Tol Energy-Driven Localization of Pal and Anchoring to the Peptidoglycan Promote Outer-Membrane Constriction

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Abstract

During cell division, gram-negative bacteria must coordinate inner-membrane invagination, peptidoglycan 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-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 inner-membrane 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 peptidoglycan and subsequently OM constriction. Our results provide evidence that the constriction of the OM is an energized process.

Introduction

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

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

The Tol–Pal system is a trans-envelope complex highly conserved among gram-negative bacteria. The core of this system is composed of five proteins organized in two sub-complexes. The IM complex is

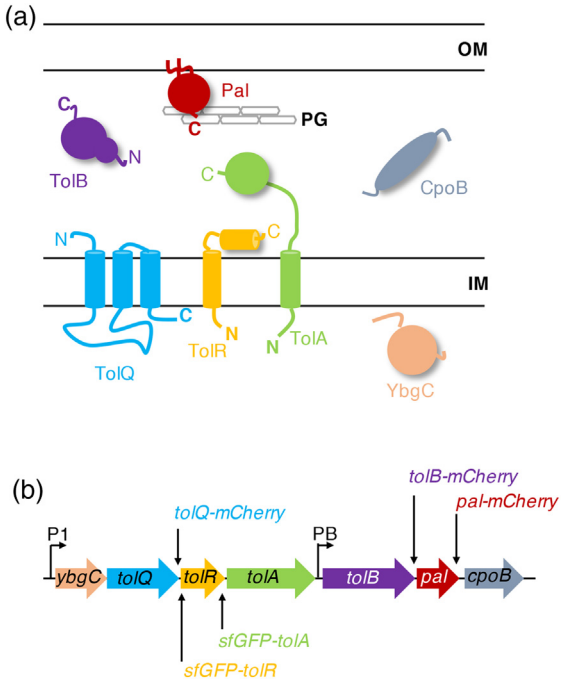

Fig. 1. The *E. coli tol–pal* gene cluster. (a) Schematic representation of the components of the Tol–Pal complex in the *E. coli* envelope. (b) Organization of the *tol–pal* operons. The genes are represented by arrowheads. The two promoters and the inserts encoding sfGFP or mCherry fusions are indicated by arrows.

composed of three proteins: the proton channel-forming TolQ, TolR and associated TolA anchored in the IM, while the OM complex is composed of TolB, a periplasmic protein, and Pal a lipoprotein anchored in the inner leaflet of the OM and able to bind the PG layer (Fig. 1a). *In vivo,* the two sub-complexes are transiently connected by TolA. TolA has a long predicted helical second domain (TolAII) that is thought to stretch across the periplasm where its C-terminal domain interacts with TolB N-terminal domain and potentially also with Pal in a proton-motive force (PMF)-dependent manner [6]. Thus, the Tol–Pal complex uses the PMF to form a link between the IM and OM. In *E. coli,* two additional proteins are encoded in the *tol–pal* cluster (Fig. 1b): the cytoplasmic thioesterase YbgC and the periplasmic protein CpoB [7].

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

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

In this study, we revisited the question of the Tol–Pal proteins localization. In order to monitor the intracellular localization and dynamic of each Tol–Pal protein during the cell cycle, we initiated a systematic fluorescence microscopy approach in *E. coli*. To further gain insight on how this machinery operates, we engineered chimeric proteins expressed from their endogenous chromosomal loci, fused to fluorescent markers such as super-folder green fluorescent protein (sfGFP) or mCherry. Only the core proteins of the Tol–Pal system that are conserved among gram-negative bacteria were studied. In the first part of this paper, we showed that all the chimeric proteins were functional and stably produced. We confirmed that the Tol–Pal proteins are dynamically recruited to the constriction sites during cell division. In addition, we showed that TolR requires either TolA or TolQ for its localization, whereas TolA and TolQ localize independently of any of the other Tol–Pal proteins. Strikingly, TolQ, TolR, TolA and TolB are absolutely required for Pal association with division sites, an active recruitment process that requires the PMF. Finally, the PG binding domain of Pal is not required for its septal localization, but absolutely require for its function and for proper OM constriction during cell division.

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

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$_{D23A}$	S	C	0	4
ΔPal: pPal-sfGFP	R	N	4	4
ΔPal: pPal$_{E102K}$-sfGFP	S	C	4	4
ΔPal: pPal$_{R104C}$-sfGFP	S	C	4	4

W3110 cells expressing different chromosomal fusions and deletions of the *tol–pal* genes were tested for level of import of colicin A (Tol dependent) and colicin D (Ton dependent), susceptibility to SDS and cell division ± NaCl. SDS sensitivity was tested in liquid LB medium containing various concentrations of SDS (R, resistant; S, sensitive). Colicin A and D sensitivity was tested on bacterial lawns by a spot dilution assay. The number indicated represents the maximal 10-fold dilution of the colicin stock (1 mg/ml) still able to kill the strain tested (0, resistant; 4: sensitive to 10^4 dilution). Cell division was observed during exponential phase after growth in LB or LB–NaCl (N, normal cell division; C, chain formation).

Results

Chromosomal-encoded fusion of the Tol–Pal components to fluorescent domains results in functional fluorescent proteins

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

We first checked that all the fluorescent Tol–Pal fusions proteins were functional. It has been previously described that Tol–Pal mutants are hypersensitive to detergents [29], form cell chains in medium of low osmolarity [25] and are resistant to colicins [30]. Here we showed that all the strains

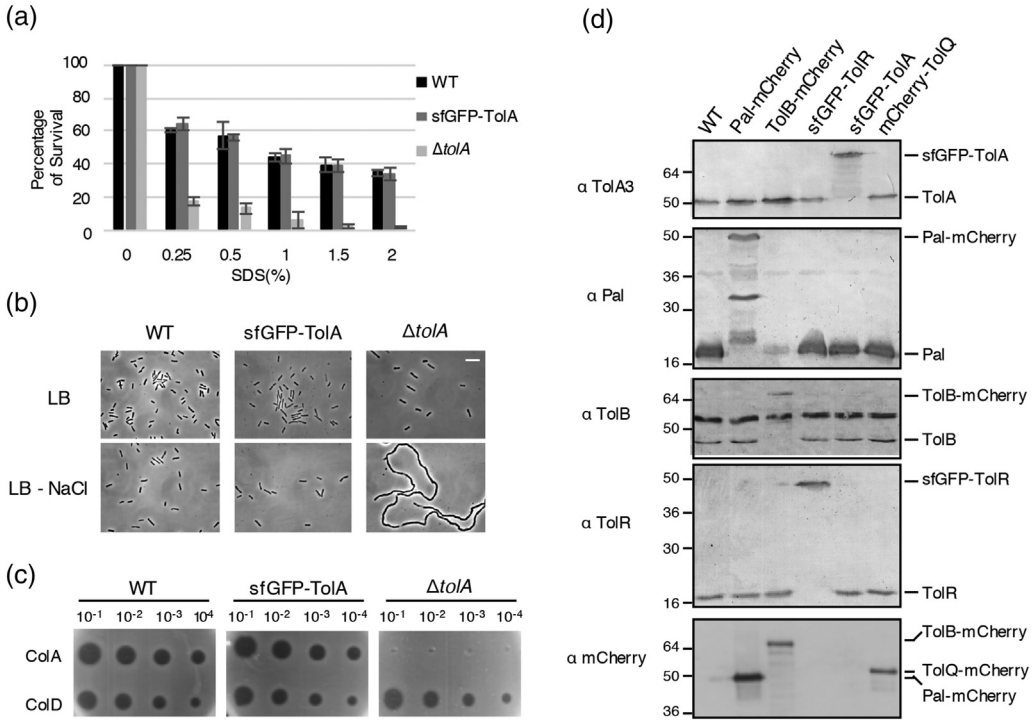

Fig. 2. Chromosomal encoded Tol–Pal fusion proteins are functional. (a) Sensitivity to SDS of WT, sfGFP-TolA and ΔTolA cells (strains are referenced in Table S1) was tested in liquid LB medium containing various concentrations of SDS at 37 °C. The percentage of surviving cells in liquid LB medium was estimated after 4 h 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 ΔTolA 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. The scale bar represents 5 μm. (c) Sensitivity of WT, GFP-TolA and ΔTolA 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.

producing a fluorescent Tol–Pal fusion protein displayed a wild-type phenotype (Table 1 and Figs. 2 and S1–3). These strains were able to grow as efficiently as the wild-type strain in presence of SDS (Figs. 2a and S1), did not form cell chains in LB medium lacking NaCl (Figs. 2b and S2), and were sensitive to different colicin concentrations (Figs. 2c and S3), in contrast to the strain W3110 deleted of the *tolA* gene (Table 1 and Figs. 2a–c and S1–3). These results clearly showed that all the fluorescent Tol–Pal fusion proteins remained functional.

The *tol–pal* genes are transcribed as two distinct operons, *ybgC tolQRA* and *tolB pal cpoB* [31] (Fig. 1b). As shown in Fig. 2d by immunoblotting analysis with the indicated antibodies, insertion of sfGFP or mCherry coding sequence in these operons did not modify the level of expression of the resulting chimeric fluorescent proteins, as well as the level of expression of the others proteins of the Tol–Pal complex. Nevertheless, the strain-producing TolB-mCherry was associated with a decrease in Pal abundance. This decrease does not interfere with Tol–Pal function because the strain producing-TolB-mCherry displayed a wild-type phenotype, absence of filamentation, growth on detergent and sensitivity to colicin A similar to the W3110 strain (Table 1 and Figs. S1–3). We therefore used that strain throughout this study.

Tol–Pal proteins are recruited to cell constriction site

The intracellular localization of each of the Tol–Pal proteins fused to a fluorescent reporter was analyzed by a live fluorescence microscopy approach. Time-lapse microscopy recordings showed that all tested fluorescent Tol–Pal proteins first appeared diffuse in the cell and were dynamically recruited to future division sites during cell division until they dispersed after daughter cell separation (Fig. 3a, left panel). Notably, Pal-mCherry remained longer than

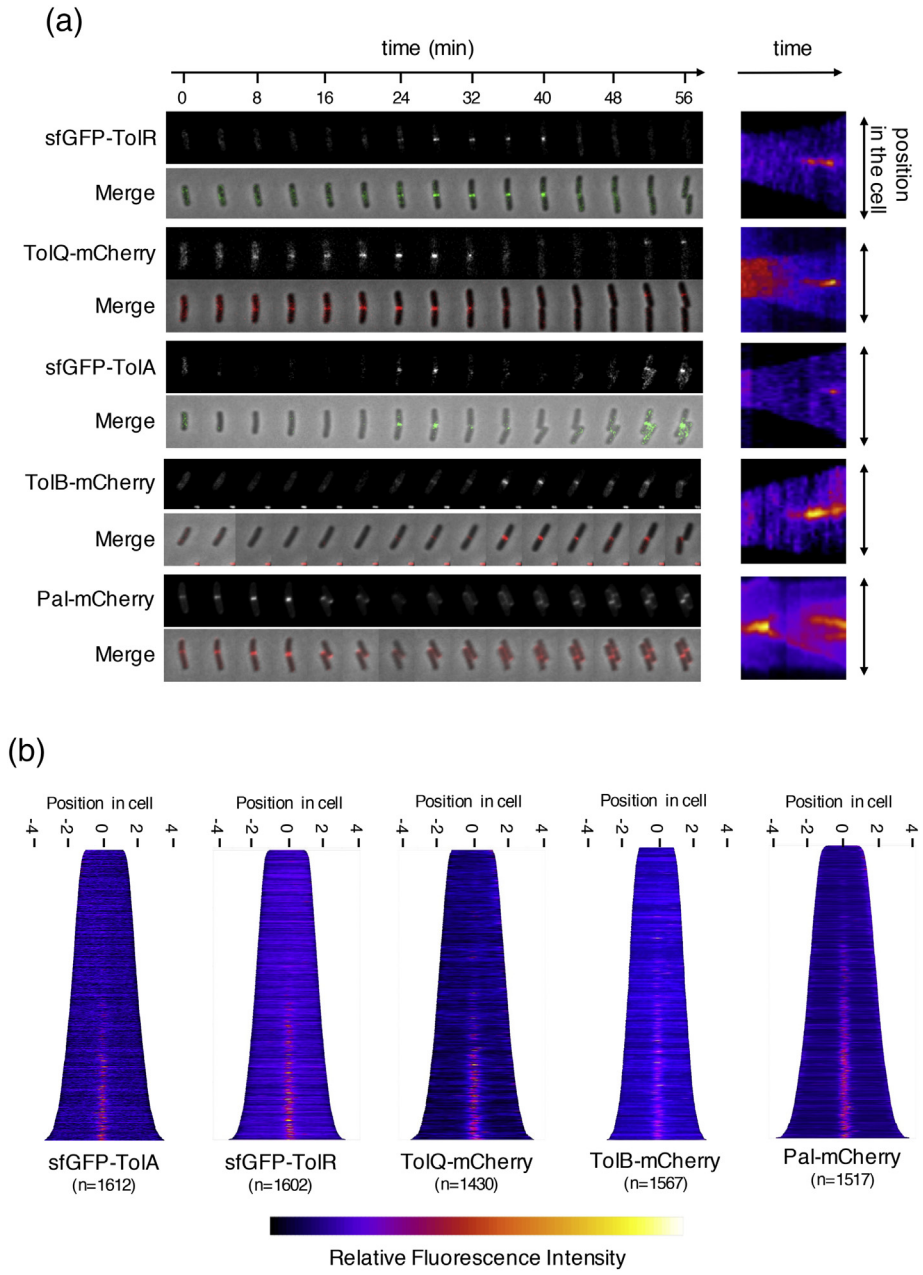

Fig. 3. The Tol–Pal proteins localize to cell septum during cell division. (a) Fluorescent microscopy time-lapse recording of *E. coli* W3110 cells expressing the fluorescent versions the Tol–Pal proteins. Individual images were taken every 4 min. Corresponding kymograph of each time-lapse is shown on 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 (low intensity) to yellow (high intensity). On the *y*-axis, cells are sorted for length, ascending from top (the shortest cell) to down (the longest cell). Each horizontal line corresponds to a single cell. The longest cells correspond to cells in the last stage of cell division. In all these experiments, *E. coli* W3110 wild-type or derivative strains were grown in M9 minimal medium supplemented with glucose and casa-amino acids at 30 °C.

the other Tol proteins at the new cell poles (Fig. 3a, right panel). These results were confirmed by Kymograph analysis (Fig. 3a, right panel) and a large-scale demograph analysis of cells (Fig. 3b). While the demograph analysis (Fig. 3b) may suggest that there is a temporal pattern in the mid-cell recruitment of the Tol proteins during cell cycle, we believe that our data cannot be used to confirm or

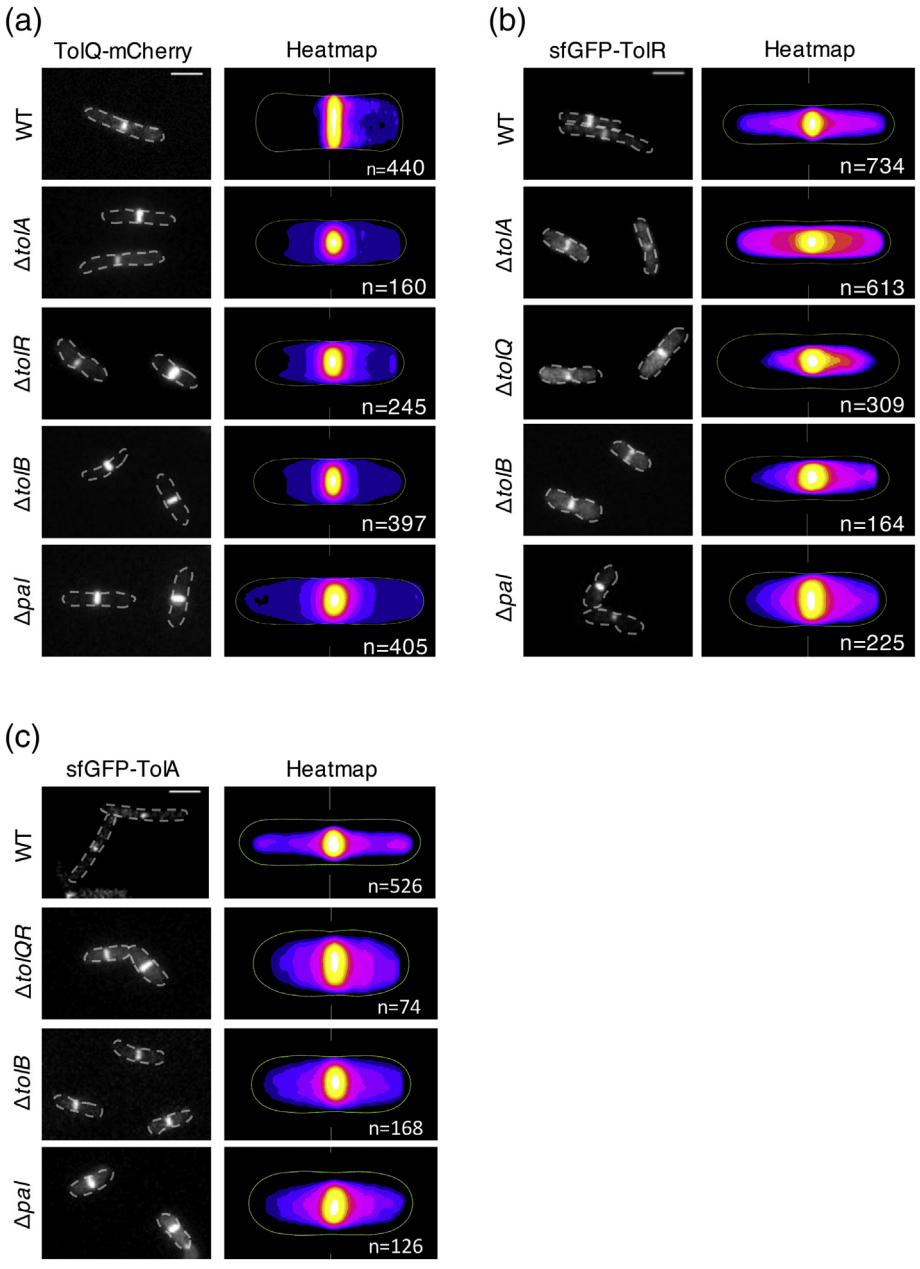

Fig. 4. Localization of the membrane Tol–Pal proteins in different genetic backgrounds. Fluorescence microscopy images of the different IM fluorescent Tol proteins were observed in each *tol–pal* gene deletion background. (a) TolQ-mCherry, (b) sfGFP-TolR and (c) sfGFP-TolA. Note that the three proteins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

infirm this hypothesis. Indeed, the number of Pal molecules per cell has been estimated between 30,000 to 40,000 copies, whereas the number of TolA molecules per cell has been estimated between 400 to 800 copies [26]. These differences of abundance make it easier, for example, to detect Pal at the septum. The precise timing interpretation of the Tol–Pal protein localization would require a more stringent analysis of cell fluorescence, specifically the analysis of dual-labeled strains. Nevertheless, based on the recordings presented on Fig. 3, our results confirm unambiguously that the Tol–Pal proteins are recruited to the septum during cell division.

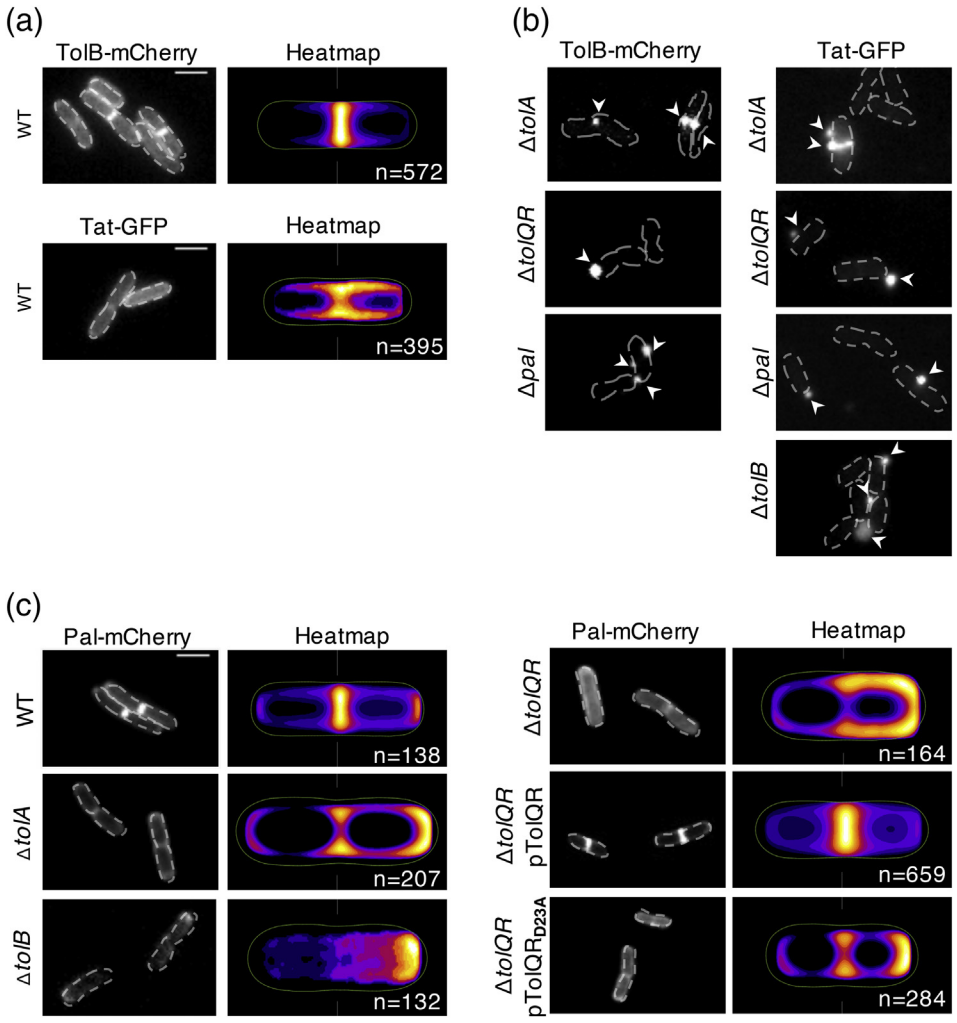

Fig. 5. Localization of the outer-membrane Tol–Pal complex in different genetic backgrounds. (a) Fluorescence microscopy images of W3110 *E. coli* cells expressing the chromosomal fluorescent fusion protein TolB-mCherry or the plasmid encoded Tat-GFP fusion protein. Tat-GFP has been previously described to be efficiently exported across the IM by the Twin-arginine transport system [46]. Tat-GFP is observed in the periplasm of the producing cells, whereas TolB-mCherry localizes at constriction sites during cell division. Heatmap distribution in cells of the fluorescent proteins tested is represented on the right of each panel, where *n* represents the number of cells for each study. The scale bar represents 2 μm. (b) Fluorescence microscopy images of *tol–pal* mutants cells producing Tat-GFP and TolB-mCherry. In *tol–pal* mutants, fluorescence is observed in vesicles at cell poles or cell septa. Accumulation of Tat-GFP or TolB-mCherry in vesicles is indicated by arrows. (c) Fluorescence microscopy images of W3110 cells expressing the chromosomal fusion protein Pal-mCherry in different genetic backgrounds. As shown previously, Pal-mCherry is observed at midcell in a wild-type background. Localization at cell septum is affected in a *ΔtolA*, *ΔtolQR and ΔtolB* mutant. In a *ΔtolQR* mutant carrying the plasmid pTolQR encoding TolQ and TolR, Pal-mCherry localization at the cell septum is restored. In a ΔtolQR mutant carrying the plasmid pTolQR$_{D23A}$ encoding TolQ and TolR$_{D23A}$, Pal-mCherry does not localize at the cell septum but appears dispersed in the cell envelope. In all these experiments, *E. coli* W3110 wild-type or derivative strains were grown in M9 minimal medium supplemented with glucose and casa-amino acids at 30 °C.

TolR requires TolA or TolQ for its localization at the cell constriction site

To determine the role of each Tol–Pal proteins in the assembly of the core complex and their recruitment to the septum, we analyzed the intracellular localization of the different chimeric fluorescent proteins in various deletion mutant backgrounds. Each of these deletion mutants, *tolA*, *tolB*, *tolQ*, *tolR*, *tolQR* and *pal,* present a classical *tol* phenotype (Table 1 and Figs. S1–3). Western blot analysis confirmed deletion of each *tol–pal* gene and showed that *tolB* and *tolQ* deletions led to a reduced production of Pal and of TolR, respectively. The level of production of the others proteins was not affected (Fig. S4) First, we analyzed the behavior of

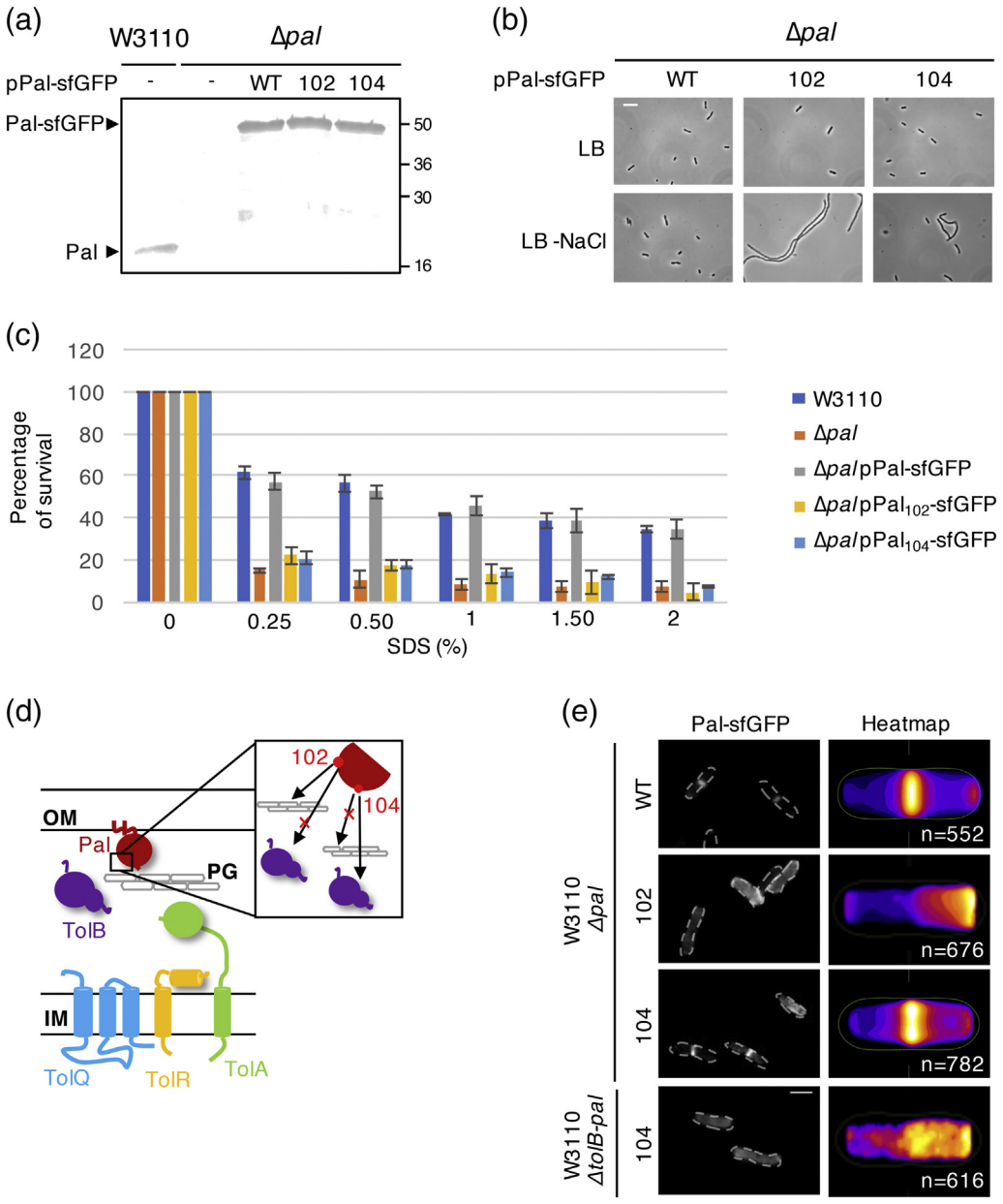

Fig. 6. Localization and function of Pal variants. (a) Western blot analysis of Pal, Pal-sfGFP, Pal_{102}-sFGP and Pal_{104}-sfGFP production. Bacteria constitutively producing or not Pal and producing plasmid-encoding Pal-sfGFP, Pal_{102}-sFGP and Pal_{104}-sfGFP were grown in LB medium at 37 °C to an A_{600} = 0.3 and induced for 60 min with 0.02% arabinose. Cell extracts (A_{600} = 0.2) were immunoblotted, and the presence or absence of the protein of interest was detected with anti-Pal antibodies. (b) Phase-contrast imaging of strains producing Pal-sfGFP, Pal_{102}-sFGP and Pal_{104}-sfGFP showing cell division after growth at 37 °C in LB (upper panel) or in LB–NaCl (lower panel). The scale bar represents 5 µm. (c) Sensitivity to SDS of W3110, Δ*pal*, and Δ*pal* strains producing Pal-sfGFP, Pal_{102}-sFGP and Pal_{104}-sfGFP was tested in liquid LB medium containing various concentrations of SDS. The percentage of surviving cells in liquid LB medium was estimated after 4 h of growth at 37 °C from the turbidity ratio of the SDS-treated and the control sample. Error bars indicate the error on triplicate samples for each condition. (d) Schematic of Pal_{E102K} and Pal_{R104C} interactions with TolB and the PG previously published [37]. Pal_{E102K} interacts with the PG but not with TolB, whereas Pal_{R104C} interacts with TolB but not with the PG. (e) A Δ*pal* strain was complemented with pPal-sfGFP, $pPal_{E102K}$-sfGFP or $pPal_{R104C}$-sfGFP. *E. coli* W3110 wild-type or derivative strains were grown in M9 minimal medium supplemented with glucose and casa-amino acids at 30 °C. Fluorescence microscopy images reveal that Pal-sfGFP and Pal_{R104C}-sfGFP localize at the cell septum during cell division in contrast to Pal_{E102K}-sfGFP. The septal localization of Pal_{R104C}-sfGFP is abolished in a Δ*tolB–pal* strain.

the three IM proteins of the Tol–Pal complex, TolA, TolQ and TolR, during cell division. Using plasmid-encoded fluorescent fusions, Gerding and co-workers [13] previously reported that GFP-TolA and TolQ-GFP, but not GFP-TolR localize at the septum in a TolQ-R-A-B-Pal mutant background. Here, we deciphered further the Tol recruitment process, showing that sfGFP-TolA, TolQ-mCherry and sfGFP-TolR accumulated at the constriction sites in all single deletion mutants tested. These data demonstrate that assembly of a functional molecular motor is not required for recruitment of the Tol–Pal proteins to the septum (Fig. 4a-c). It has been previously described by crosslinking experiments that TolR can interact with TolQ in absence of TolA and with TolA in absence of TolQ [9,32]. Thus, although TolA and TolQ do not require any Tol–Pal protein for their septal location, and based on previous observations [13], it is likely that TolR requires either TolA or TolQ for its recruitment to the septum (Fig. 4b).

Pal requires the TolQ, TolR, TolA and TolB proteins and a functional TolQ–TolR molecular motor for its localization at the cell constriction site

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

The intracellular localization of Pal in the different mutant backgrounds revealed that Pal-mCherry was not recruited at the constriction sites during cell division but remained spread in the OM (Fig. 5c). This result demonstrates that recruitment of Pal-mCherry to division sites requires all the Tol proteins. TolA interacts with Pal in a PMF-dependent manner (directly or *via* TolB, see below), and this interaction is regulated by the molecular motor TolQR. It has been previously described that a single D23A mutation in the transmembrane segment of TolR deactivates the TolQR motor without affecting TolQR–TolA complex assembly [36]. When we complemented, a *tolQR* strain with a plasmid-encoding TolQR, Pal-mCherry was recruited to the septum (Fig. 5c). However, introduction of a plasmid-encoding $TolQR_{D23A}$ failed to restore septal localization and Pal-mCherry was dispersed along the periphery of the cell (Fig. 5c). These results show that a functional TolQR motor is absolutely required to maintain Pal at the septum.

Key role of Pal at the septum

Previous studies demonstrated that Pal interacts both with TolB and the PG, and that these two interactions are mutually exclusive [10,11]. To analyze the role of Pal at the septum, we first constructed a plasmid encoding the fluorescent hybrid protein Pal-sfGFP under the control of an arabinose-inducible promotor and verified that this construction was correctly expressed, functional (Fig. 6a–c and Table 1) and localized at the constriction sites during cell division (Fig. 6e, WT panel). Then, we constructed two mutated version of Pal-sfGFP (Fig. 6d). A Pal (E102K) variant that prevents Pal–TolB interaction but does not affect PG-binding and a Pal (R104C) variant that interacts with TolB but does not bind PG [37]. The two mutated Pal-sfGFP proteins (Pal_{102}-sfGFP and Pal_{104}-sfGFP) were correctly expressed (Fig. 6a) but as previously shown were not able to complement a *Δpal* strain [37] (Fig. 6b, c and Table 1). Thus, *Δpal* strain producing either Pal_{102}-sfGFP or Pal_{104}-sfGFP fusion protein showed a high sensitivity to detergents (Fig. 6c) and showed a chaining phenotype under low osmolarity growth conditions indicative of a division and cell separation defect (Fig. 6b). The Pal_{102}-sfGFP did not localize at the sites of constriction during cell division, indicating that interaction with TolB is essential for Pal recruitment (Fig. 6e). In contrast, Pal_{104}-sfGFP localized at the septum normally, indicating that once at the septum, Pal must interact with the PG to perform its function. Finally, in a *ΔtolB–pal* strain, Pal_{104}-sfGFP showed no septal localization during cell division (Fig. 6e). These results clearly indicate that TolB is absolutely necessary for functional Pal localization and that Pal binding to PG is absolutely necessary to the function of the Tol–Pal system in cell division.

Discussion

Cell division in gram-negative bacteria is orchestrated by the divisome and requires the coordinated constriction of the three cell envelope layers. Despite

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

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

Interestingly, we observe that TolQ and TolA accumulate at the constrictions site independently of the other Tol–Pal proteins and that Pal absolutely requires all the Tol proteins to be associated with the cell septum (Figs. 4, 5). Based on these results and previously published results, it seems likely that the IM TolQRA complex is first recruited by either FtsN for TolQ [38] or an active divisome complex for TolA [7,13,39]. TolR is able to directly interact with TolA or TolQ, and therefore, its septal localization is likely dependent of one of these two proteins. Once in mid-cell, the TolQRA complex and TolB drive the recruitment of Pal at the division site. However, from our results, it is not possible to conclude decisively whether the TolQRA complex recruits a TolB–Pal OM complex or whether it first recruits TolB, which then recruits Pal. TolA could also interact directly with Pal, although this interaction cannot overcome the requirement for TolB. In fact, previous papers indicate contradictory results about the TolA–Pal interaction. Based on a co-immunoprecipitation approach, TolA and Pal have been described to interact *in vivo* through a PMF-dependent mechanism [6,40]. However, such interaction was not detected *in vitro* with purified proteins by isothermal calorimetry [28], suggesting that the TolA–Pal interaction could be indirect (i.e., mediated *via* TolB). In a previous paper [13], the authors speculated that TolB could reverse Pal–PG interactions to permit lateral movement of the lipoprotein in the OM and then its interaction with TolA at the division site. However, this is unlikely because we show here that the mutated Pal_{R104C} that does not bind the PG and is free to diffuse in the OM is not localized at the division site in the absence of TolB (Fig. 6e). Thus, TolB is required for Pal to localize to the septum likely *via* direct recruitment.

In addition, we demonstrate that the energy provided by a functional TolQR complex is absolutely required for a correct localization of Pal during the cell cycle. Indeed, in the absence of TolQR or in the presence of a defective $TolQR_{D23A}$ complex, unable to energize TolA, Pal localized all around the cell envelope and does not accumulate at the constriction site during cell division (Fig. 5c). Thus, the septal recruitment of Pal is a PMF-dependent process. Based on these results, we propose a model for Tol–Pal function during cell division. First, the IM TolQRA complex is recruited to the division site by divisome proteins (i.e., FtsN and others). Second, the motor becomes active and energizes TolA to recruit the OM TolB–Pal complex. Such transient ternary complex has been previously observed although in low yield by *in vitro* cross-linking experiment with purified proteins [28]. It is possible that the mechanical action of the TolQR motor allows TolA to pull the TolB–Pal complex inward, drawing the OM invagination over to the PG and IM layers. Completing this cycle, TolA would dissociate TolB from Pal, thus permitting Pal–PG interaction. Indeed, the large differences of affinity between the TolB–Pal (nM) and TolA–TolB (μM) interactions [28] are not in favor of a TolA–TolB association. In conclusion, this cycle of exclusive TolA, TolB and Pal interactions and the contractile activity of Tol–Pal complex regulated by the energy state of the TolQR motor would maintain sufficient contact between the three layers of the envelope for efficient invagination. In addition, the energy state of the Tol–Pal complex has already been involved in the regulation of the PBP1B-LpoB machine involved in PG synthesis at constriction sites [7]. Thus, the Tol–Pal complex appears to regulate and coordinate septal PG synthesis and OM constriction. Surprisingly, *in E. coli*, despite all the phenotypes

associated with the *tol* mutations and the role of the Tol–Pal complex in the cell division, this complex is not essential for viability in standard laboratory conditions, suggesting that the Tol–Pal function, at least in the cell division, could be compensated by another trans-envelope complex. Indeed two global approaches have shown that mutations in Tol–Pal complex are synthetically lethal when combined with mutations in the trans-envelope complex, LpoB-PBP1B [41]. This complex also seems to participate in tethering the OM either to the PG or the IM and localizes at constriction sites, likely promoting OM constriction during cell division even when Tol–Pal is absent, in standard laboratory conditions [41]. In low osmolarity media, where Tol–Pal is essential, the energy provided by the TolQR motor and the elongated TolAII domain could prevent local expansions of the periplasmic space, which is harmful to complete the cell division.

Although the Tol–Pal localization at the septum during the cell cycle is clearly established, many questions remain unanswered. Which proteins recruit the Tol–Pal complex at the septum? FtsN is a good candidate for TolQ and has been previously described to be involved in the recruitment of TolA [13,38]. However, the role of FtsN in the recruitment of TolA could be indirect, because a suppressor mutation in FtsA can restore the correct localization of TolA and function of the Tol–Pal system in a FtsN-depleted strain [39]. Similarly, the direct recruitment of TolQ by FtsN is questionable. Another major interrogation concerns the timing and sequence of the interactions between TolA, TolB, Pal and the PG driven and cycled as function of the energy state of the system. Understanding and deciphering these interaction networks would greatly improve our knowledge on the Tol–Pal system function at the septum during the cell division.

Material and Methods

Bacterial strains, plasmids and growth

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 or at 30 °C in M9 minimum medium, supplemented with antibiotics if necessary (ampicillin 100 µg/ml, kanamycin 50 µg/ml and/or chloramphenicol 30 µg/ml).

Plasmid construction

PCRs were performed in a Biometra thermocycler, using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). Oligonucleotides were synthesized by Sigma. Plasmids pTolQR and pPal were constructed by a double PCR technique [42], allowing amplification of the *tolQR* and *pal* genes, flanked by extensions annealing to the target vector (pBad/HisC). The product of the first PCR was then used as oligonucleotides for a second PCR with the target vector as a template. Plasmid pPal-sfGFP was constructed by a double PCR technique, allowing amplification of the *sfGFP* gene, flanked by extensions annealing to the target vector (pPal). Site-directed substitutions were introduced by QuikChange mutagenesis PCR using complementary pairs of oligonucleotides.

Cloning and expression of *E. coli* Tol–Pal proteins

The *E. coli* W3110 strains carrying the chromosomal gene deletions or tags of interest were obtained using a modified protocol from Datsenko and Wanner [43] as described previously [44]. Briefly, for each gene deletion, a kanamycin cassette was amplified from the vector pKD4 using a pair of oligonucleotides carrying 50-nucleotide extensions homologous to regions that are adjacent to the target gene. After electroporation of the PCR product into *E. coli* W3110 cells carrying the pKOBEG vector, kanamycin-resistant clones were selected and confirmed by colony PCR. The kanamycin cassette was then excised using vector pCP20 and gene deletions were verified by PCR. The same procedure was performed to introduce the mCherry or sfGFP coding sequences upstream the stop codon (pKD4-*sfgfp* or pkD4-*mCherry*) or downstream from the start codon (p*gfp*-KD4 or p*mCherry*-KD4), resulting in strains producing fusion proteins from their native chromosomal loci.

Western blots and protein production

Protein production from gene expression at chromosomal loci was checked by Western blot experiments on overnight cultures. Heat treated samples (A_{600} = 0.2) were loaded on 12,5% SDS-PAGE and analyzed by Western blot immunodetection using specific antibodies. When necessary, plasmid multicopy expression was induced with arabinose 0.02% for 1 h.

Fluorescence microscopy

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

Time-lapse fluorescent experiments

For each time-lapse experiment, four independent fields were manually defined with a motorized stage (Prior Scientific) and stored (X, Y, Z, PFS-offset) in a custom automation system designed for time-lapse experiments. Fluorescence and phase-contrast images were captured every 2 min using an automated and inverted epifluorescence microscope TE2000-E-PFS (Nikon, France) equipped with Perfect Focus System (PFS) for 2 h. PFS automatically maintains focus in order to keep in sharp focus the point of interest at all times and despite mechanical or thermal perturbations. Images were recorded with a CoolSNAP HQ 2 (Roper Scientific, Roper Scientific SARL, France) and a 100×/1.4 DLL objective. Excitation light was emitted by a 120-W metal halide light. The sfGFP images were recorded by using the ET-GFP filter set (Chroma 49002) using an exposure time of 500–700 ms, and the mCherry images were recorded by using the ET-mCherry filter set (Chroma 49008) using an exposure time of 100–700 ms. Phase-contrast and fluorescence channels were adjusted and merged using the Fiji software (http://rsb.info.nih.gov/ij/). Some slight movements of the whole field during the experiments were corrected by registering individual frames using 'StackReg' and 'Image Stabilizer' plugins for ImageJ.

One-shot acquisitions

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

Statistical analysis

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 (http://www.microbej.com). To avoid false positive, each event was manually controlled in the original raw data. Kymograph representations were made using the "KymoResliceWide" plugin from Fiji and demograph or Heatmap representations were made with the 'Demograph' or "Heatmap" options from MicrobeJ.

Phenotypic studies

Phenotypic studies have been performed in order to visualize effects of the fluorescent markers chromosomal insertion and gene deletions onto the *tol–pal* operon. Three assays were achieved to check the OM integrity, the Tol–Pal complex assembly and the cell division process.

Colicin tolerance assay

Colicin A and D lethal activities were checked by the presence of halos on a cell lawn of the strain to be tested, as previously described [45]. Data are reported in Table 1 as the maximal dilution of the colicin stock sufficient for inhibiting cell growth and are shown in Fig. S3.

SDS sensitivity assay

Sensitivity to sodium dodecyl sulfate (SDS) was determined by measuring the level of survival of the strain after 4 h of incubation at 37 °C in liquid LB medium containing various concentration of SDS (from 0.5% to 2%). Data are reported in Table 1 as "S" for sensitive strains or "R" for resistant strains and also in Fig. S1 and S5.

Cell division assay

Cells were grown for 4 h at 37 °C in LB (171 mM NaCl) or LB–NaCl (0 mM NaCl) liquid medium. Then cells were fixed on fresh poly-L-lysine (0.1% w/v in H_2O) coated microscope slides and were observed with an optical microscope (Eclipse 50i, Nikon, France).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2019.05.039.

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

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