The *Caulobacter* Tol-Pal Complex Is Essential for Outer Membrane Integrity and the Positioning of a Polar Localization Factor^{∇†}

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Cell division in *Caulobacter crescentus* involves constriction and fission of the inner membrane (IM) followed about 20 min later by fission of the outer membrane (OM) and daughter cell separation. In contrast to *Escherichia coli*, the *Caulobacter* Tol-Pal complex is essential. Cryo-electron microscopy images of the *Caulobacter* cell envelope exhibited outer membrane disruption, and cells failed to complete cell division in TolA, TolB, or Pal mutant strains. In wild-type cells, components of the Tol-Pal complex localize to the division plane in early predivisional cells and remain predominantly at the new pole of swarmer and stalked progeny upon completion of division. The Tol-Pal complex is required to maintain the position of the transmembrane TipN polar marker, and indirectly the PleC histidine kinase, at the cell pole, but it is not required for the polar maintenance of other transmembrane and membrane-associated polar proteins tested. Coimmunoprecipitation experiments show that both TolA and Pal interact directly or indirectly with TipN. We propose that disruption of the *trans*-envelope Tol-Pal complex releases TipN from its subcellular position. The *Caulobacter* Tol-Pal complex is thus a key component of cell envelope structure and function, mediating OM constriction at the final step of cell division as well as the positioning of a protein localization factor.

The cell envelope of *Caulobacter crescentus* and other Gram-negative bacteria consists of a peptidoglycan layer positioned between the inner membrane (IM) and the outer membrane (OM). *Caulobacter* cell division is implemented by the constrictive IM-associated Z-ring, a polymeric structure of the highly conserved tubulin-like FtsZ protein positioned at the division plane. In *Caulobacter*, FtsZ localizes to the incipient division plane at the time of chromosomal origin duplication and segregation to the cell poles (45), well before a cell constriction is visible in the light microscope (45). In the early stages of cell division, the inner and outer membranes are constricted simultaneously. However, late in the cell division process, the IM and peptidoglycan layers constrict faster, creating a separation of the inner and outer membranes near the division plane (27). Fission of the IM and the peptidoglycan layer occurs about 20 min before cell division, creating a cell containing two inner membrane and peptidoglycan-bound cytoplasmic compartments surrounded by a single continuous outer membrane (27). Since *Caulobacter* OM invagination is temporally and spatially separated from peptidoglycan and IM invagination, separate mechanisms must drive the two processes. Hydrolysis of short membrane-bound FtsZ filaments that affects their curvature has been suggested as the mechanism for generation

of the constrictive force for invagination of the IM (32, 40). However, the mechanism that implements the delayed constriction of the OM layer of the cell envelope is poorly understood.

The Tol-Pal complex of Gram-negative bacteria is widely conserved and plays multiple physiological roles, including maintaining OM interaction with the peptidoglycan, expressing lipopolysaccharide surface antigens and virulence factors, facilitating infection by filamentous DNA phage, and reducing sensitivity to detergents (3, 12, 13, 18, 33, 34). In many bacteria, *tol-pal* mutants form cell chains with lateral membrane blebs in low-osmolarity or high-ionic-strength medium, suggesting that Tol-Pal plays a role in completing cell division under conditions of membrane stress in these organisms (3, 11, 46). In *Escherichia coli*, TolA, TolQ, and TolR are inner membrane proteins (Fig. 1A), and the TolA transmembrane domain interacts with the transmembrane domain of TolQ and TolR (14, 20). Pal, an abundant outer membrane lipoprotein, is thought to interact with the peptidoglycan layer through a conserved α -helical motif (4, 6, 28, 30), while TolB is a periplasmic protein that interacts with Pal, the Lpp murein lipoprotein, and OmpA (5, 10, 43, 49). Thus, the Tol-Pal system bridges the three layers of the cell envelope via multiple interactions, including the interaction of the C-terminal periplasmic domain of TolA with Pal and TolB (8, 15, 21, 48). In *E. coli*, the peptidoglycan-associated Lpp protein is a structural protein that is involved in maintaining the integrity of the cell envelope structure. *Caulobacter* does not have an Lpp homolog. The Lpp protein is found only in enteric and endosymbiont bacteria (<http://string-db.org>), suggesting that adaptation to survival in

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FIG. 1. The *Caulobacter crescentus tol-pal* gene cluster. (A) Predicted organization of the components of the Tol-Pal complex in the *E. coli* (35) and *Caulobacter* cell envelopes. TolQ, TolR, and TolA are integral IM proteins, and Pal is an OM protein that interacts with the periplasmic TolB protein. The *Caulobacter* TipN polar marker is an IM protein (25, 29). (B) Schematic of the gene organization of the *Caulobacter tol-pal* components. Arrows indicate the direction of transcription and putative position of promoters. The +1 transcriptional start site is at -49 of the *tolQ* coding sequence, as indicated (36). (C) mRNA expression patterns of genes encoding the components of the Tol-Pal complex over the course of a cell cycle. *pal* expression peaks in the swarmer cell and drops thereafter, while expression of the other genes is not cell cycle dependent. (D) Western blot analysis of relative Pal protein levels (upper panel; arrows indicate the Pal protein) during the cell cycle starting with a synchronous population of wild-type swarmer cells. The FtsZ protein (lower panel) is shown as a loading control and quality control for the synchrony. (E) Normalized abundance of Pal and FtsZ protein levels over the course of the cell cycle. The Pal protein level does not exhibit significant changes during the course of the cell cycle.

a high-osmolarity environment may explain the significant differences between the *E. coli* and *Caulobacter* Tol-Pal systems.

Here, we report that the *Caulobacter* Tol-Pal complex is concentrated at the division plane and following cell division it remains at the new poles, where it plays a role in maintaining the subcellular positioning of the TipN polar localization factor. *Caulobacter* strains depleted of TolA, TolB, or Pal components of the essential Tol-Pal complex exhibited surface bleb formation at both the division plane and the cell poles and defects in invagination during the final stages of *Caulobacter* cell division.

MATERIALS AND METHODS

Bacterial strains, synchronization, growth conditions, and cloning. All *Caulobacter* strains were derived from CB15N and grown at 28°C in peptone-yeast extract (PYE) with selected antibiotics. Strains and plasmids are listed in Table S1 of the supplemental material, and details on their construction are also given in Table S1. For the Pal, TolA, and TolB depletion constructs, cells were grown overnight in PYE medium containing 0.3% xylose (PYEX), washed with PYE

medium, and then resuspended in PYE medium containing 0.2% glucose (PYEG).

Fluorescence microscopy. Cells were immobilized using a thin layer of agarose in M2G medium. For localization studies, 0.3% xylose or 0.5 mM vanillate (pH 7.5) was used to induce expression of fluorescent protein fusions from the *xytX* or *vanA* promoters for at least 2 h, respectively. Two $\mu\text{g}/\text{ml}$ *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64; Molecular Probes) was added to the agarose to fluorescently label membranes. Differential interference contrast (DIC) and fluorescence microscopy images were obtained using a Leica DM 6000 B microscope with an HCX PL APO 100 $\times$, 1.40 numerical aperture, oil PH3 CS objective, Hamamatsu 16 EM-CCD C9100 camera, and a custom-designed microscope control and image analysis software package called KAMS (9).

Scanning electron microscopy. Samples were fixed in 2% glutaraldehyde and 4% formaldehyde in a 0.1 M sodium cacodylate buffer (pH 7.3) overnight, allowed to adhere onto poly-L-lysine-coated 12-mm coverslips, and moved to 4°C. Subsequently, samples were dehydrated with 50%, 70%, 95%, and 100% ethanol for 10 min each and then coated with gold-palladium. All scanning electron microscopy (SEM) images were taken on a Hitachi S-3400N VP-SEM.

Cryo-EM. Cells were grown overnight in liquid PYE medium containing 0.3% xylose at 28°C, washed with unsupplemented PYE medium, and then resuspended in PYE medium containing 0.2% glucose for 11 h until reaching an

optical density at 660 nm (OD_{660}) of approximately 0.4. Aliquots of 5 μ l were then taken directly from the culture and placed onto glow-discharged carbon grids (Ted Pella 01881). The grids were blotted and plunged as described previously (27). All cryo-electron microscopy (cryo-EM) images were acquired with a JEOL-3100-FEF electron microscope at the Lawrence Berkeley National Laboratory.

Coimmunoprecipitation experiments. Coimmunoprecipitation (co-IP) was performed as previously described (26). Strains LS4536 and LS4529 were grown in PYE medium containing 0.3% xylose and were harvested in log phase. LS4538 and wild-type strains were induced with 0.3% xylose for 3 h before being harvested. One liter of cell culture in PYE medium was washed in IP buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, and 20% glycerol), treated with the membrane-permeable cross-linking agent formaldehyde (Fisher Biotech), at a final concentration of 1%, and then quenched with 0.125 M glycine. Cells were lysed by passage through a French press at 15,000 lb/in² three times. The cell lysate was then incubated with anti-FLAG-conjugated beads (FLAGIT-1 kit; Sigma) overnight. Subsequently, the beads were washed, bound proteins were eluted by incubating with 3 $\times$ FLAG peptides, and the supernatant was collected.

Immunoblot analysis. Immunoblot assays were performed as previously described (6, 50), and the blots were developed for chemiluminescently. Western blotting to monitor DivJ, PleC, FtsZ, and PopZ was performed as described previously (6, 37, 50). Antibody dilutions were as follows: anti-green fluorescent protein (anti-GFP; Roche) at 1/1,000; anti-Pal at 1/50,000; anti-FLAG monoclonal antibody (Sigma) at 1/10,000.

RESULTS

Genomic organization of the *Caulobacter tol-pal* gene cluster.

The cell envelope configuration of the protein components of the *E. coli* and *Caulobacter* Tol-Pal complexes are shown schematically in Fig. 1A, and the genomic organization of *Caulobacter tol-pal* homologs (CC3233 to CC3229) is shown in Fig. 1B. The *Caulobacter* gene CC3231, originally predicted to encode a hypothetical protein (39), shares only 27% identity with the *tolA* from *E. coli*. However, *tolA* represents a highly divergent gene family with a strongly species-dependent sequence (44). The predicted TolA protein contains a signature single transmembrane helix at the N terminus and a large periplasmic domain at the C terminus. As observed in other Gram-negative bacteria, the *tolA* gene is adjacent to the predicted *tolQ*, *tolR*, and *tolB* homologs. Based on its chromosome location and conserved structure features, we designated *Caulobacter* gene CC3231 as *tolA*. Microarray assays of the gene expression temporal pattern over the cell cycle indicated that *tolA*, *tolB*, *tolR*, and *tolQ* are not cell cycle regulated (Fig. 1C) (36). In contrast, *pal* is cell cycle regulated with high transcript levels in swarmer cells that drop thereafter (Fig. 1C) (36). To determine if the Pal protein level is also cell cycle dependent, we raised a polyclonal antibody against purified recombinant Pal and used the antibody to probe synchronized wild-type CB15N cell lysate samples taken at various times after synchronization. This immunoblot analysis revealed that the level of Pal was roughly constant over the cell cycle (Fig. 1D and E). The quality of the synchrony was monitored by immunoblot analysis of the FtsZ protein (42, 45), and it was low in swarmer cells, increased in stalked cells, and peaked at the onset of cell division, as expected (Fig. 1D and E).

Proteins of the Tol-Pal cluster accumulate at the incipient division site. To examine the cellular localization of the proteins in the Tol-Pal complex, we constructed fusions of each gene to a fluorescent protein coding sequence and placed the fusion construct under the control of a xylose-inducible promoter at the chromosomal *xylX* locus. In each case, the wild-type copy of the gene was retained at its native chromosomal

site, creating a merodiploid strain. We constructed a *yfp* fusion to the 3' end of *tolQ*, *gfp* fusions to the 5' end of *tolR* and *tolA*, and *mCherry* fusions to the 5' end of *tolB* and to the 3' end of *pal*. Individual strains, each containing one of the five fusion constructs, were incubated in the presence of 0.3% xylose to induce the expression of the fluorescently tagged proteins. All of the fusion proteins exhibited significant accumulation at the division plane in predivisional cells and were retained at the new poles of the daughter swarmer and stalked cells (Fig. 2A). In contrast to *E. coli* (19), however, a significant fraction of the components of the Tol-Pal complex also displayed a punctate (TolQ, TolR, and TolA) or peripheral (TolB and Pal) localization pattern throughout the cell envelope, including the stalk.

To investigate the localization dynamics of TolA, we followed the subcellular positioning of GFP-TolA over the course of the cell cycle. We isolated swarmer cells of strain LS4519 (*P_{xyl}-gfp-tolA*) and imaged the cells by fluorescence microscopy as they grew synchronously on agarose pads supplemented with 0.3% xylose (Fig. 2B). Following separation of the two daughter cells, GFP-TolA remained at the new poles. When the swarmer cell differentiated into a stalked cell, an additional focus appeared at mid-cell. Later, predivisional cells had a single focus at the cell division site. We demonstrated that the GFP-TolA fusion protein was functional by transducing the *P_{xyl}-gfp-tolA* allele into the TolA depletion strain, LS4525, replacing the wild-type *tolA* gene at the *xylX* locus with *gfp-tolA* to create strain LS4522. This strain has the native *tolA* gene deleted, and the only full-length copy of the gene is *gfp-tolA*. Upon induction with xylose, the localization of GFP-TolA was similar to that observed in wild-type cells (see Fig. S1A in the supplemental material), and the cellular morphology and growth rate were indistinguishable from wild type. Thus, the GFP-TolA fusion protein alone complemented the TolA deletion phenotype. The Pal-mCherry fusion also fully complemented the Pal depletion phenotype (see Fig. S1A). To verify that the expected fusion proteins were produced, cell lysates from the above strains were probed with antibodies that recognize GFP and Pal (see Fig. S1B and C in the supplemental material).

To determine the temporal order of the Tol-Pal complex and divisome assembly, we compared the localization dynamics of TolA and TolQ with those of FtsZ, FtsA, and FtsI by using *in vivo* time-course microscopy. The localization timing of FtsZ-Venus, Venus-FtsA, Venus-FtsI, GFP-TolA, and TolQ-yellow fluorescent protein (YFP) near mid-cell (Fig. 2C) showed that both TolQ and TolA localize to mid-cell after FtsZ is assembled at the division plane, well before Venus-FtsA and Venus-FtsI.

The dependency localization pattern at the division plane. Since the division plane localization of GFP-TolA and TolQ-YFP occurs later than FtsZ, we asked if the localization of the components of the Tol-Pal complex is dependent on the prior localization of FtsZ. Accordingly, we constructed a strain (LS4546) with *P_{van}-yfp-tolA* on a high-copy-number plasmid in a background in which the only functional copy of *ftsZ* is under the control of the xylose-inducible promoter. Cells grown in the absence of xylose were depleted of FtsZ (data not shown) and formed smooth filamentous cells. In these cells, YFP-TolA was diffuse (Fig. 2D). FtsZ was then allowed to accumulate in

FIG. 2. Subcellular localization of the proteins encoded by the *tol-pal* cluster. (A) Fusions of TolQ, TolR, TolA, TolB, and Pal to GFP, YFP, or mCherry, at either N or C terminals, were generated and their cellular locations were observed by fluorescence microscopy. LS4517 (*P_{xylX}-tolQ-yfp*), LS4518 (*P_{xylX}-gfp-tolR*), LS4519 (*P_{xylX}-gfp-tolA*), LS4520 (*P_{xylX}-mCherry-tolB*), and LS4521 (*P_{xylX}-pal-mCherry*) strains were each incubated in the presence of 0.3% xylose for 2 h to induce the expression of the fluorescently tagged fusions in a merodiploid cell containing the wild-type gene. All of the fusion proteins tested localized to both the cell pole (upper panel) and to the division plane (lower panel). (B) A synchronized LS4519 swarmer cell population, producing GFP-TolA, was suspended in PYE medium containing 0.3% xylose and allowed to proceed through the cell cycle. Samples were visualized at 20-min intervals by phase-contrast and fluorescence microscopy. A schematic of *Caulobacter* cells showing the dynamic localization of TolA as a function of the cell cycle is shown below the micrographs. The accumulation of

these cells by incubating them in PYE containing 0.3% xylose for 0.5 h. The localization of TolA to the division plane was restored upon FtsZ accumulation (Fig. 2D, arrows), indicating that TolA requires FtsZ for its positioning at the division site. As the recruitment of FtsA to the division site is significantly later than TolA and TolQ (Fig. 2C), we examined the localization patterns of YFP-TolA in cells that had been depleted of FtsA. Interestingly, the recruitment of YFP-TolA to the divisome was independent of FtsA, a protein reported to be immediately downstream of FtsZ in the *E. coli* assembly hierarchy (22). YFP-TolA localized to discrete bands in filamentous cells depleted of FtsA (Fig. 2D, arrows). Similar to the localization pattern observed for YFP-TolA, all other proteins of the Tol-Pal complex were also robustly recruited to the division site in the absence of FtsA (data not shown), arguing that FtsA is not required for Tol-Pal localization in *Caulobacter*. We also examined the localization pattern of YFP-TolA in cells depleted of FtsN, the last known essential protein recruited to the divisome in the *E. coli* assembly hierarchy (1). YFP-TolA foci were observed at several sites in the FtsN-depleted filamentous cells (Fig. 2D, arrows), suggesting that the recruitment of TolA to the divisome occurs independently of FtsN.

In *E. coli*, division site localization of TolQ and TolA is independent of any of the other four Tol-Pal proteins, while TolR requires TolQ, and possibly TolB, and Pal requires TolA to accumulate at the division plane (19). To determine which of the remaining components of the Tol-Pal complex were able to localize to the division site in the *Caulobacter tolA* and *pal* depletion strains, we examined the localization patterns of TolQ-YFP, YFP-TolR, Pal-mCherry, and YFP-TolA in the *tolA* and *pal* mutant strains. In the absence of Pal, TolQ-YFP, YFP-TolR, and YFP-TolA were localized to the division plane (Fig. 2E, arrows), indicating that Pal is not required to position the other proteins of the Tol-Pal complex to the division site. Both TolQ-YFP and YFP-TolR were localized to the division site in the absence of TolA (Fig. 2E, arrows), but Pal-mCherry failed to localize to a subcellular site (Fig. 2E). Thus, Pal requires TolA for its localization to the division plane.

TolA and Pal are required for the polar positioning of TipN and PleC. Because the Tol-Pal complex remains at the new poles in swarmer and stalked daughter cells after cell division, we asked if transmembrane polar proteins are mislocalized in mutant strains lacking components of the Tol-Pal system. We constructed Pal or TolA depletion strains containing either

tipN-gfp, *pleC-gfp*, *divJ-yfp*, or *Pvan::popZ-yfp* in place of each wild-type gene. In wild-type cells, TipN-GFP accumulates at the cell division plane and then, following division, it remains as a single focus at the new poles of the daughter cells (25, 29). In cells depleted of Pal by growth in PYEG for 11 h, 46% of the cells ($n = 297$) exhibited TipN-GFP foci that were aberrantly placed throughout the cell (Fig. 3A). Similarly, when TolA was depleted by growth in PYEG for 11 h, we observed an aberrant placement of TipN-GFP (Fig. 3B). Since TipN contributes to the polar placement of the PleC histidine kinase (29), we examined the localization of PleC in the absence of either Pal or TolA. In a localization pattern similar to the pattern observed for TipN-GFP in cells depleted of either Pal or TolA, PleC-GFP foci were aberrantly positioned throughout the cell (Fig. 3A and B).

The cellular localization of two additional polar proteins, the DivJ-YFP histidine kinase (50) and the membrane-associated PopZ-YFP centromere anchor (6, 16), was maintained in depletion strains of Pal and TolA (Fig. 3A and B). Thus, membrane integrity mediated by the Tol-Pal complex is specifically required to localize a subset of polar proteins. Of the polar proteins imaged, only the TipN protein first accumulates at the division plane and then subsequently at the new cell poles in wild-type strains, suggesting that in the TolA and Pal depletion strains TipN is not properly positioned at the division plane and consequently is mislocalized at the cell poles. After 11 h of either TolA or Pal depletion, 80% of the cells remained viable, and in the TolA and Pal depletion strains, there were no significant changes in the levels of either TipN or PleC after 11 h of growth in the absence of xylose (Fig. 3C). Thus, the mislocalization of TipN in the depletion strains was not due to effects in dying cells or elimination of TipN. We examined the localization of FtsZ and divisome components ZapA and FtsL in cells depleted of TolA or Pal, and all localized normally in these strains (data not shown).

To determine if TipN interacts with TolA and Pal, we used strain LS4536 (*tipN-gfp ΔtolA PxylX-tolA-m2*), in which the native *tolA* coding sequence was deleted and replaced with a chromosomal xylose-inducible *tolA* gene carrying a C-terminal FLAG-M2 tag. When strain LS4536 was grown in the presence of xylose, inducing expression of *tolA-m2*, cell morphology was similar to that of the wild type (data not shown). As a control, an isogenic strain, *tipN-gfp ΔtolA PxylX-tolA* (LS4529), was generated. Formaldehyde, a membrane-permeable cross-

GFP-TolA at the division plane is indicated by arrows. (C) Cells of strains EG052 (*PxylX-ftsZ-venus*), EG055 (*PxylX-venus-ftsA*), EG051 (*PxylX-venus-ftsI*), LS4517 (*PxylX-tolQ-venus*), and LS4519 (*PxylX-gfp-tolA*) were grown to exponential phase in PYE medium, induced for 1 h with 0.3% xylose, synchronized, and resuspended in PYE medium supplemented with 0.3% xylose. Cells were withdrawn from the cultures at 10-min intervals and visualized by phase-contrast and fluorescence microscopy to determine if the fluorescently labeled proteins were positioned at the incipient division plane. At least 150 cells were analyzed per time point. (D) Localization of YFP-TolA in strain LS4546 depleted of FtsZ for 3 h (–FtsZ) and when FtsZ was depleted for 3 h and then was FtsZ added back by incubation in the presence of the xylose inducer for 0.5 h. In both cases, cultures were incubated in the presence of 0.5 mM vanillate to induce the expression of *yfp-tolA*. Localization of YFP-TolA in strain LS4547 depleted of FtsA after growth in PYE medium with glucose (PYEG) for 8 h is shown. White arrows indicate foci of YFP-TolA. Localization of YFP-TolA in strain LS4626 (*xylX::PxylX-yfp-tolA ΔftsN vanA::Pvan-ftsN*) depleted of FtsN after growth in PYE medium without vanillate for 14 h is also shown. Two hours before analysis, expression of YFP-TolA was induced by addition of 0.3% xylose. YFP-TolA requires FtsZ, but not FtsA or FtsN, to form fluorescent foci. (E) Fluorescence microscopy of Pal depletion strains carrying plasmid-borne *Pvan-tolQ-yfp*, *Pvan-yfp-tolR*, or *Pvan-yfp-tolA* incubated in the absence of xylose (–Pal). Cells were grown in PYEX, washed, and incubated in PYEG for 11 h to deplete Pal. TolA depletion strains bearing the plasmid *Pvan-tolQ-yfp* or *Pvan-yfp-tolR* were incubated in the absence of xylose (–TolA) for 11 h. A TolA depletion strain, with *Pvan-pal-mCherry* integrated at the chromosomal *vanA* locus, was incubated in the presence of 0.5 mM vanillate to induce Pal-mCherry and in the absence of xylose for 11 h (–TolA). Cells were imaged by using phase-contrast and fluorescence microscopy.

FIG. 3. Mislocalization of the TipN-GFP and PleC-GFP polar proteins in the absence of the Pal or TolA proteins. (A) Fluorescence microscopy of Pal depletion strains bearing TipN-GFP, PleC-GFP, DivJ-YFP, or PopZ-YFP grown in the presence of xylose to induce Pal accumulation or in the presence of glucose to deplete Pal. Strain LS4528 (*tipN-gfp Δpal PxlX-pal*) was imaged after either 9 or 11 h of incubation in the absence of xylose to deplete Pal. The upper panels show the subcellular location of TipN-GFP, PleC-GFP, and DivJ-YFP in cells grown in the presence of xylose to induce Pal, compared to cells shown in the lower panels incubated with glucose to deplete Pal. Strain LS4534 (*Δpal PxlX-pal Pvan-popZ-yfp*) was incubated for 1 h in the presence of 0.5 mM vanillate to induce PopZ-YFP in the presence of either xylose (+Pal) or glucose (−Pal). (B) The same fusion proteins were examined by fluorescence microscopy in TolA depletion strains LS4529 (*tipN-gfp ΔtolA PxlX-tolA*), LS4531 (*pleC-gfp ΔtolA PxlX-tolA*), LS4533 (*divJ-yfp ΔtolA PxlX-tolA*), and LS4535 (*ΔtolA PxlX-tolA Pvan-popZ-yfp*) incubated for 11 h in the presence of either xylose (+TolA) or glucose (−TolA). Arrows indicate polar localization of DivJ-YFP and PopZ-YFP in the absence of either Pal or TolA. (C) Immunoblot analysis of Pal, TipN-GFP, and PleC levels in cell extracts of TolA depletion and Pal depletion cultures. Neither TipN-GFP nor PleC levels were affected after 11 h of growth in the absence of xylose. (D) The predicted localization dependency pathway derived from experiments shown in this figure and in Fig. 2.

linking agent, was added prior to cell lysis, and then cultures of LS4529, without the M2 epitope tag, and LS4536, carrying TolA-M2, were immunoprecipitated with anti-M2-coupled beads. Analysis of the purified products by Western blotting showed that TipN-GFP, FtsZ, and Pal interacted directly or indirectly with the TolA complex, while neither DivJ, PleC,

nor PopZ interacted with TolA-M2 (Fig. 4A). PleC was found not to interact with TolA-M2 (Fig. 4A), suggesting that the observed mislocalization of PleC in Pal and TolA mutants is likely an indirect effect. To obtain additional evidence for an interaction between TipN and components of the Tol-Pal complex, we immunoprecipitated TipN-M2 from a strain bearing

FIG. 4. The Tol-Pal complex interacts with TipN-GFP. (A) *In vivo* coimmunoprecipitation of TolA-M2 in strain LS4536 (*tipN-gfp* Δ *tolA* *P_{xylX}-tolA-m2*) and LS4529 (*tipN-gfp* Δ *tolA* *P_{xylX}-tolA*). Western blots of whole-cell extracts (lysate) and eluted samples were probed with the indicated antibodies (anti-M2 to probe TolA-M2 and anti-GFP to probe TipN-GFP, anti-Pal, anti-FtsZ, anti-DivJ, anti-PleC, and anti-PopZ). TipN, FtsZ, and Pal, but not DivJ, PleC, or PopZ, were observed to interact, directly or indirectly, with TolA-M2. (B) Coimmunoprecipitation of Pal with TipN-M2 in strain LS4538 (*P_{xylX}-tipN-m2*) and in wild-type cells. Western blots of eluted samples were probed with anti-M2 to probe TipN-M2, anti-Pal, and anti-PopZ. Pal but not PopZ was observed to interact with TipN-M2.

xylose-inducible *tipN-m2* at the chromosomal *xylX* locus (LS4538). Immunoblots of the immunoprecipitated samples were then probed with anti-Pal or anti-PopZ antibodies (Fig. 4B). A band corresponding to the size of Pal was detected in the immunoprecipitated TipN-M2 sample, while a control protein, PopZ, was not. These results, together with the localization data, support the conclusion that TipN directly or indirectly interacts with both TolA and Pal.

The localization dependencies described in this and the preceding section are diagrammed in Fig. 3D.

The Pal, TolA, and TolB components of the Tol-Pal complex are essential and are required for OM invagination during cell division. (i) **Phenotype of a Pal depletion strain.** It was not possible to disrupt the *pal* gene without complementing with a copy of the gene, suggesting that *pal* is essential in *Caulobacter*, as recently reported (2). We constructed a *pal* depletion strain, LS4524, by deleting the majority of the native *pal* coding region (amino acids [aa] 13 to 177) and placing a full-length *pal* gene under the control of a xylose-inducible promoter at the chromosomal *xylX* locus (see Fig. S2A in the supplemental material). Immunoblot analysis showed that the levels of Pal were reduced in Pal depletion strain LS4524 after 9 h of growth in the absence of xylose (see Fig. S2B), and this coincided with a decline in CFU.

Depletion of Pal by growth of strain LS4524 in PYEG for 10 h resulted in the accumulation of chains of cells (Fig. 5A, right), unlike strain LS4524 grown in the presence of xylose to induce *pal* expression (Fig. 5A, left), suggesting that Pal is required for the completion of cell separation. To deter-

mine the effect of Pal depletion on membrane invagination, LS4524 cells were stained with the lipophilic fluorescent styryl dye FM4-64 and examined by fluorescence microscopy. Strain LS4524 grown in the presence of xylose appeared similar to wild type (Fig. 5A, left), but those cells incubated with glucose to deplete Pal initially grew as chains and then exhibited extensive membrane blebs (blebbing phenotype, 88.6% [$n = 120$ cells]), predominantly at division sites and cell poles (Fig. 5A, right).

SEM images of cells depleted of Pal (grown in PYEG for 12 h) had large, irregular blebs both laterally and at the cell poles which were not observed in wild-type cells or the Pal depletion strain grown in the presence of the xylose inducer (Fig. 5B). In the absence of the xylose inducer, cell growth arrested and a portion of the cells lysed, while in the presence of inducer, both growth rate and cell morphology were indistinguishable from wild type (see Fig. S2B in the supplemental material).

To visualize the IM and OM in the presence and absence of Pal, plunge-frozen cells of the wild type and the Pal depletion strain LS4524 were observed by cryo-EM. A cryo-EM image of a dividing wild-type cell clearly showed the IM, peptidoglycan (PG), OM, and S-layer and the fissioned IM and PG layers that create two cellular compartments within an OM envelope (Fig. 5C, left). When strain LS4524 was grown in xylose to induce Pal, the constriction and the space between the IM and OM in the region of the division plane were indistinguishable from those of wild-type cells (data not shown). However, in the absence of Pal, OM invagination was disrupted and daughter cell separation did not reach completion, in many cases yielding chains of cells. In these cells, large OM membrane extrusions were observed at the division site (Fig. 5C, center). Notably, the OM was also abnormally separated from the IM at the cell poles (Fig. 5C, right). Cumulatively, these results demonstrate that *Caulobacter* Pal is required for the maintenance of OM-peptidoglycan integrity and for mediating invagination of the OM during cytokinesis.

(ii) **Phenotypes of TolA and TolB depletion strains.** We constructed a *tolA* depletion strain, LS4525, with a chromosomal deletion of *tolA* (see Fig. S3A in the supplemental material) by deleting the native *tolA* coding region (aa 13 to 259) and integrating a full-length *tolA* gene at the chromosomal *xylX* locus. A *tolB* depletion strain was constructed by incorporating a truncated version of *tolB* at the native chromosomal site and a second, full-length copy under the control of the xylose-inducible promoter, creating the strain LS4526 (see Fig. S3A). Strains LS4525 and LS4526 exhibited impaired cell separation and OM defects (see Fig. S3C and D) and remained viable only in the presence of the xylose inducer (see Fig. S3B). Thus, TolA and TolB are essential for viability. It was reported previously that genes encoding two other components of the *Caulobacter* Tol-Pal complex, *tolQ* and *tolR*, are essential (17).

Scanning electron microscopy of strains LS4525 and LS4526 incubated in the presence or absence of the xylose inducer showed that with the depletion of either TolA or TolB, envelope blebs appeared at the poles of both strains, and cells exhibited cell division and polar structural defects (Fig. 6A and B). Although cells depleted of TolB exhibited outer membrane blebs primarily at the site of cell division and the cell poles, cells depleted of TolA also exhibited extensive OM blebs at

FIG. 5. Phenotypes of Pal depletion strains. (A) To visualize cell morphology in cells depleted of Pal (right panels), compared to cells containing Pal (left panels), strain LS4524 was grown in PYEX to induce *pal* expression. Cells were then washed and grown in PYEG for 10 h to deplete Pal. Cells grown in either PYEX or PYEG were stained with FM4-64 and visualized by light microscopy. Bar, 2 μ m. When strain LS4524 was grown in PYEG for 10 h, the cells exhibited a late-stage cell division defect and polar blebs (white arrows). (B) Scanning EM images of the Pal depletion strain LS4524 incubated in the presence of either xylose or glucose for 12 h. Wild-type cells and strain LS4524 grown in the presence of xylose to induce expression of Pal were similar. However, in the absence of Pal, surface blebs were visible across the lateral cell surface, at the cell poles, and at the division plane (indicated by arrows). (C) A cryo-EM image of wild-type cells exhibiting a well-defined IM, PG layer, OM, and S-layer. Visualization of strain LS4524 after Pal depletion by growth in PYEG for 10 h showed aberrant OM structures at the cell pole and at the site of division.

lateral surfaces of the cell. Cryo-EM images of these strains, grown in the presence of glucose for 11 h to deplete TolA or TolB, exhibited disruptions of the OM (Fig. 6C and D). OM invagination was impaired, with large OM bulges visible at the cell division plane and also at the cell poles. Notably, depletion of either TolA or TolB caused the outer membrane to bleb outwards, while the cytoplasmic membrane remained intact. The absence of TolA induced more extensive lateral OM defects (over 82% of the cells [$n = 35$ cells]) than in either Pal or TolB depletion strains (Fig. 5C). Cumulatively, the phenotypes of strains depleted of either Pal, TolA, or TolB suggest that these proteins all participate in both cell division and polar membrane integrity.

The Tol-Pal complex attaches IM and OM to the peptidoglycan layer. Since the Tol-Pal complex in *E. coli* is known to span the periplasmic space (31), and *Caulobacter* depletion mutants of both TolA and Pal have large OM regions that are substan-

tially separated from the IM (Fig. 5 and 6), we used cryo-EM to observe the position of the peptidoglycan layer with respect to each membrane layer in both TolA and Pal depletion strains (Fig. 7). In the Pal depletion strain, the peptidoglycan layer (Fig. 7A, arrows) adhered solely to the IM over large areas of the OM blebs at the cell pole (13/14 cells examined). In contrast, in the TolA depletion strain, the peptidoglycan layer (Fig. 7B, arrows) adhered to the OM and did not attach to the IM over large areas at the cell pole in 35% of cells ($n = 20$ cells). The same phenomenon was observed for the peptidoglycan layer along the lateral cell surface (Fig. 7C and D).

DISCUSSION

The *Caulobacter crescentus* Tol-Pal complex is required for proper construction of the cell envelope and for completion of OM invagination in the late stage of cell division, as is the case

FIG. 6. Phenotype of TolA and TolB depletion strains. (A) SEM images of the TolA depletion strain LS4525 grown in the presence of either xylose or glucose for 12 h (to deplete TolA). (B) SEM images of the TolB depletion strain LS4526 grown for 12 h in the presence of either xylose or glucose (to deplete TolB). Depletion of either TolA or TolB yielded cells with extensive surface blebs. Bar (A and B), 1 μ m. (C) Cryo-EM images of strain LS4525 grown for 11 h in glucose to deplete TolA, showing significant OM disruptions both laterally and at the cell poles (arrows). (D) Cryo-EM images of strain LS4526 grown for 11 h in glucose to deplete TolB show OM defects at both the cell poles and the division plane (arrows). Bar (C and D), 100 nm.

FIG. 7. Structural relationship of the peptidoglycan layer and the IM and OM in TolA and Pal depletion strains. (A) Cryo-EM of strain LS4524 after Pal depletion by growth in PYEG for 10 h showed an OM polar bleb with the peptidoglycan layer (arrows) dissociated from the OM but adhering to the IM. (B) Cryo-EM of strain LS4525 grown for 11 h in glucose to deplete TolA showed an OM polar bleb with the peptidoglycan layer (arrows) adhering to the OM but separated from the IM. (C) Lateral cell envelope of strain LS4524 depleted of Pal with the OM separated from the peptidoglycan layer (arrows). (D) Lateral cell envelope of strain LS4525 depleted of TolA, with the peptidoglycan layer separated from the IM.

in *E. coli* (19). Unlike *E. coli*, however, the *Caulobacter* Tol-Pal complex is essential for viability. Although the Tol-Pal complex is not essential in *E. coli*, it contributes to the invagination of the OM during cell division and is localized to the division site (19), and it also helps to maintain the integrity of the cell wall by connecting the OM and the peptidoglycan network (7). *E. coli* primarily employs Lpp, a lipoprotein, to maintain the cell envelope integrity via generally distributed linkages between the OM and peptidoglycan layer (49). *Caulobacter* does not have a Lpp homolog. Instead, we suggest that *Caulobacter* uses Tol-Pal to maintain cell envelope integrity, performing a function analogous to Lpp in *E. coli*. In support of this, we observed that all of the fusion proteins of the Tol-Pal complex localized to the division site and a significant fraction accumulated throughout the cell envelope (Fig. 2A). The overproduction of Pal or TolA in *E. coli* complements the OM integrity defect of an *lpp* mutant strain (7), suggesting that Pal and TolA play an analogous though less important role than Lpp in *E. coli*. In *Caulobacter*, both the TolA and Pal proteins are required to maintain the subcellular location of the TipN polar marker (25, 29), which plays a critical function in cell asymmetry and polar development.

TolA and Pal accumulation at the incipient division site is dependent on FtsZ assembly. The TipN polar localization protein and the TolA, Pal, TolB, TolR, and TolQ components of the Tol-Pal complex (Fig. 2) localize to the cell division plane

and then remain at the new poles upon completion of cell division. TolA localizes to the division plane well after the accumulation of the FtsZ protein at the incipient cell division site, and TolA is dependent on FtsZ for its positioning at that site. We found that the recruitment of FtsA to the division plane followed arrival of TolA and, as expected, the recruitment of TolA, and all the other components of the Tol-Pal complex, to the division plane was independent of FtsA. The finding parallels that of the FtsZ-dependent and FtsA-independent mid-cell localization of DipM, which is a putative peptidoglycan endopeptidase required for peptidoglycan remodeling during cell division (23, 38, 41). We found, however, that the recruitment of Pal to the division plane required TolA (Fig. 2E), as is the case in *E. coli* (19), while the recruitment of the other components of the Tol-Pal complex was independent of both TolA and Pal. Thus, the mid-cell localization of the TolA and Pal components of the Tol-Pal complex depends on the formation of the Z-ring, as does the mid-cell positioning of the TipN protein (25, 29).

The subcellular positioning of TipN is dependent on, and TipN forms a complex with, the TolA and Pal proteins. In wild-type cells, TipN, a polar protein with two transmembrane domains and a short periplasmic domain, transiently localizes to the division plane significantly later than the appearance of TolA at mid-cell. TipN is then maintained at the new cell poles following cell separation (25, 29), at the same position as polar

TolA and Pal. We found that TipN mislocalized in both Pal and TolA depletion strains. Although the retention of TipN at the division plane and at the cell poles required both the TolA and Pal proteins, it is possible that cell envelope defects induced by inactivation of the Tol-Pal complex disrupt the interaction between TipN and a possible unknown positioning factor. However, we showed that TipN interacts, directly or indirectly, with both TolA and Pal in coimmunoprecipitation assays (Fig. 4), suggesting that complex formation with TolA and Pal contributes to the subcellular localization of TipN. Moreover, it was recently reported that FtsN can interact with both TolR and TipN in a bacterial adenylate cyclase two-hybrid assay (38). Our observation that the TipN polar landmark protein appears to interact with Pal in the outer membrane, and that it also associates with the TolA inner membrane protein, suggests *trans*-envelope subcellular localization of the TolA/Pal/TipN complex at the cell pole.

The PleC histidine kinase was also partially mislocalized in cells depleted of TolA and Pal, but it was not found to be part of the TolA complex, indicating that the mislocalization of PleC is probably a downstream effect of TipN mislocalization. Notably, other than the PleC, whose polar positioning is dependent on TipN (29), the polar localization of other polar transmembrane proteins, including DivJ and PopZ, was unaffected by depletion of the TolA or Pal proteins. We cannot exclude the possibility that mislocalization of PleC in TolA/Pal depletion depends on PodJ, since PodJ is also required for the polar localization of PleC. Cumulatively, these observations suggest the localization dependency pathway shown in Fig. 3D.

The Tol-Pal complex is necessary for OM-peptidoglycan integrity. In TolA, TolB, and Pal depletion strains, the OM extrudes outward to form widespread blebs (Fig. 5C and 6C and D), suggesting that these OM blebs probably formed due to a deficiency of Tol-Pal connections between the OM and the peptidoglycan layer in the mutant strains. Interestingly, depletion of *dipM* causes peptidoglycan thickening and the formation of cell surface blebs similar to that seen in Tol-Pal depletion strains (23). The physical contact between TolA and Pal may be disrupted in DipM-depleted cells due to peptidoglycan thickening.

Cryo-EM images revealed that in cells depleted of TolA, the peptidoglycan layer sometimes failed to attach to the IM while adhering to the OM (Fig. 7B and D). Similar disruptions in envelope organization were not detected in either Pal or TolB depletion strains. Instead, the peptidoglycan layer adhered only to the IM in the blebs of those mutant strains (Fig. 7A and C). The depletion of *tolA* also caused the formation of OM blebs along the cell sidewalls. In accordance with phenotypes exhibited by *E. coli* TolA mutants (35), the absence of TolA in *Caulobacter* induced more extensive OM defects than in either *pal* or *tolB* mutant strains. Thus, these observations suggest TolA plays the larger role in maintaining general cell envelope integrity.

In wild-type *Caulobacter*, there is a short stretch of unsupported OM that maintains its integrity and withstands internal pressure during the late stages of cell division when the IM and peptidoglycan layer are constricting and ultimately under fission to form two cell compartments before completion of OM invagination (Fig. 5C, WT cell) (27). In both Pal and TolB depletion strains, the OM-peptidoglycan integrity is disrupted, predominately at newly synthesized areas of the cell wall, such as the division plane and recently created poles. Thus, the OM

in these areas appears to be poorly anchored, resulting in localized OM bulging (Fig. 5C, middle). Newly synthesized Pal is inserted at the site where new peptidoglycan is incorporated (2), consistent with the Tol-Pal complex playing a vital role in OM membrane integrity by mediating OM-peptidoglycan contacts during growth and division.

Owing to the temporal separation of the constriction of the IM-peptidoglycan and the OM in *Caulobacter*, there is a physical tension between the OM and the peptidoglycan layer as the IM-peptidoglycan invagination approaches the terminal stage of fission. Upon fission of the IM and peptidoglycan layer, the OM is left unsupported, as shown in Fig. 5C (WT cell). The final stages of IM fission leading to cell compartmentalization probably occur extremely rapidly (27). Thereafter, over a period of about 20 min, the periplasmic gap between the IM and the peptidoglycan layer is closed by progressive attachment of the OM to the peptidoglycan layer, presumably by action of Tol-Pal complex molecules, at the point of OM and peptidoglycan layer divergence, so that the region of untethered OM becomes smaller and smaller (27). At some point, when the unattached region is small enough that the OM curvature reaches a critical point, the OM also undergoes fission and reforms over the new cell poles of the daughter cells. In Pal depletion strains, the region of OM-peptidoglycan disconnection appears to be significantly larger and the subsequent reconnection of OM and peptidoglycan fails to occur, leading to the blown-out sections shown in Fig. 5C (middle). Some of the cell poles in the Pal depletion strain exhibit the peptidoglycan layer adhered to the IM and the OM blown out in a large bleb-like structure, unable to make contact with the PG and IM (Fig. 7A). These cells are probably those that completed cell division earlier in the depletion process when there was still a partially functional Tol-Pal complex.

The fitness advantage to *Caulobacter* of separating the time of IM fission from final OM constriction and cell separation is a matter of conjecture. In the extended time interval when the cell is compartmentalized, but not separated, free diffusion can occur through the continuous periplasmic space surrounding both compartments. The stalk acts to collect phosphorus efficiently in low-phosphate environments (24, 47), so a possible fitness benefit is that this cell division method provides more reliable development of the nascent swarmer daughter cell under low environmental phosphate situations. A time interval between compartmentalization and cell separation may also ensure the assembly of the chemotaxis control system before the swarmer cell is physically separated from the stalked cell. The delayed completion of OM constriction would be a necessary feature of late cell division in this scenario.

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