilitate an active mechanism of OM constriction  
17 during cell division

18 Here, we revisited the localization of the Tol-Pal system during cell division and  
19 provided information on the impact of the TolQR motor and the Pal PG binding site in  
20 Pal localization and function. To overcome possible artefacts resulting from  
21 overexpression of chimeric fluorescent proteins encoded by plasmids, we engineered  
22 strains producing super-folder GFP (sf-GFP) or mCherry fused to the N- or C-  
23 terminus of the Tol-Pal proteins (Table S1). All these chromosomal constructs were  
24 introduced at the native, original loci. We first checked that all constructions were  
25 functional and expressed at their original level (Fig. 2 and Table 1). Time lapse  
26 experiments clearly indicate a dynamic septal recruitment of the Tol-Pal proteins  
27 during cell division (Fig. 3a). In non-constrictive cells, each of the Tol-Pal protein  
28 appears dispersed in the cell envelope, then begins to accumulate at the septum  
29 during cells constriction and finally disperses again after cell separation. Demograph  
30 analysis of cells producing the different fluorescent chimeric Tol-Pal proteins confirm  
31 this result (Fig. 3b).

32 Interestingly we observe that TolQ and TolA accumulate at the constrictions site  
33 independently of the other Tol-Pal proteins and that Pal absolutely requires all the Tol  
34 proteins to be associated with the cell septum (Fig. 4, 5). Based on these results and

1 previously published results, it seems likely that the IM TolQRA complex is first  
2 recruited by either FtsN for TolQ [37] or an active divisome complex for TolA  
3 [7,13,38]. TolR is able to directly interact with TolA or TolQ and therefore its septal  
4 localization is likely dependent of one of these two proteins. Once in mid-cell, the  
5 TolQRA complex and TolB drive the recruitment of Pal at the division site. However,  
6 from our results, it is not possible to conclude decisively whether the TolQRA  
7 complex recruits a TolB-Pal OM complex or whether it first recruits TolB, which then  
8 recruits Pal. TolA could also interact directly with Pal, although this interaction cannot  
9 overcome the requirement for TolB. In fact, previous papers indicate contradictory  
10 results about the TolA-Pal interaction. Based on a co-immunoprecipitation approach,  
11 TolA and Pal have been described to interact *in vivo* through a PMF-dependent  
12 mechanism [6,39]. However, such interaction was not detected *in vitro* with purified  
13 proteins by isothermal calorimetry (ITC) [28] suggesting that the TolA-Pal interaction  
14 could be indirect (*i.e.*, mediated via TolB). In a previous paper [13], the authors  
15 speculated that TolB could reverse Pal-PG interactions to permit lateral movement of  
16 the lipoprotein in the OM and then its interaction with TolA at the division site.  
17 However, this is unlikely because we show here that the mutated Pal<sub>R104C</sub> that does  
18 not bind the PG and is free to diffuse in the OM is not localized at the division site in  
19 the absence of TolB (Fig. 6e). Thus, TolB is required for Pal to localize to the septum  
20 likely via direct recruitment.

21 In addition, we demonstrate that the energy provided by a functional TolQR  
22 complex is absolutely require for a correct localization of Pal during the cell cycle.  
23 Indeed, in the absence of TolQR or in the presence of a defective TolQR<sub>D23A</sub>  
24 complex, unable to energize TolA, Pal localized all around the cell envelope and  
25 does not accumulate at the constriction site during cell division (Fig. 5c). Thus, the  
26 septal recruitment of Pal is a PMF-dependent process. Based on these results, we  
27 propose a model for Tol-Pal function during cell division. First, the IM TolQRA  
28 complex is recruited to the division site by divisome proteins (*i.e.*, FtsN and others).  
29 Second, the motor becomes active and energizes TolA to recruit the OM TolB-Pal  
30 complex. Such transient ternary complex has been previously observed although in  
31 low yield by *in vitro* cross-linking experiment with purified proteins [28]. It is possible  
32 that the mechanical action of the TolQR motor, allows TolA to pull the TolB-Pal  
33 complex inwards, drawing the outer membrane invagination over to the PG and IM  
34 layers. Completing this cycle, TolA would dissociate TolB from Pal, thus permitting

1 Pal-PG interaction. Indeed, the large differences of affinity between the TolB-Pal  
2 (nM) and TolA-TolB ( $\mu$ M) interactions [28] are not in favor of a TolA-TolB association.  
3 In conclusion, this cycle of exclusive TolA, TolB and Pal interactions and the  
4 contractile activity of Tol-Pal complex regulated by the energy state of the TolQR  
5 motor, would maintain sufficient contact between the three layers of the envelope for  
6 efficient invagination. In addition, the energy state of the Tol-Pal complex has already  
7 been involved in the regulation of the PBP1B-LpoB machine involved in PG synthesis  
8 at constriction sites [7]. Thus, the Tol-Pal complex appears to regulate and  
9 coordinate septal PG synthesis and OM constriction. Surprisingly, *in E. coli*, despite  
10 all the phenotypes associated with the *tol* mutations and the role of the Tol-Pal  
11 complex in the cell division, this complex is not essential for viability in standard  
12 laboratory conditions, suggesting that the Tol-Pal function, at least in the cell division,  
13 could be compensated by another trans-envelope complex. Indeed two global  
14 approaches, have shown that mutations in Tol-Pal complex are synthetically lethal  
15 when combined with mutations in the trans-envelope complex, LpoB-PBP1B [40].  
16 This complex also seems to participate in tethering the OM either to the PG or the IM  
17 and localizes at constriction sites, likely promoting OM constriction during cell division  
18 even when Tol-Pal is absent, in standard laboratory conditions [40]. In low osmolarity  
19 media, where Tol-Pal is essential, the energy provided by the TolQR motor and the  
20 elongated TolAll domain could prevent local expansions of the periplasmic space  
21 which is harmful to complete the cell division.

22 Although the Tol-Pal localization at the septum during the cell cycle is clearly  
23 established, many questions remain unanswered. Which proteins recruit the Tol-Pal  
24 complex at the septum? FtsN is a good candidate for TolQ and has been previously  
25 described to be involved in the recruitment of TolA [13,37]. However, the role of FtsN  
26 in the recruitment of TolA could be indirect, because a suppressor mutation in FtsA  
27 can restore the correct localization of TolA and function of the Tol-Pal system in a  
28 FtsN-depleted strain [38]. Similarly, the direct recruitment of TolQ by FtsN is  
29 questionable. Another major interrogation concerns the timing and sequence of the  
30 interactions between TolA, TolB, Pal and the PG driven and cycled as function of the  
31 energy state of the system. Understanding and deciphering these interaction  
32 networks would greatly improve our knowledge on the Tol-Pal system function at the  
33 septum during the cell division.

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

## 1 **Material and Methods**

### 3 **Bacterial strains, plasmids, and growth**

5 Strains, and plasmids used for this study are listed in Table S1. *E. coli* strains were  
6 grown aerobically at 37°C in Lysogeny Broth medium or at 30°C in M9 minimum  
7 medium, supplemented with antibiotics if necessary (ampicillin 100 µg/ml, kanamycin  
8 50 µg/ml and/or chloramphenicol 30 µg/ml).

### 10 **Plasmid construction**

12 PCRs were performed in a Biometra thermocycler, using Pfu Turbo DNA  
13 polymerase (Stratagene, La Jolla, CA). Oligonucleotides were synthesized by Sigma.  
14 Plasmids pTolQR and pPal were constructed by a double PCR technique [41],  
15 allowing amplification of the *tolQR* and *pal* genes, flanked by extensions annealing to  
16 the target vector (pBad/HisC). The product of the first PCR was then used as  
17 oligonucleotides for a second PCR with the target vector as a template. Plasmid  
18 pPal-sfGFP was constructed by a double PCR technique, allowing amplification of  
19 the *sfGFP* gene, flanked by extensions annealing to the target vector (pPal). Site-  
20 directed substitutions were introduced by QuikChange mutagenesis PCR using  
21 complementary pairs of oligonucleotides.

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

25 The *E. coli* W3110 strains carrying the chromosomal gene deletions or tags of  
26 interest were obtained using a modified protocol from Datsenko & Wanner [42] as  
27 described previously [43]. Briefly, for each gene deletion, a kanamycin cassette was  
28 amplified from the vector pKD4 using a pair of oligonucleotides carrying 50-  
29 nucleotide extensions homologous to regions that are adjacent to the target gene.  
30 After electroporation of the PCR product into *E. coli* W3110 cells carrying the  
31 pKOBEG vector, kanamycin-resistant clones were selected and confirmed by colony-  
32 PCR. The kanamycin cassette was then excised using vector pCP20 and gene  
33 deletions were verified by PCR. The same procedure was performed to introduce the  
34 mCherry or sfGFP coding sequences upstream the stop codon (pKD4-*sfgfp* or pKD4-



1 *mCherry*) or downstream from the start codon (*pgfp*-KD4 or *pmCherry*-KD4),  
2 resulting in strains producing fusion proteins from their native chromosomal loci.

### 4 **Western Blots and protein production**

6 Protein production from gene expression at chromosomal loci was checked by  
7 western blot experiments on over-night cultures. Heat treated samples ( $A_{600} = 0.2$ )  
8 were loaded on 12,5% SDS-PAGE and analyzed by western-blot immunodetection  
9 using specific antibodies. When necessary, plasmid multicopy expression was  
10 induced with arabinose 0,02% for 1 hour.

### 12 **Fluorescence microscopy**

14 Overnight cultures of *E. coli* W3110 wild-type or derivative strains were diluted to  
15  $A_{600} \sim 0.1$  in M9 minimum medium supplemented with glucose and casa-amino acids  
16 and grown at 30°C with agitation to reach an  $A_{600} \sim 0.6$ . Cells were washed twice and  
17 resuspended in the same medium to an  $A_{600} \sim 0,6$  and spotted on a cover slip and  
18 covered with a thin pad of 1.5% agar in M9 minimum medium supplemented with  
19 glucose and casa-amino acids.

### 21 **Time-lapse fluorescent experiments**

23 For each time-lapse experiment, four independent fields were manually defined  
24 with a motorized stage (Prior Scientific) and stored (X, Y, Z, PFS-offset) in a custom  
25 automation system designed for time-lapse experiments. Fluorescence and phase  
26 contrast images were captured every 2 minutes using an automated and inverted  
27 epifluorescence microscope TE2000-E-PFS (Nikon, France) equipped with Perfect  
28 Focus System (PFS) during 2 hours. PFS automatically maintains focus in order to  
29 keep in sharp focus the point of interest at all times and despite mechanical or  
30 thermal perturbations. Images were recorded with a CoolSNAP HQ 2 (Roper  
31 Scientific, Roper Scientific SARL, France) and a 100X/1.4 DLL objective. Excitation  
32 light was emitted by a 120-watt metal halide light. The sfGFP images were recorded  
33 by using the ET-GFP filter set (Chroma 49002) using an exposure time of 500–700  
34 ms and the mCherry images were recorded by using the ET-mCherry filter set

1 (Chroma 49008) using an exposure time of 100–700 ms. Phase contrast and  
2 fluorescence channels were adjusted and merged using the Fiji software  
3 (<http://rsb.info.nih.gov/ij/>). Some slight movements of the whole field during the  
4 experiments were corrected by registering individual frames using ‘StackReg’ and  
5 ‘Image Stabilizer’ plugins for ImageJ.  
6

## 7 **One-shot acquisitions**

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9 Fluorescence and phase contrast images were captured and recorded with the  
10 same devices described previously, using an exposure time of 1500 ms for mCherry  
11 and sfGFP images.  
12

## 13 **Statistical analysis**

14  
15 First, noise and background were reduced using the ‘Subtract Background’ plugin  
16 from Fiji. Cells and fluorescent foci were automatically detected with MicrobeJ plugin  
17 for ImageJ (<http://www.microbej.com>). To avoid false positive, each event was  
18 manually controlled in the original raw data. Kymograph representations were made  
19 using the ‘KymoResliceWide’ plugin from Fiji and demograph or Heatmap  
20 representations were made with the ‘Demograph’ or ‘Heatmap’ options from  
21 MicrobeJ.  
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## 23 **Phenotypic studies**

24  
25 Phenotypic studies have been performed in order to visualize effects of the  
26 fluorescent markers chromosomal insertion and gene deletions onto the *tol-pal*  
27 operon. Three assays were achieved to check the OM integrity, the Tol-Pal complex  
28 assembly and the cell division process. *Colicin tolerance assay*: Colicin A and D  
29 lethal activities were checked by the presence of halos on a cell lawn of the strain to  
30 be tested, as previously described [44]. Data are reported in Table 1 as the maximal  
31 dilution of the colicin stock sufficient for inhibiting cell growth and are showed in Fig.  
32 S3). *SDS sensitivity assay*: Sensitivity to Sodium Dodecyl Sulfate (SDS) was  
33 determined by measuring the level of survival of the strain after 4 hours of incubation  
34 at 37°C in liquid LB medium containing various concentration of SDS (from 0.5% to  
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1 2%). Data are reported in Table 1 as “S” for sensitive strains or “R” for resistant  
2 strains and also in Fig S1 and S5. *Cell division assay*: Cells were grown for 4 hours  
3 at 37°C in LB (171 mM NaCl) or LB–NaCl (0 mM NaCl) liquid medium. Then cells  
4 were fixed on fresh Poly-L-Lysine (0,1% w/v in H<sub>2</sub>O) coated microscope slides and  
5 were observed with an optical microscope (Eclipse 50i, Nikon, France).  
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**Fig. 1.** The *Escherichia coli tol-pal* gene cluster. (a) Schematic representation of the components of the Tol-Pal complex in the *Escherichia coli* envelope. (b) Organization of the *tol-pal* operons. The genes are represented by arrowheads. The two promoters and the inserts encoding sfGFP or mCherry fusions are indicated by arrows.

**Fig. 2.** Chromosomal encoded Tol-Pal fusion proteins are functional. (a) Sensitivity to SDS of WT, sfGFP-TolA and  $\Delta$ TolA cells (strains are referenced in Table S1) was tested in liquid LB medium containing various concentrations of SDS at 37°C. The percentage of surviving cells in liquid LB medium was estimated after 4 hours from the turbidity ratio of the SDS-treated and the control sample. Error bars indicate the error on triplicate samples for each condition. (b) Phase contrast imaging of WT, sfGFP-TolA and  $\Delta$ TolA strains showing cell division defect or not after growth at 37°C in LB (upper panel) or in LB without NaCl (lower panel). Any of the *tol-pal* gene deletion leads to cell septation defect under hypo-osmolarity conditions. Scale Bar: 5  $\mu$ m. (c) Sensitivity of WT, GFP-TolA and  $\Delta$ TolA strains to colicin A (Tol dependent) and colicin D (Ton dependent) lethal activities was tested using dilutions from  $10^{-1}$  to  $10^{-4}$  of colicins spotted on freshly seeded lawns. Zones of clearance indicate colicins activity against the strain. (d) Western blot analysis was used to check native and chimeric Tol-Pal protein production in each *tol-pal* chromosomal fusion strain. The antibodies used are indicated.

**Fig. 3.** The Tol-Pal proteins localize to cell septum during cell division. (a) Fluorescent microscopy time-lapse recording of *E. coli* W3110 cells expressing the fluorescent versions the Tol-Pal proteins. Individual images were taken every 4 min. Corresponding kymograph of each time-lapse is shown on left panel. Dark areas are outside the cell. Note that protein fluorescence re-localizes to future division sites. (b) Fluorescent profiles (demographs) of *E. coli* W3110 cells expressing the fluorescent versions the Tol-Pal proteins 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

1 (low intensity) to yellow (high intensity). On the y-axis, cells are sorted for length,  
2 ascending from top (the shortest cell) to down (the longest cell). Each horizontal line  
3 corresponds to a single cell. The longest cells correspond to cells in the last stage of  
4 cell division. In all these experiments, *E. coli* W3110 wild-type or derivative strains,  
5 were grown in M9 minimal medium supplemented with glucose and casa-amino  
6 acids at 30°C.

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8 **Fig. 4.** Localization of the membrane Tol-Pal proteins in different genetic  
9 backgrounds. Fluorescence microscopy images of the different IM fluorescent Tol  
10 proteins were observed in each *tol-pal* gene deletion background. (a) TolQ-mCherry,  
11 (b) sfGFP-TolR, (c) sfGFP-TolA. Note that the three proteins are able to localize at  
12 constriction sites during cell division independently of their Tol-Pal partners. Heatmap  
13 distribution in cells of the fluorescent proteins tested is represented on the right of  
14 each panel where n represents the number of cells for each study. Scale Bar: 2  $\mu$ m.  
15 In all these experiments, *E. coli* W3110 wild-type or derivative strains, were grown in  
16 M9 minimal medium supplemented with glucose and casa-amino acids at 30°C.

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19 **Fig. 5.** Localization of the outer-membrane Tol-Pal complex in different genetic  
20 backgrounds. (a) Fluorescence microscopy images of W3110 *E. coli* cells  
21 expressing the chromosomal fluorescent fusion protein TolB-mCherry or the plasmid  
22 encoded Tat-GFP fusion protein. Tat-GFP has been previously described to be  
23 efficiently exported across the IM by the Twin-arginine transport system [45]. Tat-  
24 GFP is observed in the periplasm of the producing cells whereas TolB-mCherry  
25 localizes at constriction sites during cell division. Heatmap distribution in cells of the  
26 fluorescent proteins tested is represented on the right of each panel where n  
27 represents the number of cells for each study. Scale Bar: 2  $\mu$ m. (b) Fluorescence  
28 microscopy images of *tol-pal* mutants cells producing Tat-GFP and TolB-mCherry. In  
29 *tol-pal* mutants, fluorescence is observed in vesicles at cell poles or cell septa.  
30 Accumulation of Tat-GFP or TolB-mCherry in vesicles is indicated by arrows. (c)  
31 Fluorescence microscopy images of W3110 cells expressing the chromosomal fusion  
32 protein Pal-mCherry in different genetic backgrounds. As shown previously, Pal-  
33 mCherry is observed at midcell in a wild type background. Localization at cell septum  
34 is affected in a  $\Delta$ *tolA*,  $\Delta$ *tolQR* and  $\Delta$ *tolB* mutant. In a  $\Delta$ *tolQR* mutant carrying the

1 plasmid pTolQR encoding TolQ and TolR, Pal-mCherry localization at the cell  
2 septum is restored. In a  $\Delta$ tolQR mutant carrying the plasmid pTolQR<sub>D23A</sub> encoding  
3 TolQ and TolR<sub>D23A</sub>, Pal-mCherry does not localize at the cell septum but appears  
4 dispersed in the cell envelope. In all these experiments, *E. coli* W3110 wild-type or  
5 derivative strains, were grown in M9 minimal medium supplemented with glucose  
6 and casa-amino acids at 30°C.

9 **Fig. 6.** Localization and function of Pal variants. (a) Western blot analysis of Pal,  
10 Pal-sfGFP, Pal<sub>102</sub>-sFGP and Pal<sub>104</sub>-sfGFP production. Bacteria constitutively  
11 producing or not Pal and producing plasmid encoding Pal-sfGFP, Pal<sub>102</sub>-sFGP and  
12 Pal<sub>104</sub>-sfGFP were grown in LB medium at 37°C to an  $A_{600} = 0.3$  and induced for 60  
13 min with 0.02% arabinose. Cell extracts ( $A_{600} = 0.2$ ) were immunoblotted and the  
14 presence or absence of the protein of interest was detected with anti-Pal antibodies.  
15 (b) Phase contrast imaging of strains producing Pal-sfGFP, Pal<sub>102</sub>-sFGP and Pal<sub>104</sub>-  
16 sfGFP showing cell division after growth at 37°C in LB (upper panel) or in LB - NaCl  
17 (lower panel). Scale Bar: 5  $\mu$ m. (c) Sensitivity to SDS of W3110,  $\Delta$ pal, and  $\Delta$ pal  
18 strains producing Pal-sfGFP, Pal<sub>102</sub>-sFGP and Pal<sub>104</sub>-sfGFP was tested in liquid LB  
19 medium containing various concentrations of SDS. The percentage of surviving cells  
20 in liquid LB medium was estimated after 4 hours of growth at 37°C from the turbidity  
21 ratio of the SDS-treated and the control sample. Error bars indicate the error on  
22 triplicate samples for each condition. (d) Schematic of Pal<sub>E102K</sub> and Pal<sub>R104C</sub>  
23 interactions with TolB and the PG previously published [36]. Pal<sub>E102K</sub> interacts with  
24 the PG but not with TolB, whereas Pal<sub>R104C</sub> interacts with TolB but not with the PG.  
25 (e) A  $\Delta$ pal strain was complemented with pPal-sfGFP, pPal<sub>E102K</sub>-sfGFP or pPal<sub>R104C</sub>-  
26 sfGFP. *E. coli* W3110 wild-type or derivative strains were grown in M9 minimal  
27 medium supplemented with glucose and casa-amino acids at 30°C. Fluorescence  
28 microscopy images reveal that Pal-sfGFP and Pal<sub>R104C</sub>-sfGFP localize at the cell  
29 septum during cell division in contrast to Pal<sub>E102K</sub>-sfGFP. The septal localization of  
30 Pal<sub>R104C</sub>-sfGFP is abolished in a  $\Delta$ tolB-pal strain.

32 **Table 1.** Phenotypic analysis of *tol-pal* chromosomal fusions and deletions strains.  
33 W3110 cells expressing different chromosomal fusions and deletions of the *tol-pal*  
34 genes were tested for level of import of colicin A (Tol dependent) and colicin D (Ton

1 dependent), susceptibility to SDS and cell division +/- NaCl. SDS sensitivity was  
2 tested in liquid LB medium containing various concentrations of SDS (R: resistant, S:  
3 sensitive). Colicin A and D sensitivity was tested on bacterial lawns by a spot dilution  
4 assay. The number indicated represents the maximal 10-fold dilution of the colicin  
5 stock (1 mg/ml) still able to kill the strain tested (0: resistant, 4: sensitive to 10<sup>4</sup>  
6 dilution). Cell division was observed during exponential phase after growth in LB or  
7 LB - NaCl (N = Normal cell division, C = Chain formation).  
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**Table 1: *E. coli* strains and phenotypes.**

Strains	OM stability SDS	Cell chaining	Colicin uptake	
			A	D
W3110	R	N	4	4
sfGFP-TolA	R	N	4	4
sfGFP-TolR	R	N	4	4
TolQ-mCherry	R	N	4	4
TolB-mCherry	R	N	4	4
Pal-mCherry	R	N	4	4
ΔTolA	S	C	0	4
ΔTolQ	S	C	0	4
ΔTolR	S	C	0	4
ΔTolQR	S	C	0	4
ΔTolB	S	C	0	4
ΔPal	S	C	4	4
ΔTolB-Pal	S	C	0	4
sfGFP-TolA:ΔTolQR	S	C	0	4
sfGFP-TolA:ΔTolB	S	C	0	4
sfGFP-TolA:ΔPal	S	C	4	4
sfGFP-TolR:ΔTolA	S	C	0	4
sfGFP-TolR:ΔTolQ	S	C	0	4
sfGFP-TolR:ΔTolB	S	C	0	4
sfGFP-TolR:ΔPal	S	C	4	4
TolQ-mCherry:ΔTolA	S	C	0	4
TolQ-mCherry:ΔTolR	S	C	0	4
TolQ-mCherry:ΔTolB	S	C	0	4
TolQ-mCherry:ΔPal	S	C	4	4
TolB-mCherry:ΔTolA	S	C	0	4
TolB-mCherry:ΔTolQR	S	C	0	4
TolB-mCherry:ΔPal	S	C	4	4
Pal-mCherry: ΔTolA	S	C	0	4
Pal-mCherry: ΔTolQR	S	C	0	4
Pal-mCherry: ΔTolB	S	C	0	4
Pal-mCherry: ΔTolQR pTolQR	R	N	4	4
Pal-mCherry: ΔTolQR pTolQR <sub>D23A</sub>	S	C	0	4
ΔPal: pPal-sfGFP	R	N	4	4
ΔPal: pPal <sub>E102K</sub> -sfGFP	S	C	4	4
ΔPal: pPal <sub>R104C</sub> -sfGFP	S	C	4	4

## 1 Supplemental information

2  
3 **Fig. S1.** Sensitivity to SDS of WT and derivative mutant strains. Sensitivity to SDS of  
4 the indicated strains was tested in liquid LB medium containing various  
5 concentrations of SDS: 0.25% to 2%. The percentage of surviving cells in liquid LB  
6 medium was estimated after 4 hours at 37°C from the turbidity ratio of the SDS-  
7 treated and the control sample. Error bars indicate the error on triplicate samples for  
8 each condition.

9  
10 **Fig. S2.** Phase contrast imaging of WT and derivative mutant strains. Phase contrast  
11 imaging of WT and modified strains showing cell division after growth at 37°C in LB  
12 (right panel) or in LB - NaCl (left panel). *tol-pal* gene deletions lead to cell septation  
13 defect in hypo-osmolarity conditions. In contrast all the strains encoding the  
14 fluorescent Tol-Pal fusion proteins present a wild-type phenotype. Scale Bar: 2.5 μm

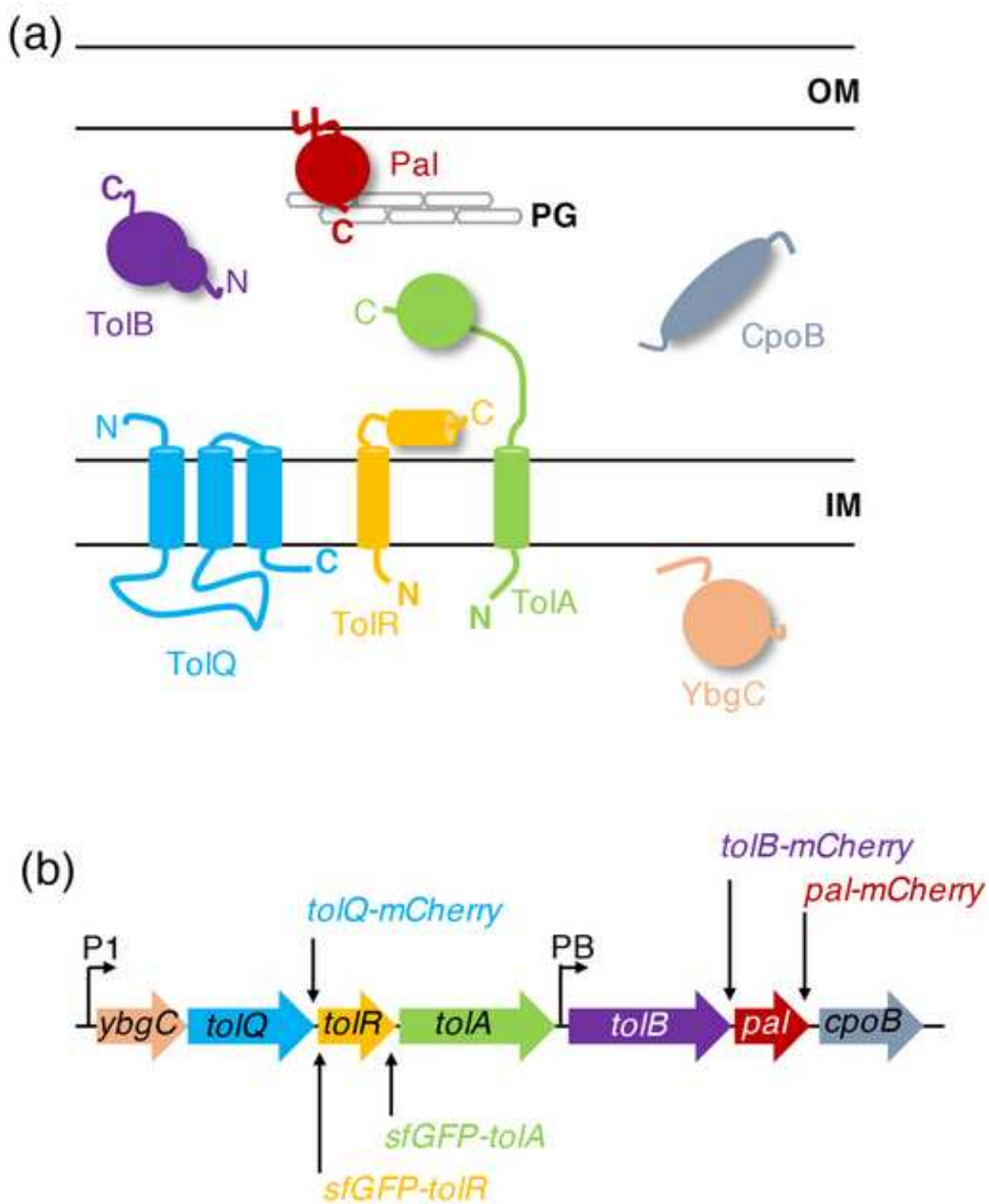
15  
16 **Fig. S3.** Sensitivity to colicins of WT and derivative mutant strains. Sensitivity of WT  
17 and modified strains to colicin A (Tol dependent) and colicin D (Ton dependent) lethal  
18 activities was tested using dilutions from  $10^{-1}$  to  $10^{-4}$  of colicins spotted on freshly  
19 seeded lawns. Zones of clearance indicate colicins activity against the strain. Colicin  
20 D uses the TonB system to enter and to kill cells and its lethal activity is not affected  
21 by a *tol-pal* deletion. Pal is not required for colicin A mode of action in contrast to the  
22 other Tol proteins. Note that all the strains encoding the fluorescent Tol-Pal fusion  
23 proteins are sensitive to the colicin A.

24  
25 **Fig. S4.** Tol-Pal proteins production in the different modified strains. Western blot  
26 analysis was used to check native and chimeric Tol-Pal proteins production in each  
27 *tol-pal* chromosomal fusion and deletion strain. The antibodies used are indicated on  
28 the left.

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

Strains or plasmids	Genotype	Source
<b>Strains</b>		
W3110	F' $\lambda$ 'IN( <i>rrnD-rrnE</i> )1 <i>rph-1</i>	[46]
sfGFP-TolA	W3110 <i>sfGFP-tolA::frt</i>	This study
sfGFP-TolR	W3110 <i>sfGFP-tolR::frt</i>	This study
TolQ-mCherry	W3110 <i>tolQ-mCherry::frt</i>	This study
TolB-mCherry	W3110 <i>tolB-mCherry::frt</i>	This study
Pal-mCherry	W3110 <i>pal-mCherry::frt</i>	This study
$\Delta$ TolA	W3110 $\Delta$ <i>tolA::frt</i>	This study
$\Delta$ TolQ	W3110 $\Delta$ <i>tolQ::frt</i>	This study
$\Delta$ TolR	W3110 $\Delta$ <i>tolR::frt</i>	This study
$\Delta$ TolQR	W3110 $\Delta$ <i>tolQR::frt</i>	This study
$\Delta$ TolB	W3110 $\Delta$ <i>tolB::frt</i>	This study
$\Delta$ Pal	W3110 $\Delta$ <i>pal::frt</i>	This study
$\Delta$ TolB-Pal	W3110 $\Delta$ <i>tolB-pal::frt</i>	This study
sfGFP-TolA: $\Delta$ TolQR	W3110 <i>sfGFP-tolA::frt, \Delta</i> <i>tolQR::kan</i>	This study
sfGFP-TolA: $\Delta$ TolB	W3110 <i>sfGFP-tolA::frt, \Delta</i> <i>tolB::kan</i>	This study
sfGFP-TolA: $\Delta$ Pal	W3110 <i>sfGFP-tolA::frt, \Delta</i> <i>pal::kan</i>	This study
sfGFP-TolR: $\Delta$ TolA	W3110 <i>sfGFP-tolR::frt, \Delta</i> <i>tolA::kan</i>	This study
sfGFP-TolR: $\Delta$ TolQ	W3110 <i>sfGFP-tolR::frt, \Delta</i> <i>tolQ::kan</i>	This study
sfGFP-TolR: $\Delta$ TolB	W3110 <i>sfGFP-tolR::frt, \Delta</i> <i>tolB::kan</i>	This study
sfGFP-TolR: $\Delta$ Pal	W3110 <i>sfGFP-tolR::frt, \Delta</i> <i>pal::kan</i>	This study
TolQ-mCherry: $\Delta$ TolA	W3110 <i>tolQ-mCherry::frt, \Delta</i> <i>tolA::kan</i>	This study
TolQ-mCherry: $\Delta$ TolR	W3110 <i>tolQ-mCherry::frt, \Delta</i> <i>tolR::kan</i>	This study
TolQ-mCherry: $\Delta$ TolB	W3110 <i>tolQ-mCherry::frt, \Delta</i> <i>tolB::kan</i>	This study
TolQ-mCherry: $\Delta$ Pal	W3110 <i>tolQ-mCherry::frt, \Delta</i> <i>pal::kan</i>	This study
TolB-mCherry: $\Delta$ TolA	W3110 <i>tolB-mCherry::frt, \Delta</i> <i>tolA::kan</i>	This study
TolB-mCherry: $\Delta$ TolQR	W3110 <i>tolB-mCherry::frt, \Delta</i> <i>tolQR::kan</i>	This study
TolB-mCherry: $\Delta$ Pal	W3110 <i>tolB-mCherry::frt, \Delta</i> <i>pal::kan</i>	This study
Pal-mCherry: $\Delta$ TolA	W3110 <i>pal-mCherry::frt, \Delta</i> <i>tolA::kan</i>	This study
Pal-mCherry: $\Delta$ TolQR	W3110 <i>pal-mCherry::frt, \Delta</i> <i>tolQR::kan</i>	This study
Pal-mCherry: $\Delta$ TolB	W3110 <i>pal-mCherry::frt, \Delta</i> <i>tolB::kan</i>	This study
<b>Plasmids</b>		
pBad/HisC	<i>amp</i>	Invitrogene
pKD4-mCherry	<i>kan</i>	[47]
psfGFP-KD4	<i>kan</i>	[48]
pCP20	<i>amp flp</i> recombinase	[42]
pKOBEG	<i>cm, \lambda</i> <i>red</i> integrase	[49]
pTolQR	<i>amp, pBad/HisC tolQR</i>	This study
pTolQR <sub>D23A</sub>	<i>amp, pBad/HisC tolQR<sub>D23A</sub></i>	This study
pPal-sfGFP	<i>amp, pBad/HisC pal-sfgfp</i>	This study
pPal <sub>E102K</sub> -sfGFP	<i>amp, pBad/HisC pal<sub>E102K</sub>-sfgfp</i>	This study
pPal <sub>R104C</sub> -sfGFP	<i>amp, pBad/HisC pal<sub>R104C</sub>-sfgfp</i>	This study
pTat-sfGFP	<i>amp, pBAD24 tat-gfp</i>	[45]

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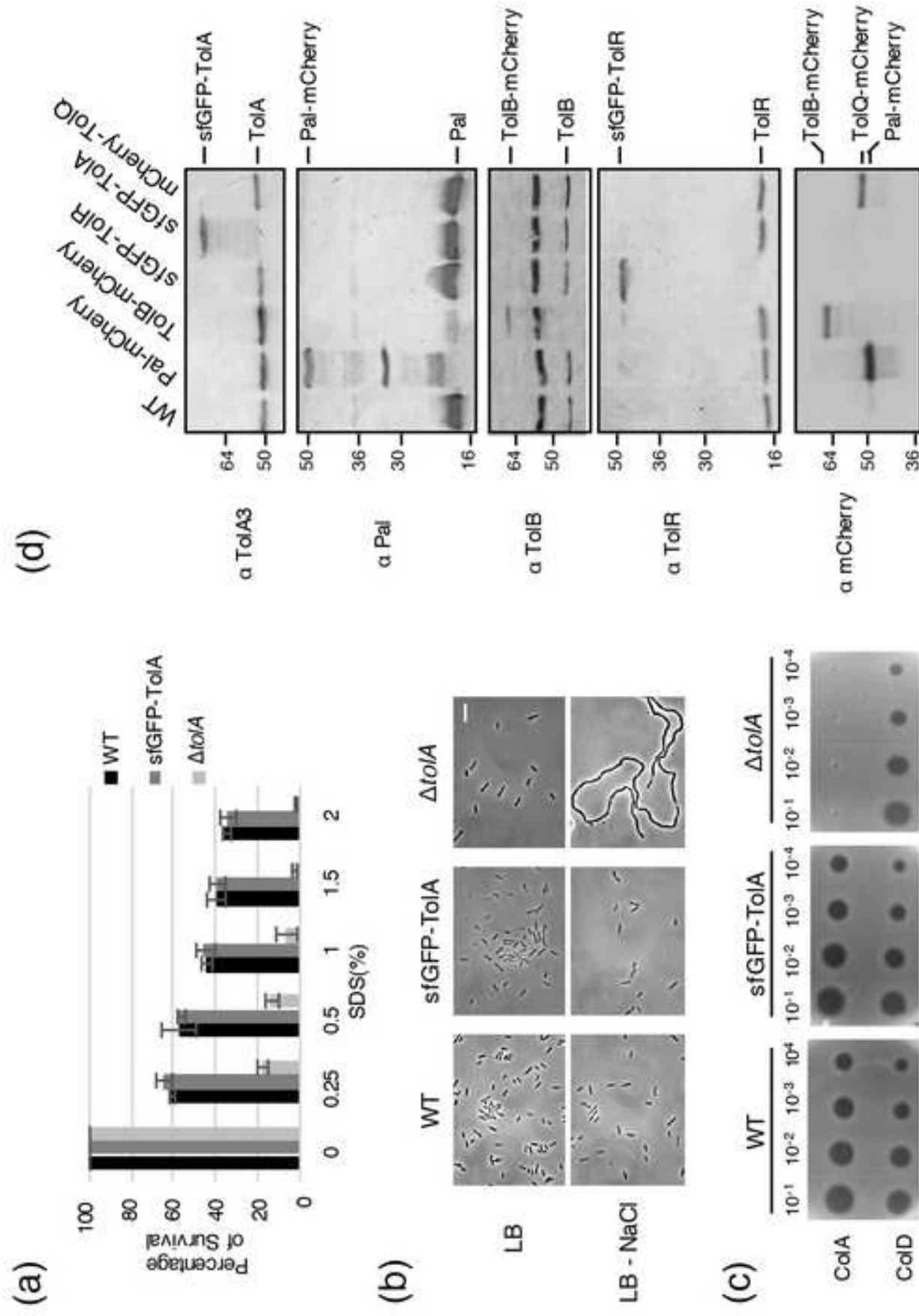


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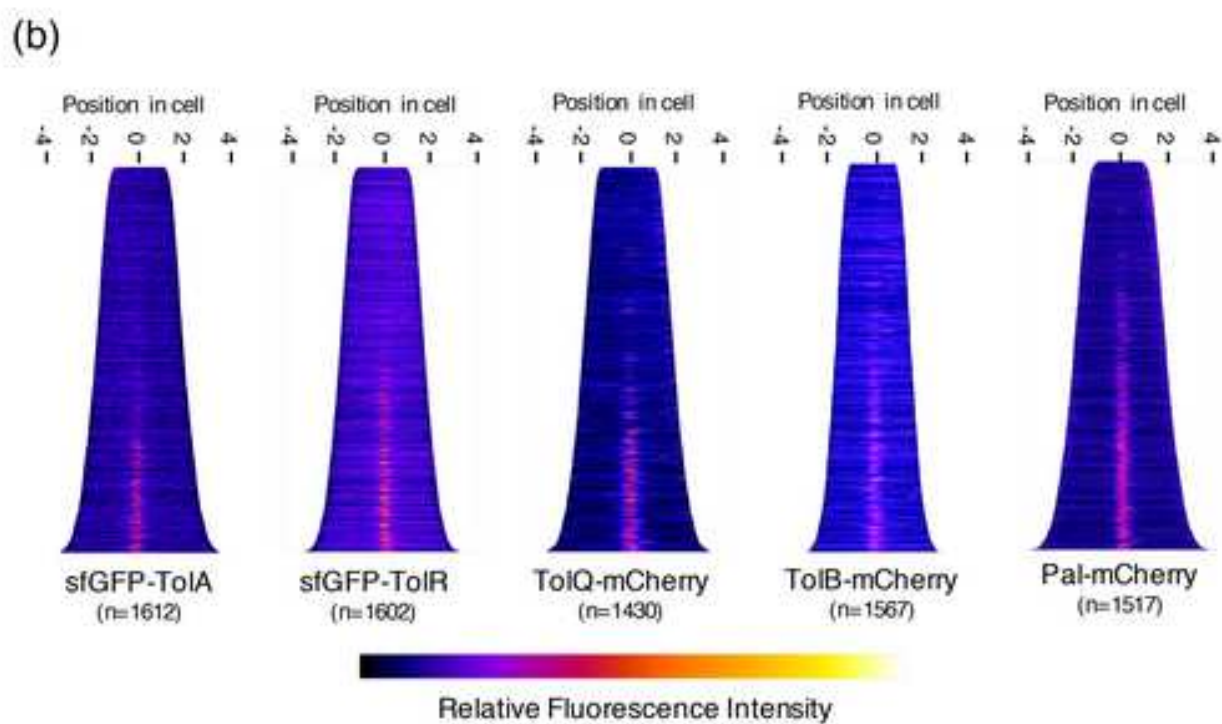
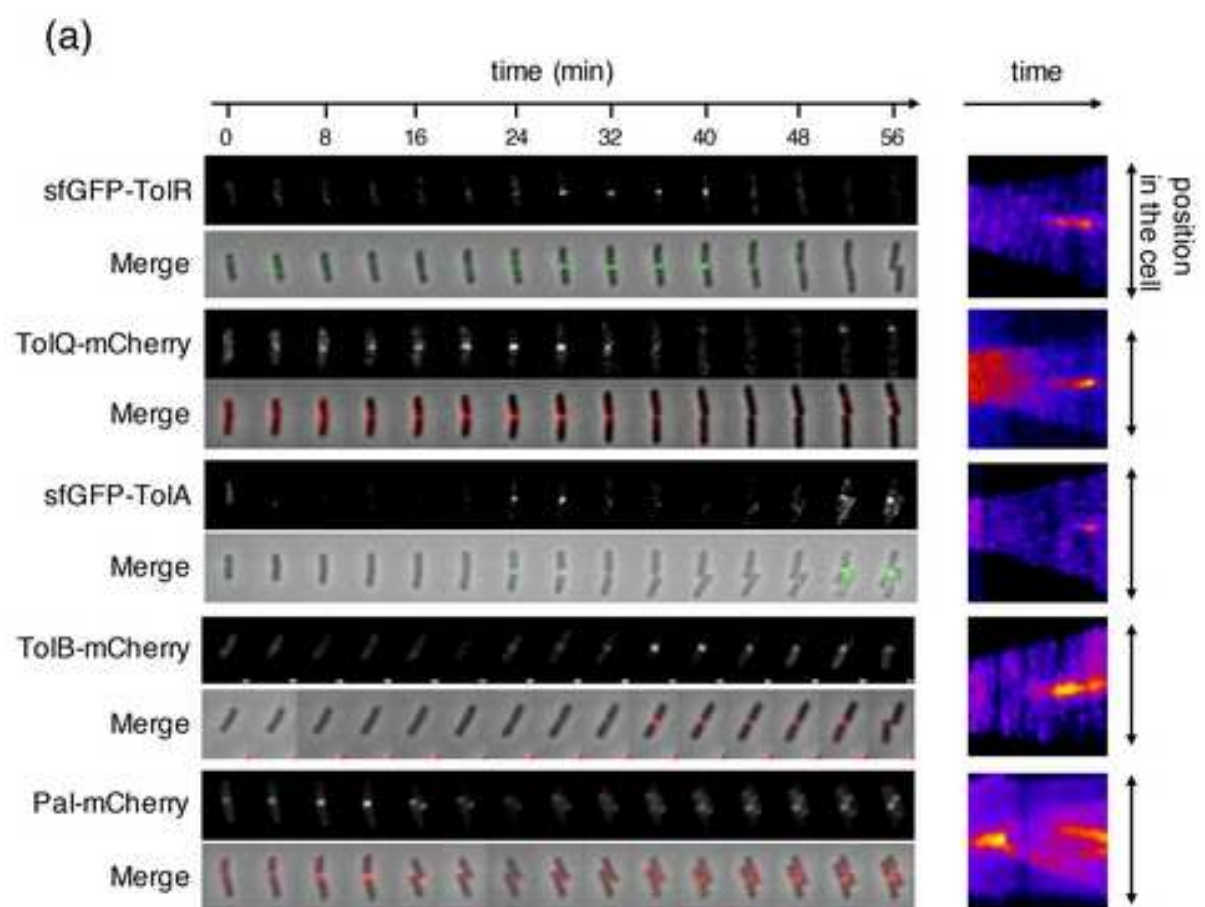


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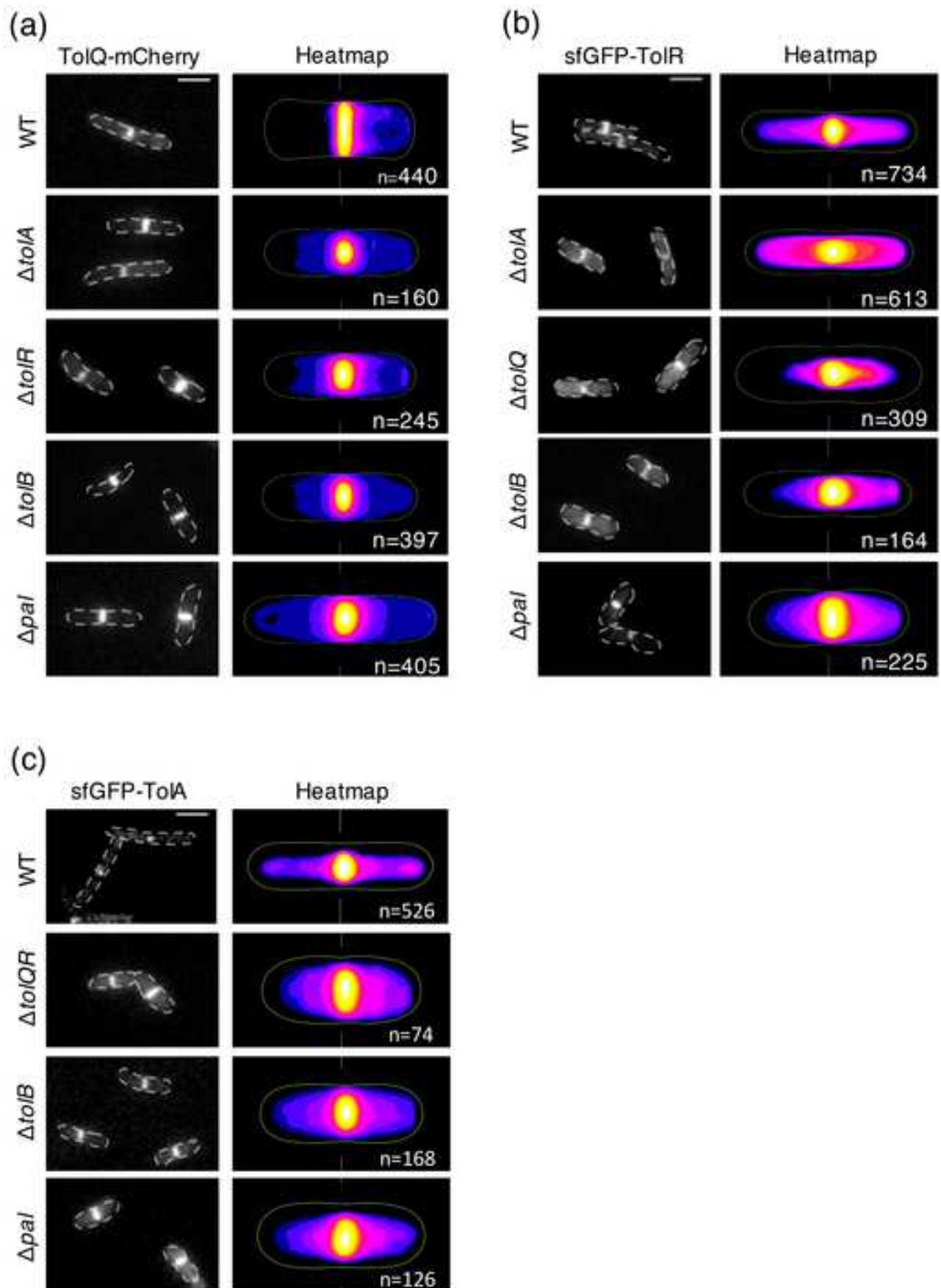
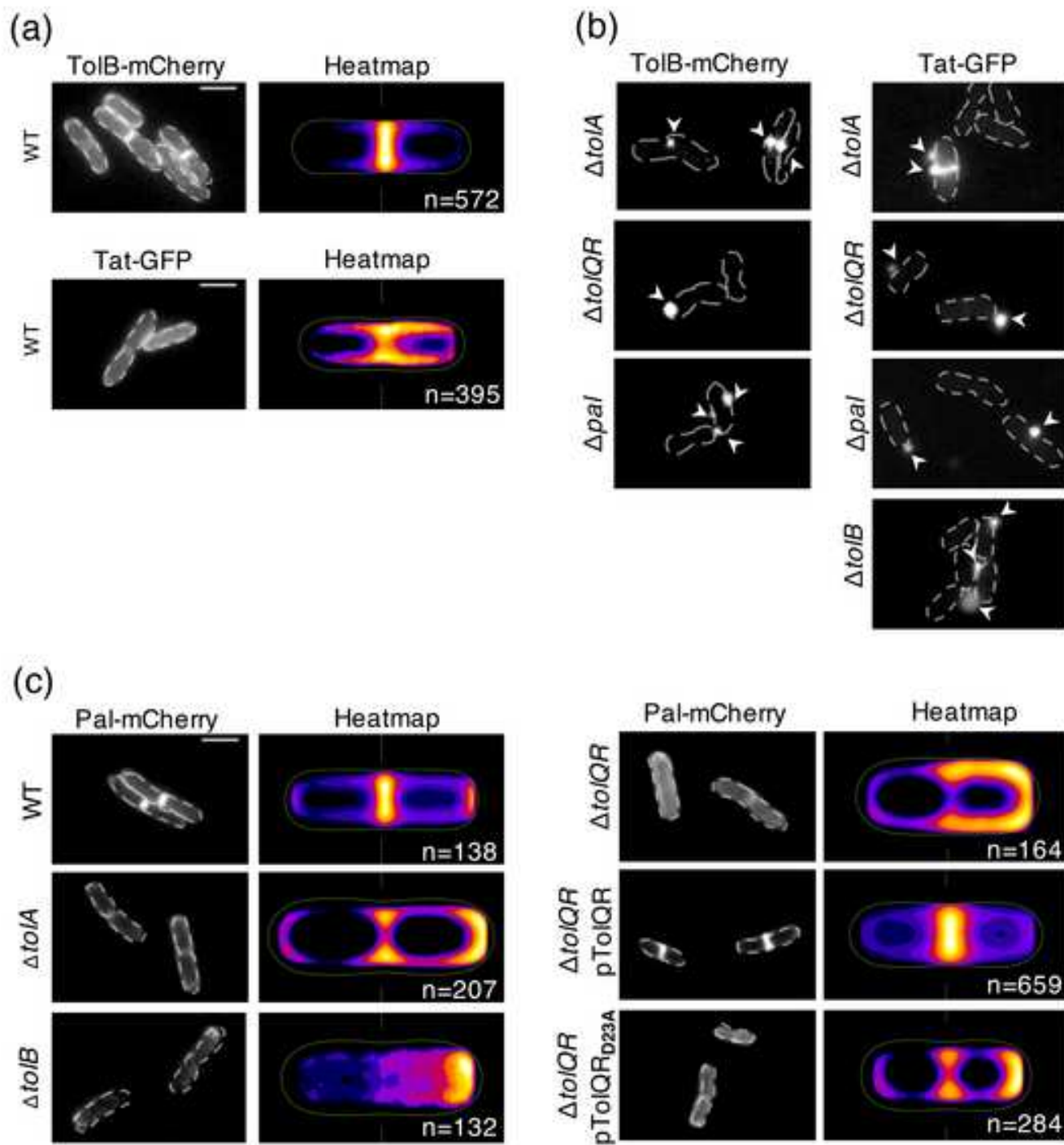


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