1 **Title:** Genetic interaction mapping highlights key roles of the Tol-Pal complex

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Abstract

The conserved Tol-Pal trans-envelope complex is important for outer membrane (OM) stability and cell division in Gram-negative bacteria. It has been proposed to mediate OM constriction during cell division via tethering to the cell wall. Yet, recent studies suggest that the complex has additional roles in OM lipid homeostasis and septal cell wall separation. How the Tol-Pal complex functions to facilitate these many processes is unclear. To gain insights into its role(s), we applied transposon insertion sequencing, and report here a detailed network of genetic interactions with the *tol-pal* locus in *Escherichia coli*. We found one positive and >20 negative strong interactions based on fitness. Disruption of genes responsible for osmoregulated periplasmic glucan biosynthesis restores fitness and OM barrier function, but not cell division defects, in *tol-pal* mutants. In contrast, deletions of genes involved in OM homeostasis and cell wall remodelling give rise to synthetic growth defects in strains lacking Tol-Pal, especially exacerbating OM barrier and/or cell division defects. Notably, the $\Delta tolA$ mutant having additional defects in OM protein assembly ($\Delta bamB$) exhibited severe division phenotypes, even under conditions where the single mutants divide normally; this highlights the possibility for OM phenotypes to indirectly influence the cell division process. Overall, our work provides insights into the intricate nature of Tol-Pal function, and reinforces the model that this complex plays crucial roles in cell wall-OM tethering, cell wall remodelling, and in particular, OM homeostasis.

Introduction

The complex Gram-negative bacterial cell envelope comprises the inner membrane (IM), the cell wall, and the outer membrane (OM) [1]. For proper growth and division, the synthesis and expansion of all three layers have to be tightly regulated and coordinated. In particular, the biosynthesis and maintenance of the cell wall and OM, being physically separated from the cytoplasm, require more specialized mechanisms. The cell wall or peptidoglycan polymer is synthesized in the periplasm by SEDS (shape, elongation, division and sporulation) proteins and PBPs (penicillin binding proteins), and is cleaved by hydrolases to facilitate remodeling during cell growth and division [2]. To build the OM, dedicated protein systems including the Lpt bridge, the Bam machine, and the Lol pathway are required to transport and assemble lipopolysaccharides (LPS), OM β -barrel proteins, and OM lipoproteins, respectively [3-5]. How bulk phospholipids (PLs) are trafficked between the membranes is less well understood [6]. In *Escherichia coli*, the cell wall and OM are also tethered covalently by lipoprotein Lpp, and non-covalently by lipoprotein Pal and β -barrel protein OmpA, for stability and function [7]. It remains unclear how cell wall and OM assembly and homeostasis are coordinated during growth and division.

The Tol-Pal complex is a conserved trans-envelope system that appears to be involved in both cell wall and OM homeostasis [8-10]. It consists of an IM complex containing TolQ, TolR, and TolA and an OM complex composed of periplasmic protein TolB and Pal. The TolQRA complex is energized by the IM proton motive force (pmf), and interacts with TolB, and possibly Pal, via the periplasmic domain of TolA [11-14]. Cells lacking the Tol-Pal complex exhibit pleiotropic phenotypes [12, 14]. Specifically, these strains exhibit hypersensitivity towards antibiotics and leak periplasmic contents, indicative of a defective OM. The same cells also bleb OM vesicles primarily from the septum, pointing at a possible cell division defect. Consistently, *tol-pal* mutants are known to form undivided chains, but only under osmotic stress, and are particularly sensitive to such stress at high temperatures [10, 15]. The complex nature of these phenotypes has made it difficult to assign Tol-Pal function. Nevertheless, it has been shown that the Tol proteins localize to the septum during cell division, and are required for Pal to accumulate at the cell division site – this enrichment of septal cell wall-OM tethering is believed to be important for coordinated OM invagination, and may account for

the OM hypervesiculation phenotype [15-17]. Recently, Tol-Pal was also suggested to modulate cell wall remodeling; chained mutant cells at low osmolarity have unseparated peptidoglycan sacculus, and overexpressing certain cell wall hydrolases can suppress this phenotype [10]. Even so, OM defects are still present in these suppressors, as is the case when cells are not osmotically stressed. Importantly, it has been demonstrated that mutants lacking an intact Tol-Pal complex accumulate excess PLs at the OM and display slower retrograde PL transport, potentially accounting for the origin of the unstable/defective OM under all growth conditions [8, 18]. Taken together, the Tol-Pal complex is seemingly involved in a plethora of diverse processes: peptidoglycan-OM tethering, cell wall remodeling, and retrograde PL transport.

The function(s) of the Tol-Pal complex remains elusive. It is believed that TolQRA dynamically controls TolB-Pal interaction to facilitate Pal binding to septal cell wall [9], but how the complex affects cell wall remodeling and PL trafficking is not clear. One possibility is that peptidoglycan-OM tethering is the main function of the Tol-Pal complex, loss of which somehow gives rise to problems in the latter two processes. However, it is known that deletion of *pal* yields milder phenotypes relative to the deletion of other *tol* genes [10], implying that the function(s) of Tol-Pal might not be centered around the ability of Pal to bind peptidoglycan but instead depends largely on TolQRA and/or TolB. The Tol proteins may possibly rely on additional interacting partner(s), or be somewhat sufficient, to facilitate cell wall remodeling and PL trafficking processes. It is conceivable that these processes are independent functions of the Tol complex. It is also possible that disruption of one process indirectly results in the other. Notably, preventing cell chaining of *tol* mutants does not alleviate OM permeability defects [10]; therefore, OM instability, likely due to defects in retrograde PL trafficking, is not a downstream effect of problems in cell wall remodeling. The molecular function(s) of the Tol-Pal complex, and the relationships between the existing phenotypes, remain to be clarified.

In this study, we adopted an unbiased genetic approach to gain additional insights into the function(s) of the Tol-Pal complex. Using high-throughput transposon insertion sequencing [19], we mapped and validated positive and negative genetic interactions, based on fitness, with the *tol-pal* locus in *E. coli*. We found only one strong positive interaction; abolishing biosynthesis of osmo-regulated periplasmic glucans (OPGs) improves fitness and OM barrier function in the $\Delta tolA$ mutant,

but does not rescue its cell division defects. In contrast, we identified >20 negative genetic interactions with *tolQ* and *tolA*. These genes are primarily involved in cell wall and OM processes, consistent with the fact that the Tol-Pal complex plays crucial roles in cell wall remodeling and OM lipid homeostasis. Interestingly, we noted that additional OM defects that are not known to impact cell division can exacerbate the division defects in $\Delta tolA$ strains, suggesting it is possible for OM phenotypes to indirectly affect cell division. Notwithstanding the complex relationships between various *tol-pal* effects in cell wall-OM tethering, cell wall remodeling and lipid homeostasis, our work reinforces the idea that the Tol-Pal complex may play a primary role in OM structure and function.

Results

***En masse* transposon directed insertion sequencing (TraDIS) reveals extensive genetic interactions with *tol-pal*.**

Transposon insertion site mapping, using methods such as Tn-seq or TraDIS, have been widely applied to study essentiality and fitness impact of genes in different organisms [20, 21]. This technique has also been extended to investigate synthetic interactions between genes via comparative analysis of transposon insertion site profiles in different strain backgrounds [22, 23]. To clarify its physiological role(s), we applied TraDIS to identify all possible genetic interactions with the *tol-pal* locus. Any gene with increased or decreased frequency of transposon insertion in the *tol-pal* but not the WT background would indicate positive or negative genetic interaction with the locus, respectively (Fig. 1A). We generated separate Tn5 transposon mutant libraries in *E. coli* WT, $\Delta tolQ$, and $\Delta tolA$ strains. Each library contains approximately one million Tn5 mutants, as estimated from the total numbers of colonies obtained from transposition. Three replicate cultures were grown from each library in LB media, and genomic DNA were isolated and prepared for sequencing using an optimized TraDIS protocol adapted from Langridge et al. [24]. From a single multiplexed sequencing run (MiSeq) of the nine samples, a total of ~6.7 million Tn5 junction-containing reads were recovered, with ~1.9, ~2.9, and ~1.7 million combined reads from the WT, $\Delta tolQ$, and $\Delta tolA$ triplicates, respectively (Fig. 1B; Table S1). Alignment of sequences adjacent to the insertion junctions allowed

pinpointing of the exact insertion sites on the genome. We identified at least 430,000 unique insertion sites in each background, with one insertion every 9-11 bp on average, indicating that our Tn5 libraries have extensive coverage of all possible non-essential genes (Figs. 1B, S1A).

To identify genes that exhibited strong interactions, we compared the total number of reads for all insertions in each gene in the $\Delta to/Q/A$ background to that in WT. Genes that contained less than ten insertion reads in both WT and mutant backgrounds were removed from analysis. In either the $\Delta to/Q:WT$ or $\Delta to/A:WT$ comparison, a number of genes displayed statistically significant (FDR corrected P-value $<10^{-5}$) $\log_2$ fold change ($\log_2FC$) value of >2.5 in either direction (Figs. S1B, C; Tables S2, S3), indicative of strong genetic interactions with *tolQ* or *tolA*. In fact, the comparative profiles were well correlated (Fig. 1C; $R^2 = 0.76$ for 3912 genes), suggesting that most genetic interactions are common, and therefore specific to overall Tol-Pal function(s). We noted that *tolR/A/B* and *tolQ/R/B* were among the genes with strongest enrichment of transposon insertion reads in $\Delta to/Q$ and $\Delta to/A$ backgrounds, respectively, relative to WT (Figs. S1B-D). This is expected given that these genes are from the same pathway, thus disruption would only affect WT but no longer have additional deleterious effects in $\Delta to/Q/A$ backgrounds. Intriguingly, insertion frequency in *pal* remained unchanged in either $\Delta to/Q$ or $\Delta to/A$ relative to WT ($\log_2FC$ of -0.08 or +0.3, respectively; statistically insignificant, Figs. S1B-D), perhaps suggesting less major contribution to overall function of the complex. Using the stringent cutoff ($|\log_2FC| > 2.5$), our TraDIS screen uncovered 2 other genes showing strong positive genetic interactions with both *tolQ* and *tolA*, and 25 genes exhibiting common negative interactions.

Disrupting biosynthesis of osmoregulated periplasmic glucans results in positive genetic interactions with *tol-pal*.

Transposon insertions in *mdoH* (*opgH*) and *mdoG* (*opgG*), two genes necessary for the biosynthesis of osmoregulated periplasmic glucans (OPGs) [25], were strongly enriched in both $\Delta to/Q$ and $\Delta to/A$ backgrounds (Figs. 1C, 2A), suggesting improved fitness. A third gene involved in but not required for OPG biosynthesis, *mdoD* (*opgD*), also exhibited positive interactions with *tolQ/A*, albeit with lower fold-enrichment in insertion frequency (Table S4). Interestingly, we have previously found

that mutations in *mdoH* may contribute to suppression of vancomycin sensitivity in *tol-pal* mutants [26]. We proceeded to validate this interaction by examining both growth and vancomycin sensitivity of a $\Delta tolA$ strain with *mdoH* deleted. The $\Delta tolA \Delta mdoH$ double mutant indeed grew better (as judged by optical density measurements), and had restored vancomycin resistance compared to the $\Delta tolA$ strain (Figs. 2B, C). Mutants lacking the Tol-Pal complex also exhibit cell division defects; however, $\Delta mdoH$ did not rescue the cell chaining nor the plating defects (at 37°C) observed in the $\Delta tolA$ strain under low osmolarity conditions (Figs. 2C, D). MdoH has a moonlighting function in growth rate regulation via sequestration of FtsZ by its N-terminal domain [27]; we showed that expression of the N-terminal domain *in trans* did not reverse the suppression (Fig. S2), suggesting that the positive genetic interaction observed was unrelated to FtsZ sequestration. We conclude that removing OPGs from the periplasm somehow confers better growth and OM barrier function in mutants lacking Tol-Pal.

Genes exhibiting negative interactions with *tol-pal* are important for outer membrane or cell wall integrity.

25 genes contained significantly fewer insertions in both $\Delta tolQ$ and $\Delta tolA$ background relative to WT, indicative of negative genetic interactions (Fig. 1C). Among these are several genes that are known to exhibit synthetic phenotypes with *tol-pal* (*bamB*, *degP*, *lpoA*, *lpoB*, *mrcA* (*PBP1A*), *mrcB* (*PBP1B*), *ompA*, *surA*) [28-30], validating our overall TraDIS approach. Generally, $\Delta tolQ$ and $\Delta tolA$ mutants appeared less tolerant of disruption in genes involved in two major processes, namely cell wall biosynthesis/remodeling and OM assembly/integrity (Table 1). For cell wall biosynthesis/remodeling, we specifically found genes involved in peptidoglycan synthesis (*dapF*, *lpoA*, *mrcA*, *lpoB*, *mrcB*), remodeling (*nlpl*, *prc*, *tatC*), recycling (*ldcA*), and undecaprenyl lipid carrier sequestration (*qseC*, *wecB*, *wecC*, *wecD*, *wecE*, *wecF*, *wzx* [31, 32]); for OM assembly/integrity, genes involved in LPS synthesis/modification (*gtrS*, *waaP*, *waaG*), β -barrel protein assembly (*bamB*, *degP*, *surA*), and OM-cell wall tethering (*ompA*) exhibited negative interactions with *tol-pal*. We successfully constructed clean deletions of each gene in the $\Delta tolA$ background, with the exception of *qseC*, where a depletion strain was constructed (Fig. S3). We demonstrated that most of these double

mutants displayed synthetic growth defects when grown in LB liquid media at 37°C (Figs. S3, S4) and many exhibited reduced plating efficiencies on M9 minimal (37°C) or LB (30°C, 37°C, 42°C) media (Figs. S3, S5). Based on the growth phenotypes, the more severe negative interactions were further characterized below.

Mutants lacking Tol-Pal are sensitive to defects in cell wall remodeling.

Among the genes important for cell wall integrity, *nlpl*, *prc*, and *tatC* exhibited the most severe negative interactions with *tol-pal* (Figs. S4, S5, 3A-C). The OM lipoprotein Nlpl physically regulates the tail-specific protease Prc to primarily degrade MepS, a peptidoglycan endopeptidase important for cell wall expansion [33]. Disruption of *nlpl* or *prc* in the $\Delta tolA$ background resulted in severe growth defects in liquid cultures (Fig. 4A). We noticed that the overnight stationary phase cultures used as inoculums had 10- to 100-fold fewer colony-forming unit (CFU) per OD, indicating that the double mutants lost viability during late stationary phase (Fig. 4B, C). Consistent with this idea, microscopic images of these cells revealed extensive amounts of cell debris, as well as morphological defects, only at late stationary phase (Fig. 5), corresponding to when the proteolytic effect of Nlpl/Prc on MepS is most crucial [33]. Incidentally, we observed slight increases in the frequency of Tn5 insertion in *mepS* in the $\Delta tolQ$ and $\Delta tolA$ backgrounds relative to WT ($\log_2FC$ of +1.16 or +1.07, respectively; Tables S2, S3), suggesting that MepS activity could be unfavorable in strains lacking Tol-Pal. The synthetic defects with $\Delta tolA$ observed here were likely due to problems related to elevated MepS activity in the absence of Nlpl and Prc.

TatC is part of the twin-arginine translocation (Tat) system important for the secretion of folded-proteins containing Tat-specific signal peptides [34, 35]. Deletion of *tatC* is known to result in cell chaining and envelope defects, largely due to impaired translocation of cell wall amidases AmiA and AmiC [34]. Due to the chaining phenotype, the $\Delta tatC$ single mutant had ~10-fold fewer CFU per OD compared to WT, despite similar growth profiles (Figs. 4D, E). The $\Delta tolA \Delta tatC$ double mutant grew drastically slower than either single mutant in LB media, and formed much longer unseparated chains, consistent with a strong negative genetic interaction (Fig. 5). The severe cell division defects likely account for why the double mutant was unable to grow on low osmolarity media at 30°C, a

condition where the single mutants can survive (Fig. 4F). We also observed that the $\Delta tolA \Delta tatC$ strain exhibited hypersensitivity towards vancomycin (Fig. 4F). TatC functions with TatB, TatA and/or TatE to transport substrates; these three proteins are structurally homologous and have partially overlapping roles [35, 36], especially TatA and TatE, possibly explaining why we did not observe negative interactions between these genes with *tolQ/A*. Given that mutants lacking the Tol-Pal complex already exhibit problems in OM invagination and/or septal cell wall separation [10, 15], it makes sense that removing TatC further exacerbates defects in cell wall remodeling during cell division, thus giving rise to the synthetic interaction.

***tol-pal* mutants cannot withstand additional OM perturbation.**

Mutants lacking functional Tol-Pal complex are known to exhibit severe OM defects likely due to lipid dyshomeostasis [8]. It appears that further disruption of the OM, particularly defects in OMP assembly, LPS synthesis, and OM-cell wall tethering, are not well-tolerated in $\Delta tolQ$ and $\Delta tolA$ strains (Fig. 3D-G). The BAM machine comprises essential β -barrel protein BamA and lipoprotein BamD, and three non-essential lipoproteins BamB, BamC and BamE [3]. We only observed strong negative interactions between *tolQ/A* and *bamB*, but not *bamC/E*, likely because removing either of the latter genes has relatively weaker impact on OMP assembly [37, 38]. Removing BamB resulted in severe growth defects in the $\Delta tolA$ background in both liquid culture and solid media (Figs. 6A, C); similar synthetic sick phenotypes were observed in $\Delta pal \Delta bamB$ mutant previously [30]. Despite increasing optical density, CFUs recoverable from the liquid culture of the $\Delta tolA \Delta bamB$ mutant were extremely low and did not increase over time (Fig. 6B). While the cellular morphology of $\Delta tolA$ and $\Delta bamB$ single mutants are largely similar to that of WT in LB media, we found that the $\Delta tolA \Delta bamB$ double mutant exhibited severe chaining phenotype (Fig 7), which may explain the disconnect between CFU and OD. This observation is somewhat surprising given that defects in OMP assembly are not known to impact cell division.

In addition to *bamB*, both *surA* and *degP*, which encode for the main OMP periplasmic chaperone and protease [3], respectively, also exhibited negative genetic interactions with *tol-pal* (Fig. 1C). We showed that the $\Delta tolA \Delta surA$ double mutant displayed a growth defect in liquid LB media at

37°C, and a severe plating defect on solid media at 30°C (Figs. 6A, C). Removing SurA did not further sensitize the $\Delta tolA$ strain towards SDS EDTA but did increase vancomycin sensitivity (Fig. S6A). Interestingly, the $\Delta tolA \Delta surA$ double mutant was somehow more vancomycin-resistant than the $\Delta surA$ mutant alone. The $\Delta tolA \Delta degP$ double mutant also displayed a growth defect in liquid LB media at 37°C (Fig. 6A); however, as previously reported, this strain could not grow on plate at 37°C (Fig. 6C; [28]). At the permissive temperature of 30°C, the $\Delta tolA \Delta degP$ double mutant was more sensitive to SDS EDTA, but not vancomycin, when compared to the $\Delta tolA$ strain (Fig. S6B). Actively growing $\Delta tolA \Delta surA$ and $\Delta tolA \Delta degP$ cells also exhibited chaining phenotypes, albeit milder than observed in the $\Delta tolA \Delta bamB$ strain (Fig. 7). Taken together, while the synthetic interactions uncovered are necessarily complex, our data indicate a clear requirement for proper OMP biogenesis and homeostasis in strains lacking Tol-Pal.

Defects in the LPS biosynthesis pathway also appear to exhibit synthetic phenotypes with *tol-pal*. Specifically, disruption of *waaG* and *waaP* resulted in slightly slower growth and exacerbated OM permeability defects, particularly vancomycin sensitivity, in the $\Delta tolA$ strain (Fig. S7A). The $\Delta tolA \Delta waaG$ mutant especially exhibited cell morphological changes in logarithmic and stationary growth phases (Figs. S7B, C). WaaG is responsible for the installation of the first glucose in the outer core of LPS [39]; $\Delta waaG$ mutants have truncated LPS structure, and thus a deep rough phenotype. WaaP is a kinase that phosphorylates the first heptose residue in the LPS, absence of which affects other phosphorylation and glycosylation events in the inner core [39]. Other genes involved in LPS core biosynthesis (*waaB*, *waaO*, *waaQ*) also had reduced Tn5 insertions in both $\Delta tolQ$ and $\Delta tolA$ background, although not meeting our initial threshold of $\log_2 FC < -2.5$ (Table S2, S3). Therefore, the OM of *tol-pal* mutants also cannot tolerate additional perturbations to LPS core structure that may affect LPS packing in the outer leaflet.

Loss of OmpA, the abundant OMP that non-covalently tethers OM to peptidoglycan [40], caused one of the worst growth defects in mutants lacking Tol-Pal (Figs. 1, S4). The $\Delta tolA \Delta ompA$ mutant grew much slower in liquid culture (Fig. 6D) and displayed lowered plating efficiency across all growth phases (Fig. 6E); these phenotypes are likely due to the gross morphological defects observed in this strain (Fig. 7). Interestingly, removing Lpp, which links the OM and cell wall covalently

[7], has no impact on growth of $\Delta tolQ/A$ strains in our TraDIS experiment (Table S2, S3), suggesting that the observed synthetic interactions were not due to general loss of OM-peptidoglycan tethering. Deletion of *ompA* causes general OM defects in the forms of reduced stiffness and increased sensitivity to antibiotics [41, 42]. We infer that *tol-pal* mutants could not tolerate disturbance to OM stability due to specific loss of OmpA (see discussion).

Discussion

The specific function(s) of the Tol-Pal complex has been difficult to assign [8, 10, 15]. Here, using TraDIS, we have mapped the complex genetic interaction network with the *tol-pal* locus (Fig. 1). We showed that in *tol-pal* mutants, disrupting biosynthesis of OPGs ($\Delta mdoG/H$) resulted in the only positive interaction (based on stringent cutoff of $\log_2 FC > 2.5$), where fitness and OM barrier, but not division defects, were largely restored (Fig. 2). In contrast, we observed multiple negative interactions with *tol-pal* almost exclusively due to further defects in either the cell wall or OM (Table 1; Figs 4-7); $\Delta tolA$ strains exhibited strong synthetic defects especially when combined with mutations in cell wall remodelling ($\Delta nlpI$, Δprc , and $\Delta tatC$) or OM assembly ($\Delta bamB$, $\Delta degP$, $\Delta surA$, $\Delta waaG$, and $\Delta waaP$) pathways. Overall, our results are consistent with the idea that the Tol-Pal complex plays important roles in OM homeostasis and cell wall remodelling, in addition to OM-peptidoglycan tethering.

An interesting observation in our TraDIS dataset is that transposon insertions in *pal* were not enriched in the $\Delta tolQ/A$ backgrounds relative to WT (Figs. S1B-D). Intuitively, deleting any additional gene in the *tol-pal* locus should be neutral in $\Delta tolQ/A$ mutants but has a strong detrimental effect in the WT strain. This is true for the other *tol* genes, as judged by the increase in insertion frequencies in these genes in the $\Delta tolQ/A$ strains over WT, but clearly not the case for *pal*. Therefore, it appears that deletion of *pal* does not impact the fitness of the WT strain in the same way as removing other *tol* genes. This is consistent with the milder phenotypes Δpal strains have displayed [10]. Consequently, septal peptidoglycan-OM tethering offered by Pal may be peripheral to the main function of the TolQRA-TolB system.

Besides the *tol* genes, it is intriguing that *mdoG/H* were the only other genes that exhibited strong positive interactions with $\Delta tol/Q/A$. Removing MdoH restored fitness and OM barrier function of mutants lacking Tol-Pal, but how the resulting loss of OPGs suppresses these phenotypes remains elusive. Nevertheless, it is known that removal of OPGs improves OM barrier function in general [26, 43, 44], suggesting that the suppression effect might not be specific to the *tol-pal* locus, and therefore less related to its direct function. At a less stringent cutoff ($\log_2FC > 1.25$), additional positive genetic interactions with both $\Delta tol/Q$ and $\Delta tol/A$ are revealed (Table S4). While we did not go on to validate these TraDIS interactions, we note that higher insertion frequencies were observed in genes involved in cell division (*minC*, *minD*, *minE*), lipid homeostasis (*cdh*, *fadR*, *kdsC*), cell wall homeostasis (*ddlB*, *elyC*, *ldtB*) and others (e.g., *wzzE*, *yhdP*). Additional characterization of these putative interactions would be warranted to understand their connections to the Tol-Pal complex.

In an earlier study, we have demonstrated that accumulation of intermediates in the enterobacterial common antigen (ECA) pathway is linked to restoration of OM barrier function in strains lacking Tol-Pal [26]. The fact that Tn5 insertions within *wzzE* were enriched in $\Delta tol/Q$ and $\Delta tol/A$ backgrounds ($\log_2FC$ of +1.68 and +1.73, respectively; Fig. 3H) is consistent with our observation that deletion of *wzzE* restores OM barrier function in the $\Delta tol/A$ strain. However, several ECA biosynthesis genes that restored OM barrier function when deleted (*wecB*, *wecC*, *wecE*, *wecF*) were found to exhibit strong negative interactions with *tol/Q/A* ($\log_2FC < -2.5$). This is not unexpected given that the build-up of ECA-related intermediates is known to result in cell wall defects via the sequestration of undecaprenyl-phosphate (und-P) [32], the lipid carrier also used in peptidoglycan biosynthesis; indeed, *wec tol* double mutants do display more severe morphological defects than the corresponding single mutants [26]. One of the strongest negative genetic interaction with *tol/Q* and *tol/A* is *qseC*, encoding the sensor kinase of the QseBC two-component system, that has recently been discovered to be important for cell wall homeostasis, especially when und-P pool is limited [31]. Interestingly, the same genetic screen for mutants sensitive to a limited und-P pool also identified *wec* and *tol-pal* genes, overall suggesting that *tol-pal* strains are sensitive to cell wall defects linked to und-P sequestration.

The observed negative interaction between $\Delta ompA$ and $\Delta tolQ/A$ is particularly strong. Since TolQRA/B contributes to localization of Pal to the septal cell wall, this genetic interaction suggests some level of redundancy between Pal and OmpA, which contain homologous peptidoglycan binding domains [40]. Consistently, synthetic interactions between Δpal and $\Delta ompA$ has previously been reported [30]. However, it is not clear how OmpA can contribute during cell division. While TolB has been shown to bind OmpA [45], the latter is not freely diffusible in the OM to facilitate recruitment to the cell septum [46]. An alternative reasoning is that cells cannot tolerate the simultaneous loss of OM-cell wall tethering at the cell septum and around the cell, respectively. Yet, in our $\Delta tolA \Delta ompA$ strains, Pal and Lpp are still present, and therefore should still be able to contribute to OM-cell wall tethering in the cell periphery in place of OmpA. We also did not observe any synthetic interactions between $tolQ/A$ and lpp ; the requirement of OmpA in tol strains is therefore puzzling. We posit that the loss of OmpA gives rise to specific OM defects that are not well tolerated in strains with existing OM lipid dyshomeostasis.

Clearly, $tolQ/A$ mutants could not tolerate deletion of genes that affect peptidoglycan biosynthesis and remodelling, or OM structure and assembly. The simplest explanation for the interactions with cell wall processes is that mutants lacking functional Tol-Pal already have cell septum separation defects during cell division, albeit only under conditions of low osmolarity. Likewise, the synthetic phenotypes with OM defective mutants are possibly due to existing OM lipid dyshomeostasis in $tol-pal$ strains. Notably, one of the strongest interactions we have observed was with OMP assembly, specifically $\Delta bamB$. The severe growth defects detected in the double $\Delta tolA \Delta bamB$ mutant are likely due to a combination of OM lipid imbalance ($\Delta tolA$), and OMP assembly defects ($\Delta bamB$). Like $\Delta tolA$, $\Delta bamB$ mutant also contains excess PLs in the OM [8], indicating the double mutant has exacerbated OM lipid dyshomeostasis. One interesting phenotype in the double mutant is the severe chaining defect in cells grown under conditions of normal osmolarity, where either $\Delta tolA$ or $\Delta bamB$ alone do not chain. These results overall indicate that exaggerated OM (lipid) problems can give rise to issues during cell division. While our data does not rule out the possibility that cell wall separation and OM lipid homeostasis may be two largely independent functions of Tol-Pal, it appears to be consistent with the idea that maintaining proper OM lipid balance could be the

335 primary function, thus indirectly affecting septa separation. Although the mechanism(s) by which Tol-
 336 Pal (or specifically TolQRA and/or TolB) facilitate these functions remains enigmatic, our work
 337 reinforces the emerging roles of Tol-Pal in ensuring proper cell wall separation during cell division
 338 and, importantly, in maintaining proper OM lipid homeostasis in Gram-negative bacteria.

Experimental procedures

Strains, plasmid, and growth conditions. All the strains used in this study are listed in Table S5. *Escherichia coli* strain MC4100 [*F*⁻ *araD*139 Δ (*argF-lac*) *U169 rpsL150 relA1 flbB5301 ptsF25 deoC1 ptsF25 thi*] [47] was used as the wild-type (WT) strain for most of the experiments. NR754, an *araD*⁺ revertant of MC4100 [48], was used as the WT strain for experiments involving arabinose-inducible vector. pBAD43-*tolA* was cloned using the EcoRI and XbaI restriction sites with primers EcoRI-*tolA*5 (ACAATGAATTCCGAGAGTGTCAAAGGCAACC) and XbaI-*tolA*3 (TACTGTCTAGATTACGGTTTGAAGTCCAATGGC). pTrc99a-*mdoH*-N pTrc99a-*mdoH*-FL were cloned using NcoI and XbaI restriction sites with the 5' primer NcoI-*mdoH*5 (TTTTCCATGGCAAATAAGACAAGTACATT) in combination with either the XbaI-*mdoH*-N (TTTTTCTAGATTAGTGATGGTGATGGTGATGTCCGCCACCACGGCGGATGGTACCGACG) or XbaI-*mdoH*-FL (TTTTTCTAGATTAGTGATGGTGATGGTGATGTCCGCCACCTTGCGAAGCCGCATCCGG), respectively. Gene deletion mutants were constructed using recombineering [49, 50]. Primers used are listed in Table S6. Whenever needed, the antibiotic resistance cassettes were flipped out as described [50]. Gene deletion cassettes were transduced into relevant genetic background strains via P1 transduction [51]. Strains were grown in Luria-Bertani (LB) broth (1% tryptone and 0.5% yeast extract, with or without 1% NaCl). Agar plates contain 1.5% agar in the corresponding media.

Transposon mutant library construction. Recipient strains of *E. coli* were grown to mid-log, washed 4 times with cold sterile H₂O, and concentrated 100x with cold sterile H₂O to generate electrocompetent cells. For each transposon mutagenesis reaction, 100μl of competent cells was mixed with 1 μl of EZ-Tn5 KAN-2 Tnp Transposome (Epicentre) and electroporated at 1800V. 1ml SOC media was added immediately after electroporation and the mixture was allowed for recovery at 37°C for 2 hours. The recovered reaction was plated onto multiple LB plates (depending on the efficiency of each strain) with 50 μg/ml kanamycin and incubated overnight at 37°C. On subsequent day, number of colonies per reaction was estimated and the colonies were scrapped and pooled in

LB with 15% glycerol and stored at -80°C. Final mutant libraries (estimated $\approx$ 1,000,000 mutants) were generated by pooling multiple libraries of the same background.

Multiplexed TraDIS. Genomic DNAs were isolated from LB culture inoculated with the respective library after 3 hours growth at 37°C. For each mutant library, three cultures were grown separately for genomic DNA isolation (triplicate). For each sample, 100 ng of genomic DNA was sheared with Covaris and then subsequently end-repaired and -adenylated following Illumina TruSeq Nano protocol. Ligation of sequencing adapters with indices specific to each sample was also done according to TruSeq Nano kit protocol. Each adapter-ligated library was enriched for Tn5 containing fragment with 2-steps PCR using KAPA Library Amplification Kit at 65°C reannealing temperature for 10 (1st amplification) or 20 (2nd amplification) cycles and 500nM final concentration for each primer. First round of PCR was done with a 24 bases forward primer that contains the exact sequence of the -47 to -24 (relative to the junction of transposon insertion) region of the Tn5 transposon and a reverse primer that is complimentary to the 3' region of the TruSeq indexed adapter. Second round of PCR was done with the same reverse primer and an 86 bases forward primer that contains, from 5' to 3', the 58 bases TruSeq universal adapter sequence, a 6 bases additional index sequence (different for each sample), and a 22 bases sequence that contains the exact sequence of the -32 to -11 region of the Tn5 transposon. Primers used for TraDIS sample preparation are listed in Table S7. Nine libraries were pooled and normalized to 10nM and 20% PhiX spike-in was added. The pooled library was loaded onto MiSeq at 8pM final concentration and sequenced using 300 cycles, 2x150 paired end protocol.

TraDIS analysis. The ESSENTIALS pipeline [52] was used to analyze the output of TraDIS sequencing. Demultiplexed R1 reads from MiSeq sequencing were uploaded to the ESSENTIALS server and analyzed using the following setting: Tn insertion = random; library size = 1,000,000; filtering non-unique sequence = yes; barcode mismatch allowed = 0; genomic sequence length after removal of barcode and transposon = 100; barcode = beginning of line; transposon inverted repeat = beginning of line; minimal sequence match for alignment = 50; strand to align = both; use truncated

version of gene = yes; remove genomic position bias using Loess = yes; normalization method = TMM; analysis = qCML; modelling of variance = tagwise; prior.n = 5; p-value adjustment = BH. Through this analysis, all reads were filtered for the correct barcode and Tn5 sequence, and 100 bases right after the Tn5 insertion junction were aligned to an annotated MC4100 reference genome (NZ_HG738867.1) [53]. Each mapped read indicates a Tn5 insertion at the exact position of the genome. The frequency of Tn5 insertion in each gene were normalized and compared between $\Delta to/Q$ or $\Delta to/A$ library against the WT control library. Genes with less than 10 reads mapped in all three replicates of both mutant and WT library were removed from the analysis and were not compared between $\Delta to/Q$ and $\Delta to/A$ backgrounds. Downstream data was inspected in Microsoft Excel and graphical visualizations of the data were plotted using the *ggplot2* package in R.

Growth curve and CFU enumeration. To evaluate growth, overnight cultures were first normalized to $OD_{600} = 0.02$ into fresh LB media. For measurement with plate reader, 200 μ l of the diluted cultures were transferred onto 96-well plate and OD_{490} at 37°C was measured at fixed interval using Victor X4 plate reader (Perkin Elmer). For manual growth curve and CFU enumeration, at 1-hour intervals, OD_{600} for the culture at 37°C were measured and a small volume of the culture were serially diluted and plated to estimate the CFU/ml at that time point.

Efficiency of plating assay. Efficiency of plating (EOP) analyses were done by spotting serially diluted culture onto different agar plates. Cells cultures were 10-fold serially diluted in 150mM NaCl on 96-well plate. Two or five microliters of each dilution were spotted onto the plates and incubated overnight at the indicated temperatures. All results shown are representative of at least three independent replicates.

Microscopy. For each strain, five microliters of overnight or mid-log cultures were spotted onto freshly prepared 1% agarose pads made with the corresponding media. Images were acquired using a Zeiss LSM710 confocal microscope at 100x magnification.

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431 **Table 1. Negative genetic interactions with the *tol-pal* locus.**

	Category	Genes
Cell wall biosynthesis or remodelling	Peptidoglycan biosynthesis	<i>dapF, lpoA, mrcA, lpoB, mrcB</i>
	Cell wall remodelling	<i>nlpl, prc, tatC</i>
	Cell wall recycling	<i>ldcA</i>
	Und-P lipid carrier related	<i>qseC, wecB, wecC, wecD, wecE, wecF, wzxE</i>
OM assembly or integrity	LPS synthesis/modification	<i>gtrS, waaG, waaP</i>
	β -barrel protein assembly	<i>bamB, degP, surA</i>
	OM-cell wall tethering	<i>ompA</i>
Other		<i>rpe, yqeH</i>

432

Figure legends

Figure 1. Saturated genetic interaction map with the *tol-pal* locus. (A) Schematic representation of the rationale of using transposon insertion site sequencing (TraDIS) to identify genetic interactions. Increased or decreased frequency of insertion within a gene in $\Delta tolQ/A$ background relative to WT represents positive or negative genetic interactions, respectively. (B) Summary of the total number of sequencing reads recovered that aligned to the MC4100 reference genome [53]. Numbers of reads for each replicate, total combined reads, and number of unique insertion sites in the genome for each background are indicated. (C) Comparison of all genetic interactions specific to $\Delta tolQ$ and $\Delta tolA$ backgrounds. Genes that exhibit strong positive or negative genetic interaction with both *tolQ* and *tolA* (arbitrary threshold of $\log_2FC > 2.5$ or < -2.5 , corrected P-value $< 10^{-5}$) are annotated.

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Figure 3. The frequencies of transposon insertions decrease significantly in genes involved in cell wall and OM homeostasis in *tol-pal* strains. Transposon insertion profiles at selected loci that exhibited strong negative interactions with *tolQ* and *tolA*, including (A) *nlpI*, (B) *prc*, (C) *tatC*, (D) *surA*, (E) *bamB*, (F) *degP*, (G) *ompA*, and (H) ECA biosynthesis gene cluster.

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forming-unit (CFU) enumeration of indicated *nlpI/prc* strains at the corresponding timepoints taken during growth curve in (A). (C) EOP of indicated *nlpI/prc* strains from either mid-log or overnight cultures on LB media at 37°C. Cultures were normalized to the same initial optical density. (D) Growth curves of indicated *tatC* strains, based on optical density at 600 nm, in LB media at 37°C. (E) CFU enumeration of indicated *tatC* strains at the corresponding timepoints taken during growth curve in (D). (F) EOP of indicated *tatC* strains on LB media without NaCl, or containing vancomycin, at 37°C.

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Figure 7. $\Delta tolA \Delta bamB$, $\Delta tolA \Delta surA$, $\Delta tolA \Delta degP$, and $\Delta tolA \Delta ompA$ double mutants exhibit morphological defects. DIC images of cells from indicated strains grown to (A) mid-log or (B) stationary phase in LB media (with NaCl) at 37°C. Scale bar represents 5 μ m.

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