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Disassembly of a medial trans-envelope structure by antibiotics during intracellular division

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Running title: The role of the chlamydial Pal-Tol complex

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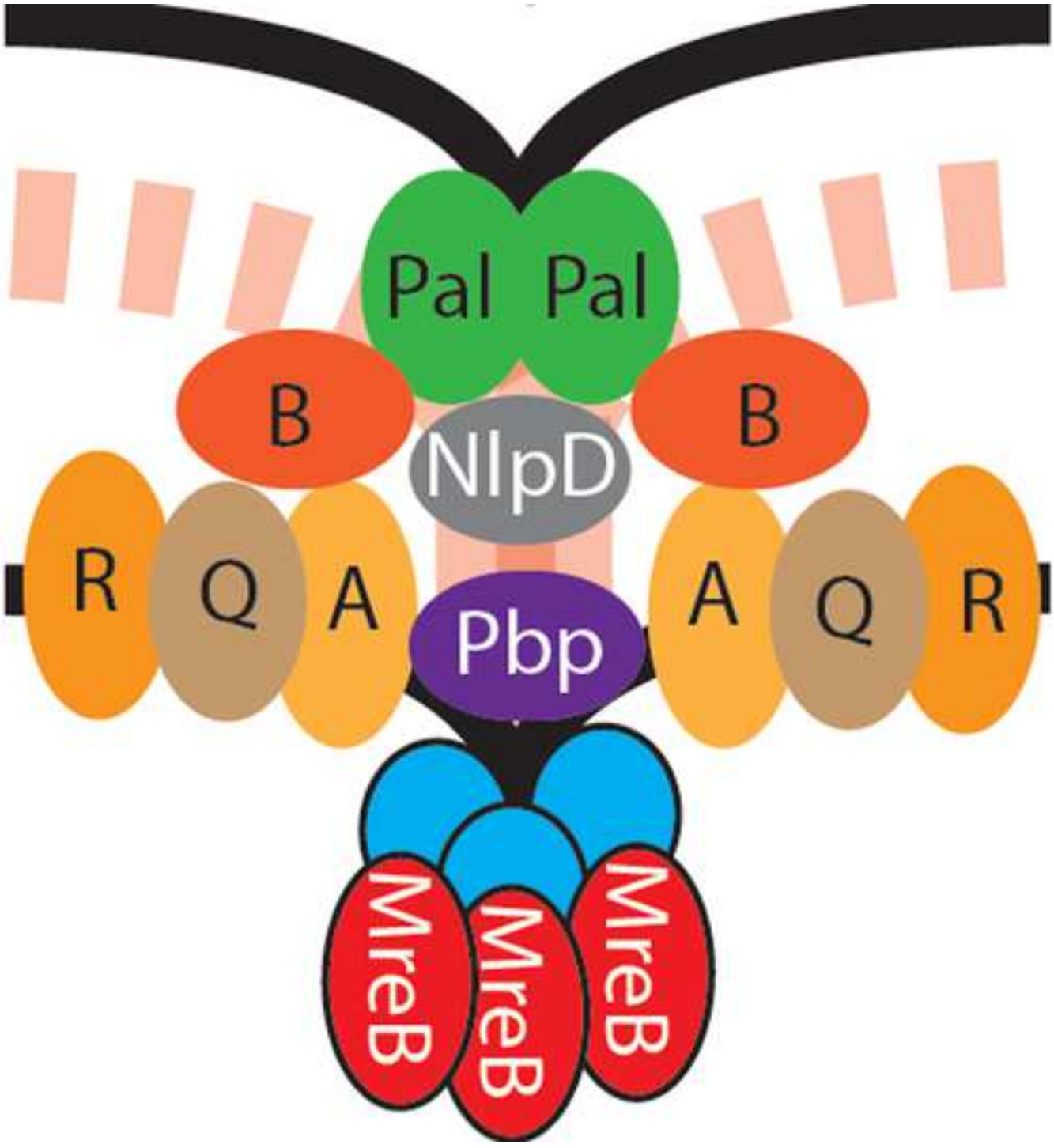
Summary

Chlamydiales possess a minimal but functional peptidoglycan precursor biosynthetic and remodeling pathway involved in the assembly of the division septum by an atypical cytokinetic machine and cryptic or modified peptidoglycan-like structure (PGLS). How this reduced cytokinetic machine collectively coordinates the invagination of the envelope has not yet been explored in *Chlamydiales*. In other Gram-negative bacteria, peptidoglycan provides anchor points that connect the outer membrane to the peptidoglycan during constriction using the Pal-Tol complex. Purifying PGLS and associated proteins from the chlamydial pathogen *Waddlia chondrophila*, we unearthed the Pal protein as a peptidoglycan-binding protein that localizes to the chlamydial division septum along with other components of the Pal-Tol complex. Together our PGLS characterization and peptidoglycan-binding assays support the notion that diaminopimelic acid is an important determinant recruiting Pal to the division plane to coordinate the invagination of all envelope layers with the conserved Pal-Tol complex even during osmotically-protected intracellular growth.


Pal Pal
B
B
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R Q A
A Q R
Pbp
MreB
MreB
MreB

Introduction

Though cell division is universal, different proteins execute this task in the two domains of life. The set of cytokinetic proteins required for bacterial division are generally very conserved and overt sequence orthologs are encoded in most (eu)bacterial lineages. One notable exception is the *Chlamydiales* order, where many (but not all) conserved division proteins are absent, suggesting that *Chlamydia* and *Chlamydia*-related bacteria have evolved a minimal division machine or that functional analogs exist that do not resemble bacterial division proteins in primary structure. Division normally begins with the assembly of membrane-anchored cytokinetic platform at midcell to which enzymes and regulatory/accessory proteins are recruited. This multicomponent machine known as the divisome governs the synthesis and remodeling of the cell wall (the peptidoglycan, PG) at the division site. The main organizer of the cytokinetic platform in most prokaryotes and many eukaryotic organelles is the protein FtsZ, an homologue of tubulin (Kirkpatrick and Viollier, 2011; Miyagishima et al., 2014). FtsZ assembles in a ring-like structure (Z-ring) at the septum and recruits a multitude of proteins (Fts proteins) responsible for the new synthesis and modification of the PG layer. The cytoplasmic membrane along with the PG layer and outer membrane invaginate in a concerted fashion through the force exerted by the divisome (For review, see (Jacquier et al., 2015)). Interestingly, all *Chlamydiales* lack a sequence homologue of FtsZ, the usual organizer of bacterial division (Jacquier et al., 2015; Miyagishima et al., 2014). However, ancillary proteins as the bacterial actin homologue MreB and RodZ (a protein tethering MreB to the membrane) are encoded in chlamydial genomes and have been localized to the septum early in the division phase, suggesting that chlamydial division relies on an alternative division mechanism (Frandi et al., 2014; Jacquier et al., 2014; Kemege et al., 2014; Miyagishima et al., 2014).

Members of the *Chlamydiales* order are very diverse in morphology and biosynthetic capacities (Omsland et al., 2014), but they all divide by binary fission (For review, see (Jacquier et al.,

2015)). These obligate intracellular bacteria present a unique biphasic developmental cycle: elementary bodies (EBs) are infectious, non proliferating and can survive outside of the host cell, and reticulate bodies (RBs) are non-infectious, proliferating and metabolically highly active. The *Chlamydiales* order comprises the well known family of *Chlamydiaceae* and the more recently discovered *Chlamydia*-related bacteria. *Chlamydiaceae* include several important human pathogens such as *Chlamydia trachomatis* and *Chlamydia pneumoniae*, which are the causative agents of trachoma, genital tract infections (*C. trachomatis*) and respiratory tract infections (*C. pneumoniae*) (Goy et al., 2009). On the other hand, some *Chlamydia*-related bacteria are emerging pathogens and have been found in very diverse niches ranging from amoebae to bovine placenta (Amann et al., 1997; Collingro et al., 2005; Corsaro et al., 2009; Henning et al., 2002; Kahane et al., 1995; Lienard et al., 2011; Rurangirwa et al., 1999; Thomas et al., 2006). *Waddlia chondrophila*, a *Chlamydia*-related bacterium, has been associated with abortion in mammals and miscarriage in humans (Baud et al., 2011; Baud et al., 2014; Baud et al., 2007; Dilbeck et al., 1990; Henning et al., 2002).

Recently a PG-like sacculus could be purified from the *Chlamydia*-related bacterium *Protochlamydia ameobophila* (Pilhofer et al., 2013) but not from *Simkania negevensis*, while in *Chlamydiaceae* a PG-like structure (PGLS) may form at the division septum (Brown and Rockey, 2000; Liechti et al., 2013). In earlier studies we could show that biosynthesis of PG precursors is required for proper localization of the septal proteins RodZ and NlpD in *W. chondrophila* (Frandi et al., 2014; Jacquier et al., 2014), although it was not tested if *W. chondrophila* produces a septal PGLS.

All chlamydia are diderm bacteria, necessitating a mechanism to draw in the outer membrane along with the invaginating inner envelope layers. PG-binding lipoproteins such as Pal are dedicated to this and a Pal-like protein is encoded in the same locus as the coding sequences for the components of the Tol system in *Chlamydiales* (Jacquier et al., 2015; Sturgis, 2001). The

Tol-Pal complex is necessary for proper invagination of the outer membrane during division (Gerding et al., 2007). Pal of *E. coli* possesses a N-terminal cleavable signal sequence, for export into the periplasm followed by a cysteine. This cysteine is lipidated and thus serves to anchor the protein to the inner leaflet of the outer membrane (Cascales and Lloubes, 2004), reviewed in (Godlewska et al., 2009). TolB is located in the periplasm, interacts with Pal and seems to regulate the association of Pal with PG (Bouveret et al., 1999). The other members of the Tol-Pal complex are the inner membrane proteins TolA, TolQ and TolR, which interact through their transmembrane domains (Cascales et al., 2001). These two subcomplexes (Pal-TolB and TolAQR) are connected through an interaction between TolA and TolB (Walburger et al., 2002).

Reasoning that the Tol-Pal complex may play an important role in division, envelope integrity and, thus, pathogenesis in *Chlamydiales*, we set out to investigate the septal localization and PG-binding properties of chlamydial Pal-Tol. By purifying PGLS from *W. chondrophila*, and recovering Pal with it, we found that Pal can bind PG from other bacteria and that *W. chondrophila* PGLS is required to recruit Pal to the division septum. As homologues of other members of the Tol-Pal complex are also recruited at the division septum, the function and properties of the Tol-Pal complex seem to be maintained across evolution including obligate intracellular bacteria of the chlamydial phylum.

Results:

Isolation and characterization of PGLS of *W. chondrophila*. As PGLS could recently be isolated from the *Chlamydia*-related bacterium *Protochlamydia amoebophila*, but not from *Simkania negevensis* (Pilhofer et al., 2013), we wanted to investigate the existence of a PGLS in *W. chondrophila*, another *Chlamydia*-related bacterium. In order to detect PG components extracted from dividing *W. chondrophila* cells, we performed high performance liquid chromatography (HPLC) following treatment with cell wall hydrolytic enzymes, including those that act on peptide crosslinks. Cell walls were extracted from Vero cell infected with *W. chondrophila* 28h post-infection (p.i.) as described, treated or not with MltA, CwlT or mutanolysin and fractionated by HPLC (at Anasyn, Tuebingen, Germany). While treatment of cell walls by digestion with mutanolysin, a muramidase, did not liberate muropeptide fragments (Fig. 1A), treatment with MltA, a lytic transglycosylase (Lommatzsch et al., 1997) (Fig. 1B), or CwlT, a bifunctional N-acetyl-muramidase and DL-endopeptidase (Fukushima et al., 2008) (Fig. 1C) provided evidence for the presence of PGLS. To confirm this notion, we repeated these cell wall extraction experiments with *W. chondrophila* that had been exposed to the PG synthesis inhibitors phosphomycin or penicillin for 4 hours. The HPLC analysis revealed a reduction in several peaks (a to f) normalized to the total of all the peaks of the sample (Figs. 1D and 1E), confirming that liberation of these fragments depends on the PG precursor biosynthesis (lipid II, that is executed by MurA) and/or PG-like transpeptidation (executed by PBPs) that can be inhibited by phosphomycin and penicillin, respectively.

As muropetides can be detected by human Nod receptors, we tested if the material extracted from *W. chondrophila* can induce the hNod1/2 reporter system in HEK-Blue cells expressing the hNod1 or hNod2 receptors, as described in Extended Experimental Procedures. We could show

using this test that *W. chondrophila* PGLS is recognized at comparable rates as *E. coli* PG (Figs.2A and B).

Co-purification of PG-binding proteins. We next sought to identify chlamydial PG-binding proteins by co-purification with *W. chondrophila* PGLS. Such proteins might provide important insight into PG biosynthesis regulation, in the anchoring of PG to inner and outer membranes and in division regulation. For this purpose, a similar PG isolation as above was again performed, but this time without proteolysis. The resulting material was then either directly resuspended in SDS-PAGE loading buffer or first washed with increasing concentrations of SDS to remove contaminating proteins not strongly bound to PG. SDS-PAGE followed by immunoblotting revealed that one known PG associated protein (AmiA) was indeed present in these preparations (Fig. 3A). Tandem mass spectrometry (MS/MS) was then used to identify other PG-associated proteins in these various samples (Tables S1 and S2). We did not further investigate PG binding of candidates that lack a secretion signal or are not conserved among *Chlamydiales*. We assume that these proteins are either contaminants of the PG preparation or that their PGLS binding has no physiological significance since they do not usually localize in the periplasm and might bind to PGLS during the lysis of the bacteria. Two of the identified proteins are conserved among *Chlamydiales* and contain a predicted secretion signal: the homologues of the protein translocase subunit SecA and the peptidoglycan-associated lipoprotein Pal. This latter is of interest, because the lipoprotein Pal is known to bind PG directly, is indirectly associated with the inner membranes through the Tol complex in *Escherichia coli* (Gerding et al., 2007) and is conserved among *Chlamydiales* (Figs. 3B and 3C). *W. chondrophila* Pal (Pal^{Wch}) is encoded in a gene cluster with predicted genes encoding the Tol-Pal system (Fig 3D). Primary sequence analysis showed that Pal^{Wch} exhibits 49% identity to *E. coli* Pal (Pal^{Eco}) and possesses an extended N-terminal region of 80 residues. However, multiple alignments indicate that residues important for Pal:PG interaction are invariant in Pal^{Wch} with

respect to the wider family of Pal-related proteins, which also comprises OmpA of *E. coli* (Figures 3B and 3C, asterisks).

Pal of *W. chondrophila* is mainly expressed in RBs

As expected for a chlamydial cell division protein, the *Pal*^{Wch} transcript was associated with the RB phase. We measured transcript levels by RT-qPCR and normalized to rRNA through the developmental cycle and observed a peak of transcription at 16h p.i, followed by a progressive decrease in mRNA levels to 72h (Fig. 4A). To confirm that Pal^{Wch} is also associated with the RB stage, we purified Pal^{Wch} protein, heterologously expressed in *E. coli*, to raise polyclonal antibodies. The antibodies detected a protein of 25 kDa, (the predicted size of Pal^{Wch}) by immunoblotting already at 16h (Fig. 4B), which is consistent with a putative role of Pal during the replicative phase of the chlamydial developmental cycle in which RBs predominate. Moreover, purified EBs exhibited only a very low ratio of Pal compared to quantities expressed by RBs at 16 and 24h p.i. (Fig. 4B). We used immunofluorescence (IF) microscopy to confirm the association of Pal with cell division. Serum taken before immunization (pre-immune, Fig. 4C) did not label any structure in infected Vero cells. In contrast, serum harvested after immunization recognized *W. chondrophila* RBs specifically (Fig. 4C, arrow) and not EBs (Fig. 4C, arrowhead). This was not affected by permeabilization of EBs with a DTT treatment, which reduces the disulfide bridges present between outer membrane proteins in EBs (Fig. 4C, +DTT), as shown for *C. trachomatis* EBs (Raulston et al., 2002). Nevertheless, low amounts of Pal might still be present in EBs, as observed by Western blotting, but could not be detected by immunofluorescence (Fig. 4B-C). The presence of Pal^{Wch}

predominantly in RBs is consistent with the important role of PG and PG-binding proteins during RBs division as proposed earlier (Jacquier et al., 2014).

Pal of *W. chondrophila* binds PG of different bacteria

PG binding of Pal^{Wch} was then investigated both *in vivo* and *in vitro*. First, we probed for the presence of Pal in the isolated *W. chondrophila* PGLS (used for MS/MS) by immunoblotting and found this to be the case. In fact the association of Pal^{Wch} to PGLS was very strong, and only removed by boiling in SDS-PAGE buffer but not by washes in 2% or 4% SDS (Fig. 3A). This is consistent with the putative OmpA-like PG binding motif found in Pal^{Wch} and in Pal of other chlamydial lineages (Figs. 3B and 3C) and its genetic linkage to other genes encoding putative PG biosynthesis and remodeling enzymes (Fig. 3D). In order to determine if recombinant Pal^{Wch} can indeed bind PG *in vitro*, we purified His_6-tagged Pal^{Wch} from *E. coli* to conduct pelleting assays with purified sacculi (polymeric PG) from various bacterial species with different structural characteristics. *E. coli* PG is characterized by the incorporation of modified amino acids, such as *m*DAP (*γ-meso*-diaminopimelic acid), as in most Gram-negative bacteria, whereas PG from Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus* have the *m*DAP replaced by D-isoglutamate or L-Lysine. Therefore we used sacculi from *E. coli*, *B. subtilis* and *S. aureus* as a template as well as purified PGLS material from *W. chondrophila*. His_6-Pal^{Wch} partitioned exclusively with the *E. coli* PG pellet in the insoluble fraction after ultracentrifugation, whereas it is in the soluble fraction in the absence of sacculi (Figure 5A). His_6-Pal^{Wch} exhibited weaker PG binding to *B. subtilis* and *S. aureus* sacculi, as indicated by the fact that His_6-Pal^{Wch} was found in both fractions when equimolar amounts of sacculi were added. As expected, His_6-Pal^{Wch} was also precipitated by the addition of *W. chondrophila* PG (Figure 5B). Since Pal^{Wch} also features the OmpA-like PG binding domain present in other Pal orthologs, we hypothesized that conserved residues in the OmpA-domain are required for PG binding of Pal^{Wch}. In order to further characterize the binding of Pal^{Wch} to PG we generated a

His_6-Pal^{Wch} mutated variant (i.e. His_6-Pal^{Wch} D180A/R195A) in residues conserved in the OmpA-like PG binding domain. Purified His_6-Pal^{Wch} D180A/R195A was detected in the soluble fraction in both the control reaction (no murein) and in the pelletting assay with *E. coli* sacculi (Fig 5C-D). Since Pal^{Wch} was strongly associated to the isolated *W. chondrophila* PGLS, we wanted to determine if this strong association with murein is maintained also *in vitro*. His_6-Pal^{Wch} was released from the insoluble pellet fraction after the first and the second washes with 4% SDS (W1, W2, Fig. 5D), suggesting that other proteins might participate to strengthen the interaction of Pal^{Wch} with PGLS *in vivo* or that the PG isolation process we used strengthened the binding of Pal^{Wch} to PGLS.

Knowing that Pal^{Wch} can bind *E. coli* sacculi *in vitro* and that there are no established methods for genetic manipulation of *W. chondrophila*, we sought a suitable *E. coli* strain that could be used as surrogate host for PG binding and/or functionality test with Pal^{Wch}. Complementation experiments revealed that, unlike *E. coli* Pal (Pal^{Eco}), Pal^{Wch} cannot correct the growth defect of the *E. coli* Δ*pal* mutant on McConkey medium (containing detergents such as the bile acid deoxycholate, to which the Δ*pal* mutant is sensitive). However, we isolated a spontaneous Δ*pal* suppressor mutant (Δpal^{Supp}) that can grow on McConkey medium and observed that expression of Pal^{Wch} was toxic under these conditions (Fig. 5E). Such toxicity did not occur when Pal^{Eco} was expressed at mild levels but was also conferred upon expression of another chlamydial Pal from *P. acanthamoebae,* Pal^{Pac} indicating that this activity is conserved among Pal of the *Chlamydia*-related bacteria. Therefore, as means to functionally characterize chlamydial Pal using this toxicity assay we isolated two mutations (G177V and R237*, introduction of a stop codon) in Pal^{Wch} that prevent toxicity in Δpal^{Supp} *E. coli* and that map to the OmpA-like PG-binding domain, suggesting that PG-binding is required for the activity of Pal^{Wch} in Δpal^{Supp}. To verify this result we engineered a double mutant in two conserved PG-binding residues (D180A/R195A) of Pal^{Wch} by site-directed mutagenesis and found that these mutations

also attenuated the growth defect of Δ*pal*^{Supp} cells harboring Pal^{Wch} (Fig. 5F). As immunoblots showed that the mutant forms are stable (Fig. 5G), we conclude that PG binding by Pal^{Wch} is also needed for its activity *in vivo*, at least in a surrogate host and thus that Pal^{Wch} can bind PG *in vitro* and *in vivo*. Interestingly, the Δ*pal*^{Supp} mutant has lower amounts of OmpA, an outer membrane protein, which interacts with PG, and which might be thus involved in the sensitivity of this mutant to Pal^{Wch} (Fig. S1).

The Pal-Tol complex is localized to the division septum in *W. chondrophila*

In order to investigate the possible role of Pal^{Wch} in chlamydial division, we observed its localization in dividing *W. chondrophila* cells by immunofluorescence microscopy. Pal^{Wch} was highly enriched at the division septum (in 56.9 ± 4.4 % of dividing bacteria, Fig. 4D). This localization is dependent on the integrity of PGLS, as treatment of infected cells with penicillin or phosphomycin 2h p.i. resulted in a partial (for penicillin, 30.7 ± 2.0 % of cells showed an accumulation of Pal^{Wch} at midcell, Fig. 4D) or nearly total dispersion of Pal^{Wch} from the midcell (for phosphomycin, 3.6 ± 5.1 % of bacteria had an accumulation of Pal at midcell, Fig. 4D). Interestingly, Pal^{Wch} localization is sensitive to disturbance of MreB by MP265 (Fig. 4D). This is consistent with earlier results showing a role of MreB in the divisome machinery recruitment in *W. chondrophila* (Jacquier et al., 2014). Moreover, vancomycin and teicoplanin, two antibiotics targeting the d-Ala-d-Ala dipeptide cause the formation of aberrant bodies in *W. chondrophila* and have a strong effect on Pal localization at mid-cell (Fig. 4D and Fig. S2A and B). Interestingly, flavomycin, an antibiotic targeting PG trans-glycosylation enzymes shows no effect on *W. chondrophila* proliferation (Fig. S2A). We also observed an effect of vancomycin and teicoplanin on the localization of other septal proteins, such as MreB, RodZ and NlpD (Fig. S2C). It is noteworthy that dispersion of Pal from division septum was not complete after drug

treatment, since Pal foci are still visible (Fig. 4D, arrowheads). These results imply that the PGLS of *W. chondrophila* contains a d-Ala-d-Ala dipeptide, which is required for proper organization of the division machinery.

TolA, TolB, TolQ and TolR, the proteins forming, in association with Pal the Tol-Pal complex are conserved in the *W. chondrophila* genome. Transcription of all members of the complex could be detected by qRT-PCR (Fig. 6A), with a maximum of RNA accumulating between 8 and 24 hours, consistent with a role of the Tol-Pal complex in maintenance of the outer membrane integrity during division. Members of the Tol complex are apparently expressed slightly earlier than Pal, since they are detected at 8h p.i. However, this might be explained by a lower sensitivity of the qRT-PCR for Pal. Our results are thus consistent with the presence of the genes coding for the Tol-Pal complex in a single operon, since their general expression profiles are similar. To investigate the localization of Tol proteins, we used a construct allowing the expression of TolA and TolR fused together. These proteins being relatively small, we assumed that a combination of them would be more immunogenic. We thus immunized rabbits against this fusion protein and could raise antibodies, which specifically recognize *W. chondrophila* by immunostaining, since the pre-immune serum does not label infected cells (Fig. 6B). The protein(s) recognized by these antibodies apparently localize(s) at division septum in a PG-dependent manner (Fig. 6C). This strongly implies an important role of the Tol-Pal complex in division, which might be conserved among all *Chlamydiales*.

Discussion

Cell division in absence of a FtsZ homologue and of a classical PG is specific to a minority of bacteria, including anammox Planctomycetes, other members, with *Chlamydiales*, of the PVC superphylum. Interestingly, a PG sacculus was recently detected in *Planctomycetes* (Jeske et

al., 2015; van Teeseling et al., 2015), and *Chlamydia*-related bacteria (Pilhofer et al., 2013). This indicates that, even in absence of FtsZ, PVC bacteria still need a septal PG-like structure (Liechti et al., 2013), which is apparently essential for proper chlamydial division (Frandi et al., 2014; Jacquier et al., 2014) and should also help coordinate the invaginations of the outer and inner membranes. Interestingly, PGLS of *Chlamydiales* and possibly *Planctomycetes* contain unconventional PG structures (Jeske et al., 2015; Pilhofer et al., 2013; van Teeseling et al., 2015), which might explain why they were not detected earlier. In contrast to *Chlamydiales*, *Planctomycetes* possess transglycosylation enzymes (Jeske et al., 2015; van Teeseling et al., 2015), indicating that the structures and/or the synthesis of their PGLS might be different.

Antibiotics targeting the PG biosynthetic pathway at different levels exhibited various effects on *W. chondrophila* growth demonstrating that PG precusors are mandatory for *W. chondrophila* DNA replication (effect of phosphomycin) and that PG transpeptidation is required for cell division but not for DNA replication (no effect of vancomycin and teicoplanin on DNA replication, but formation of aberrant bodies). Moreover, the lack of effect of flavomycin on *W. chondrophila* proliferation might indicate that transglycosylation might not happen in chlamydial PG, thus explaining the non canonical structure of PG observed in Chlamydia-related bacteria (Pillhofer *et al*., 2013) and the absence of genes encoding homologs of transglycosylation enzymes in chlamydial genomes (Ghuysen and Goffin, 1999).

We found here that *W. chondrophila* Pal is able to bind PGs purified from both Gram-positive and Gram-negative bacteria, and PGLS from *W. chondrophila*, showing that the PG-binding site of Pal^{Wch} is not specific to a modified chlamydial PG. In Gram-negative bacteria, Pal specifically binds to mDAP, a modified peptide, which is a component of all Gram-negative PGs (Parsons et al., 2006). By contrast, the PG of *Staphylococcus aureus*, which has no mDAP, but a D-iso-Glutamine is less well bound by Pal^{Wch} (Fig 4). This suggests that PG of *W. chondrophila* contains mDAP, as suggested by its seemingly functional m-DAP biosynthetic pathway encoded

in chlamydial genomes (Jacquier et al., 2015). Moreover, potential modifications of chlamydial PGLS are likely not required for binding of Pal^{Wch} , since this protein efficiently binds to Gram-negative PG. Nevertheless, these modifications might play a role in the resistance of the chlamydial PGLS towards degradation by different lytic enzymes from the host cell. This would be consistent with our observations that *W. chondrophila* PGLS is resistant to mutanolysin digestion (Fig. 1). The fact that cell division in *W. chondrophila* can be inhibited with vancomycin or teicoplanin, drugs specifically binding to d-Ala-dAla dipeptides of the PG is bolsters the findings that fluorescent d-Ala-dAla dipeptide derivatives could be localized to the *C. trachomatis* division septum (Liechti et al., 2013), that PG biosynthetic enzymes are conserved among *Chlamydiales* (Reviewed in (Jacquier et al., 2015)) and that they are required for cytokinesis and assembly of the septal division machine in this phylum.

The septal localization of Pal in *W. chondrophila* is consistent with its role in maintenance of cellular integrity during division, a time when all three envelope layers must indent together (Fig. 7). Outer membrane proteins might play an important role in this process as anchoring site for septal PG and the subsequent recruitment of the Pal-Tol complex. It would then be of high interest to investigate the localization of such outer membrane lipoproteins during chlamydial division. The Pal-Tol complex was recently shown to be physically and functionally connected to septal PG biosynthesis (Pbp1b-LpoB complex) via the protein YbgF (CpoB) (Gray et al., 2015). This protein is conserved in *Chlamydiales* (wcw_0348 in *W. chondrophila*), but is not encoded in the Tol-Pal operon and might play a role in the coordination of outer membrane invagination and new synthesis of PGLS, even in absence of chlamydial Pbp1-LpoB homologues.

The discovery of a complete and functional Pal-Tol complex in *Chlamydiales* is of highest interest in the field of drug development because targeting this complex with specific inhibitors

might completely block the chlamydial division and disturb cell integrity. In addition, since the Pal-Tol complex of *E. coli* mediates colicin sensitivity (Housden and Kleanthous, 2012), the chlamydial Pal-Tol complex might also, by analogy, represent a specific target to allow the entry of anti-chlamydial microbial peptides. Finally, given the immunogenicity of this Pal-Tol complex and the importance of Pal in septic shock caused by *E. coli* (Godlewska et al., 2009; Hellman et al., 2002), the chlamydial Tol-Pal complex might be involved in severe immune recognition leading to inflammation, as commonly observed following *C. trachomatis* infection, where inflammation is well known to induce the trachoma lesions (Burton et al., 2011), as well as tubal infertility (Hvid et al., 2007), extra-uterine pregnancy (Daponte et al., 2012) and miscarriage (Baud et al., 2008).

Significance

This study identified the role of the Pal-Tol complex in chlamydial division, highlighting the importance of peptidoglycan-binding proteins to ensure cell wall integrity during cell division of distantly related descendants of the Gram negative lineage and possibly eukaryotic organelles, which possess a minimal division machinery, lacking the main organizer of bacterial division FtsZ, but that maintained a peptidoglycan layer and peptidoglycan-binding proteins, which are essential for division. This is a further step towards the understanding of the exact mechanism of the division of these obligate intracellular pathogens.

Experimental procedures:

Strains and growth conditions

E. coli strains and mutants were grown in Luria Bertani broth (LB). *W. chondrophila* ATCC VR-1470^T was grown in the amoeba *Acanthamoeba castellanii* ATCC 30010 cultivated in 25 cm^2

cell-culture flasks containing 10 ml of peptone-yeast extract-glucose medium (PYG) incubated for 6 days at 28°C (Jacquier et al., 2013). Cell suspension was then harvested and filtered using a 5 μm-pore filter to purify bacteria from intact ameobae in the flow-through. Strains, plasmids and antibodies used in this study are described in the supplemental experimental procedures.

Cell culture and bacterial infection

Vero cells (ATCC CCL-81) were grown and infected by *Waddlia chondrophila* as described earlier (Jacquier et al., 2014). Shortly, Vero cells were grown in 75 cm^3 flasks containing 20 ml DMEM supplemented with 10% fetal calf serum at 37°C in presence of 5% CO_2. Cells were diluted to 10^5 cells/ml, grown overnight and infected with a 2000x dilution of *W. chondrophila*. The infected cells were then centrifuged for 15 minutes at 1790 x *g*, incubated 15 minutes at 37°C and washed once with PBS before addition of fresh media.

Cell wall analysis

Vero cells were infected as described above, collected 28 h p.i. and washed with PBS. 20 mg of infected cells (containing about 10^{10} *Waddlia* cells) were collected by centrifugation for 10 minutes at 10'000 x *g* and were incubated for 1h at 95°C in order to heat-inactivate the bacteria. Cell wall isolation, digestion and HPLC was performed by Cecolabs (Tuebingen, Germany), using a protocol adapted from (de Jonge et al., 1992a, b). Briefly, cells were boiled in sodium dodecyl sulfate (SDS) and the cell wall was harvested by centrifugation and broken with glass beads. Broken cell wall was then

digested with mutanolysin, MltA or CwlT. The analysis was done on a C18 column (MZ-Analysentechnik, Mainz, Germany) on a Agilent 1200 system (Agilent, Santa Clara, CA) using a 150 minutes gradient from 5% to 30% methanol in sodium phosphate buffer. Quantitative analysis of selected peaks was done by integration of the peak area using the trapezoidal rule. The area of each peak was then used to derive the ratio of cell wall components among the different strains, by normalizing each peak area to the total of the peaks present in each HPLC analysis run.

Detection of *Waddlia* PG by hNod expressing cells

Isolated *Waddlia* PG was tested using HEK-Blue hNod1 and hNod2 kits (InvivoGen, San Diego, CA), following the manufacturer's indications. Briefly, 20 µl of standards and isolated PG were added in 96 well plate to $3 \cdot 10^5$ cells expressing hNod1 (resp. hNod2) resuspended in HEK-Blue detection medium. The suspension was then incubated for 16h and activation of Nod receptors was detected at 620 nm using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany).

Characterization of PG-binding proteins by mass spectrometry

Cell wall of *W. chondrophila* was isolated as described above, but in absence of protease treatments. This cell wall was then washed by successive boilings in 2% and 4% SDS. Samples taken before and after the washes were analyzed by mass spectrometry (Protein analysis facility of the University of Lausanne, Switzerland). Ratio of peptide abundance between both samples was calculated and proteins showing enrichment after washing were selected as PG-binding candidates.

Murein (sacculi) pull-down assay

Pal^{Wch}-His$_6$ was overproduced in *E. coli* Rosetta (λDE3)/pLys. Protein was purified by nickel affinity chromatography as described above, concentrated by ultrafiltration in Amicon 3K columns (Millipore, Darmstadt, Germany) and stored at -80°C in binding buffer (20 mM Tris-HCl, 1 mM $MgCl_2$ 30 mM NaCl, 0.05% Triton X-100, pH 6.8) containing 50% glycerol. Bradford assay was used to determine the protein concentration in each sample. *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* mureins (sacculi) were purchased from Anasyn (Tuebingen, Germany) and resuspended in binding buffer at a concentration of 10 mg/ml. Pal^{Wch}-His$_6$ (6µg) was added to 1 mg of murein in a total volume of 0.2 ml and incubated on ice for 30 minutes. Murein from samples was collected by centrifugation using a Beckman SW55Ti rotor at 303648 × *g* for 30 min at 4°C. Sedimented murein was resuspended in 0.1 ml of cold Binding buffer and centrifuged again. Murein pellets were resupended in 0.1 ml of cold binding buffer. The supernatant of the first centrifugation step (S), the supernatant of the washing step (W) and the pellet (P) were analysed by SDS-PAGE followed by immunoblot with anti-His$_6$ antiserum (1:2000 dilution, Cell Signaling, Danvers, MA) (see supplemental experimental procedures for details).

Immunofluorescence labeling

Immunofluorescence was performed as described (Croxatto and Greub, 2010; Jacquier et al., 2014). Infected Vero cells on coverslips were fixed by incubation for 5 minutes in ice-cold methanol for 5 minutes. Cells were then washed 3 times with PBS and subsequently blocked and permeabilized for a minimum of 1 h in blocking buffer (PBS, 0.1% saponin, 1% BSA). Coverslips were then incubated with primary antibodies directed against bacteria in blocking buffer for 1 h at room temperature, washed 3 times with PBS supplemented with 0.1% saponin and incubated for 1 h with secondary antibodies in blocking buffer containing DAPI (Molecular Probes). Coverslips were then washed 3 times with PBS containing 0.1% saponin, once with

PBS and a last time with distilled water, before they were mounted onto glass slides using Mowiol as mounting medium (Sigma-Aldrich).

Confocal and fluorescence microscopy

Confocal microscopy pictures were taken using a Zeiss LSM 510 Meta (Zeiss, Oberkochen, Germany). Images treatment and quantification were then performed using the ImageJ software (http://www.macbiophotonics.ca).

Quantitative PCR

Infection was quantified by real-time PCR, as described (Jacquier et al., 2014). Cells were resuspended by scrapping at different time points after infection. Genomic DNA was extracted from 50 µl of cell suspension using the Wizard SV Genomic DNA purification system (Promega, Madison, WI) and eluted in 200 µl of water. 5 µl of DNA were then mixed with iTaq supermix with ROX (BioRad, Hercules, CA), 200 nM of primers WadF4 and WadR4 and 100 nM of probe WadS2(Goy et al., 2009). Quantitative PCR conditions were 3 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Amplification and detection of the PCR products were performed using a stepOne Plus Real-time PCR System (Applied Biosystems, Carlsbad, CA).

RNA extraction, cDNA synthesis and qPCR

RNA quantification was performed as described(Jacquier et al., 2014). Briefly, 500 µl of infected cell were harvested at the given time points and mixed with 1 ml of RNA Protect (Qiagen, Venlo, Netherlands). The suspension was then incubated for 5 minutes at room temperature and then centrifuged for 10 minutes at 5000 x *g*. The resulting pellet was frozen at -80°C. RNA was extracted from the pellet using the RNeasy Plus kit (Qiagen). DNA was selectively digested by DNAse, using Ambion DNA-free kit (Life technologies, Grand Island, NY).

cDNA was then reverse transcribed using a Goscript Reverse Transcription System (Promega, Fishburg, WI). qPCR was performed on 4 μl of cDNA with addition of 10 μl of iTaq Universal SYBR Green mix (BioRad, Hercules, CA), 4.8 μl of water and 0.6 μl of each specific forward and reverse primers targeting the 16S rRNA encoding gene and the *pal, tolA, tolB, TolQ or TolR* genes. Cycling conditions were 3 min at 95°C followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. A stepOne Plus Real-time PCR System (Applied Biosystems, Carlsbad, CA) was used for amplification and detection of the PCR products.

Author contributions

All authors participated in the design of experiments and in the manuscript writing. NJ performed the peptidoglycan-binding protein analysis and experiments of expression and localization of the Pal-Tol complex in *W. chondrophila*. AF did the complementation studies in *E. coli* and the *in vitro* PG binding assays.

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Figure legends

Figure 1: Known cell wall hydrolytic enzymes liberate cell wall fragments from *W. chondrophila*. Vero cells infected with *W. chondrophila* in presence of the indicated antibiotics, were harvested 28h p.i. and washed with PBS. Bacteria were heat-inactivated at 95°C for 1 h. The bacterial cell wall was isolated and digested with mutanolysin (A), MltA (B) and CwlT (C-D-E). Cell wall fragments were separated by HPLC. (a,b,c,d,e) peaks showing a decrease after treatment with penicillin or phosphomycin, percentages shown in red.

Figure 2: Peptidoglycan isolated from *W. chondrophila* is recognized by Nod1 and Nod2 receptors. HEK cells expressing the hNod1 receptor (A) or the hNod2 receptor (B) were incubated with the indicated concentration in μg/ml of the following standards: C12-iE-DAP (acylated derivative of iE-DAP), iE-DAP (D-γ-Glu-mDAP), MDP (MurNAc-L-Ala-D-isoGln), L18-MDP (synthetic derivative of MDP), M-TriDAP (MurNAc-L-Ala-D-γ-Glu-mDAP), M-TriLYS (MurNAc-Ala-D-isoGln-Lys), murabutide (MurNAc-Ala-D-isoGlnOBu), TriDAP (L-Ala-γ-D-Glu-

mDAP), PG^{Eco} (PG of *E. coli*), PG^{Sau} (PG of *S. aureus*). The indicated concentrations in μg/ml of PG^{Wch} (PG of *W. chondrophila*) were used. Highest response is normalized as 100 %. Error bars show standard deviation of 3 independent experiments.

Figure 3: Pal is conserved among *Chlamydiales*. A) Pal strongly binds to isolated PG of *W. chondrophila*. PG was isolated from Vero cells infected with *W. chondrophila* 28h p.i. in absence of proteolysis. Resulting material was washed by incubating successively in 2% and 4% SDS at 37°C for 30 minutes. Samples of washes and washed PG were then resuspended in loading buffer and proteins were detected by Western blotting. B) Predicted domain organization of *W. chondrophila* Pal: SS, Signal Sequence and, in green, OmpA-like PG binding domain PF00691. C) The features of the OmpA-like PG binding domain are conserved. Amino acid sequences of the OmpA-like PG binding domain of PF00691 (ompAEco), Pal^{Eco}, Pal^{Wch}, and Pal^{Pac} were aligned using MUSCLE. Amino acids are highlighted for their conservation and charge. Point mutation obtained and used in the experiments in figure 2 are marked with asterisks.D) Tol-pal locus organization in *Waddlia chondrophila* (*Wch*), *Simkania negevensis* (*Sne*), *Parachlamydia acanthoamoebae* (*Pac*) and *Chlamydia trachomatis* (*Ctr*). Orthologs proteins were searched by BBH using as query *W. chondrophila* Tol-Pal protein sequences. Arrows orientations do not represent the actual strand orientation of the tol-pal locus in the genome.

Figure 4: Pal is transcribed and expressed in *W. chondrophila*. A) Transcription of Pal is maximal 16 h p.i.. RNA was extracted from Vero cells infected with *W. chondrophila* at the indicated time points p.i.. Pal RNA was then quantified by qRT-PCR and normalized by comparison with the 16S rRNA. Error bars represent standard deviations of two independent experiments. B) Pal protein is expressed in *W. chondrophila*. Antibodies raised against purified heterogously expressed Pal recognize a protein of 29kDa in protein extracts of *W. chondrophila*-infected vero cells at the given time points by Western blotting. An antibody raised against the

whole inactivated *W. chondrophila* was used as control (Wad). Corresponding bands were quantified using Image J and fold increase of Pal in comparison with Wad is represented. Error bars represent the standard deviation of two independent experiments. C) Pal expression is restricted to RBs. Pre-immune control serum and serum harvested after immunization were used for immunofluorescence staining of Pal. Only RBs (arrow) and not EBs (arrowhead) contain a detectable amount of Pal. Addition of DTT to increase EBs permeability does not increase EBs labeling (Bar = 20 μm). D) Pal localizes at the division septum in a PG and MreB-dependent manner. Vero cells infected by *W. chondrophila* were treated with the indicated antibiotic 2h p.i., fixed 24h p.i. and processed for immunofluorescence (Bar = 1 μm). Enrichment of Pal at mid-cell was quantified and results are provided in percentage for each condition. Standard deviation of two independent experiments is also given. The few aberrant bodies (<10%) still exhibiting some accumulation of Pal at mid-cell are likely aberrant bodies presenting some degree of invagination at mid-cell. Arrowheads show foci of Pal accumulation.

Figure 5: Pal^{Wch} binds PG in vitro and its PG binding activity can be measured in a *E. coli* system. A-B) Pal^{Wch} binds preferentially to Gram-negative PG *in vitro*. His$_6$-Pal^{Wch} was incubated with or without *E. coli, B. Subtilis*, *S. aureus* PG, or *W. chondrophila* PGLS. PG was then pelleted by ultracentrifugation and washed once with buffer. His$_6$-Pal^{Wch} was detected by immunoblotting in the supernatant (S), the wash fraction (W) and the pellet fraction (P). The size markers are indicated in kDa. C-D) Mutations in conserved residues (D180A/R195A) mapping in the OmpA-like PG binding domain of Pal^{Wch} abolish the binding to E. coli murein. Immunoblotting with antibodies against His$_6$-tag was used to reveal His$_6$-Pal^{Wch} and the mutant variant in the supernatant (S), the wash fraction (W), the pellet fraction (P) and the supernatant

of the E. coli cells lysate (L). The insoluble pellet fraction resulting from the co-pelletting assay of His_6-Pal^{Wch} with *E. coli* murein were washed thrice with 4% SDS and the resulting washing steps (W1, W2 and W3) were loaded on the SDS-PAGE gel without previous boiling. The size markers are indicated in kDa. E) Effect of *pal*^{Wch} orthologs expression in Δ*pal*^{supp} on McConkey supplemented with 0.5% Glucose (McCG). Basal expression of Pal^{Wch} and Pal^{Pac} are sufficient to restrict the growth of Δ*pal*^{supp} on McCG, conversely basal level of Pal^{Eco} supports the growth of ΔPal^{supp}. F) Point mutations in the OmpA-like PG binding domain of *pal*^{Wch} prevent the letal effect of wild type *pal*^{Wch} expression on McCG. G) Pal^{Wch} point mutants accumulate at levels similar to that of Pal^{Wch} WT. Overnight cultures were diluted in fresh LB media supplemented or not with 1 mM IPTG to an OD = 0.1 and grown for 4h at 30°C. OD were measured at t= 4h and adjusted to 0.5 for all the cultures. Proteins in the resulting cell extracts were detected by immunoblotting with anti-Pal^{Wch} antiserum.

Figure 6: Members of the Tol complex are expressed in *W. chondrophila* and localize to the division septum in a PG-dependent manner. A) Transcription of TolA,B,Q and R are maximal between 8 and 16 h p.i.. qPCR was performed as explained earlier. Error bars represent standard deviations of two independent experiments. B) TolAR antibody is specific. TolAR antibodies were produced by immunization of a rabbit with a TolAR fusion protein. Serum before and after immunization were used for immunofluorescence as described earlier (Bar = 20 μm). C) Tol complex localizes to the division septum in a PG-dependent manner. Immunofluorescence was performed after treatment or not with the indicated antibiotics (Bar = 1 μm).

Figure 7: Model of the maintenance of membrane integrity by the Pal-Tol complex during division. A) early organization of the division site. B) early invagination and divisome organization. C) Late components of the divisome. OM: outer membrane, PG: peptidoglycan, IM: inner membrane. TolA,B.Q and R are depicted by their last letter only.

Figure S1: OmpA protein levels are lower in the ∆pal^{supp} mutant. Steady-state levels of *E. coli* OmpA (OmpA^{Eco}, arrowhead) in *E. coli* WT, ∆pal, ∆pal^{supp} and ∆ompA cell lysates (mutants shown in duplicate) determined by immunoblotting using polyclonal antibodies targetting OmpA^{Eco} (upper panel). The same relative amounts of cell lysates were resolved by SDS-PAGE and stained using Coomassie Brilliant Blue to confirm that same amounts of total proteins were loaded in each lane (lower panel).

Figure S2: *W. chondrophila* is partially sensitive to vancomycin and teicoplanin. A) Vancomycin and teicoplanin, but not flavomycin induce the formation of aberrant bodies in *W. chondrophila*. Vero cells infected with *W. chondrophila* were treated with the indicated antibiotic 2 h p.i. and then processed for immunofluorescence. Host cells were stained with concanavalin A (Conc. A) (Bar = 10 μm). B) Vancomycin and teicoplanin did not affect the replication of bacterial DNA, despite these antibiotics strongly affected *W. chondrophila* proliferation. Infected Vero cells were treated or not with the indicated antibiotic at the given concentrations (μg/ml) 2h p.i. and harvested at the given time points. DNA was extracted and *W. chondrophila* 16S DNA was quantified by qPCR. C) Vancomycin and teicoplanin treatment disturb the proper localization of important septal proteins RodZ, MreB and NlpD. Infected Vero cells were treated as in (A) and the indicated proteins were detected (green) (Bar = 2 μm).

Table S1: Proteins enriched in washed PG. PG isolated from *W. chondrophila* in absence of proteolysis was successively washed by boiling in 2% and 4% SDS. PG was then harvested and samples before and after washing were analyzed by mass spectrometry. Proteins enriched after washing are presented in this table.

Name	Description	Cell wall	Washed cell wall	Enrichment	Signal sequence
ClpC	ATP-dependent Clp protease ATP-binding subunit	0.10	0.34	3.35	
Mdh	Malate dehydrogenase	0.10	0.25	2.51	
GyrA	DNA gyrase subunit A	0.15	0.36	2.42	
DnaJ	Chaperone protein	0.15	0.31	2.05	
Pnp	Polyribonucleotide nucleotidyltransferase	0.40	0.70	1.75	
AcpP	Acyl carrier protein	0.10	0.17	1.68	
SecA	Protein translocase subunit	0.30	0.50	1.68	Y
ClpX	ATP-dependent Clp protease ATP-binding subunit	0.15	0.22	1.49	
TrxA	Thioredoxin	0.15	0.22	1.49	
wcw_1035	Uncharacterized protein	0.10	0.14	1.40	
wcw_1672	ABC-type transporter, ATPase subunit	0.10	0.14	1.40	
Gnd	6-phosphogluconate dehydrogenase	0.10	0.14	1.40	
GroES3	10 kDa chaperonin	0.25	0.34	1.34	
RpoC	DNA-directed RNA polymerase subunit beta'	2.00	2.66	1.33	
PepF	Oligoendopeptidase F	0.25	0.31	1.23	

	RpoB	DNA-directed RNA polymerase subunit beta	2.15	2.57	1.20	
	Tig	Putative trigger factor	0.30	0.34	1.12	
	Efp3	Elongation factor P	0.25	0.28	1.12	
	Pal	**Peptidoglycan-associated lipoprotein**	**0.15**	**0.17**	**1.12**	**Y**
	DnaN	DNA polymerase III subunit beta	0.75	0.81	1.08	
	NusA	Transcription elongation protein	0.50	0.53	1.06	
	MreB	Actin-like ATPase involved in cell morphogenesis	0.45	0.48	1.06	
	NrdA	Ribonucleoside-diphosphate reductase	2.15	2.27	1.05	
	ClpP3	ATP-dependent Clp protease proteolytic subunit	0.30	0.31	1.02	
	FbaB	Fructose-bisphosphate aldolase	0.25	0.25	1.01	

Table S2: Proteins only detected in washed PG. PG was treated as described for table S1. Proteins detected only in washed PG are presented in this table.

Name	Description	Washed cell wall	Signal sequence	Conserved in *Chlamydiaceae*
wcw_0501	Uncharacterized protein	0.56	Y	Y
wcw_0969	Uncharacterized protein	0.48		Y
GreA	Transcription elongation factor	0.36		Y
PheT	Phenylalanine--tRNA ligase beta subunit	0.31		Y
GltX	Glutamate--tRNA ligase	0.25		Y
FtsH	ATP-dependent zinc metalloprotease	0.22		Y
GatA	Glutamyl-tRNA(Gln) amidotransferase subunit A	0.22		Y
GspD	Putative general secretion pathway protein D	0.22	Y	Y
AccC	Biotin carboxylase	0.20		Y
ProS	Proline--tRNA ligase	0.20		Y
PyrG	CTP synthase	0.17	Y	Y
wcw_1595	SWI/SNF helicase 2 family protein	0.14		Y
wcw_0579	Proline amido peptidase	0.14		Y
ClpB	ATP-dependent Clp protease, ATP-binding subunit ClpB	0.08		Y
PolA	DNA polymerase I	0.06		Y

Figure 1

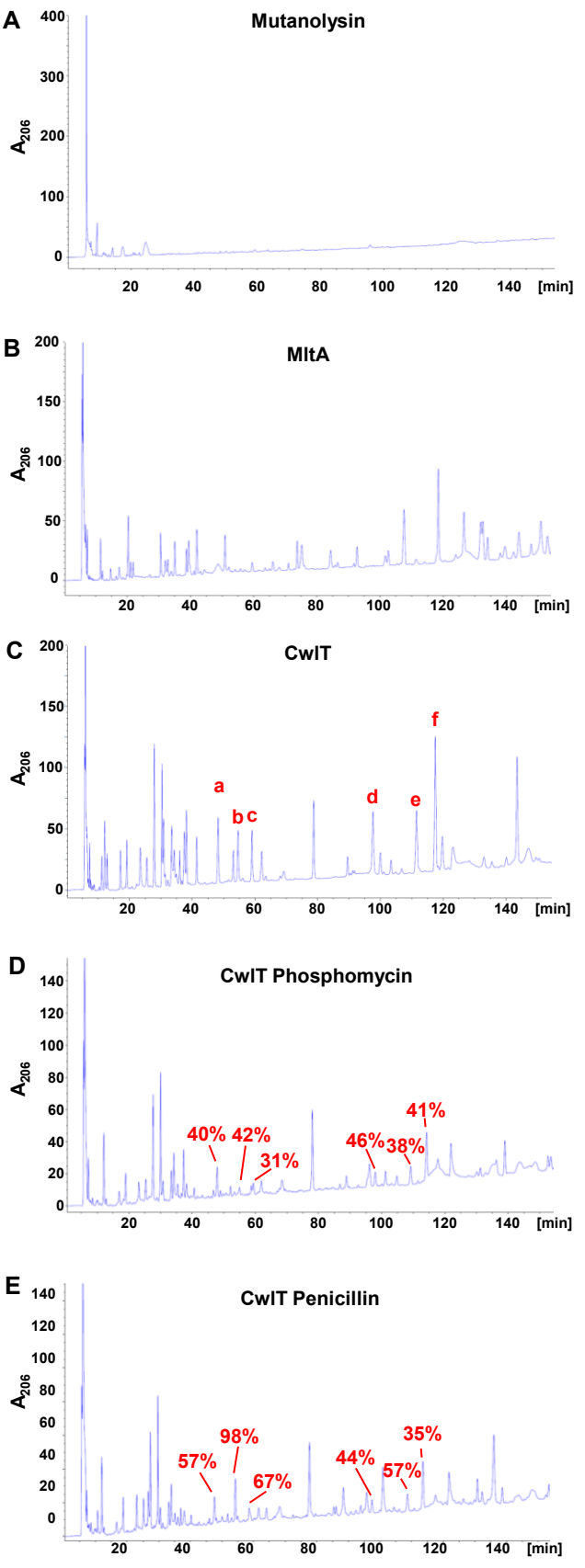
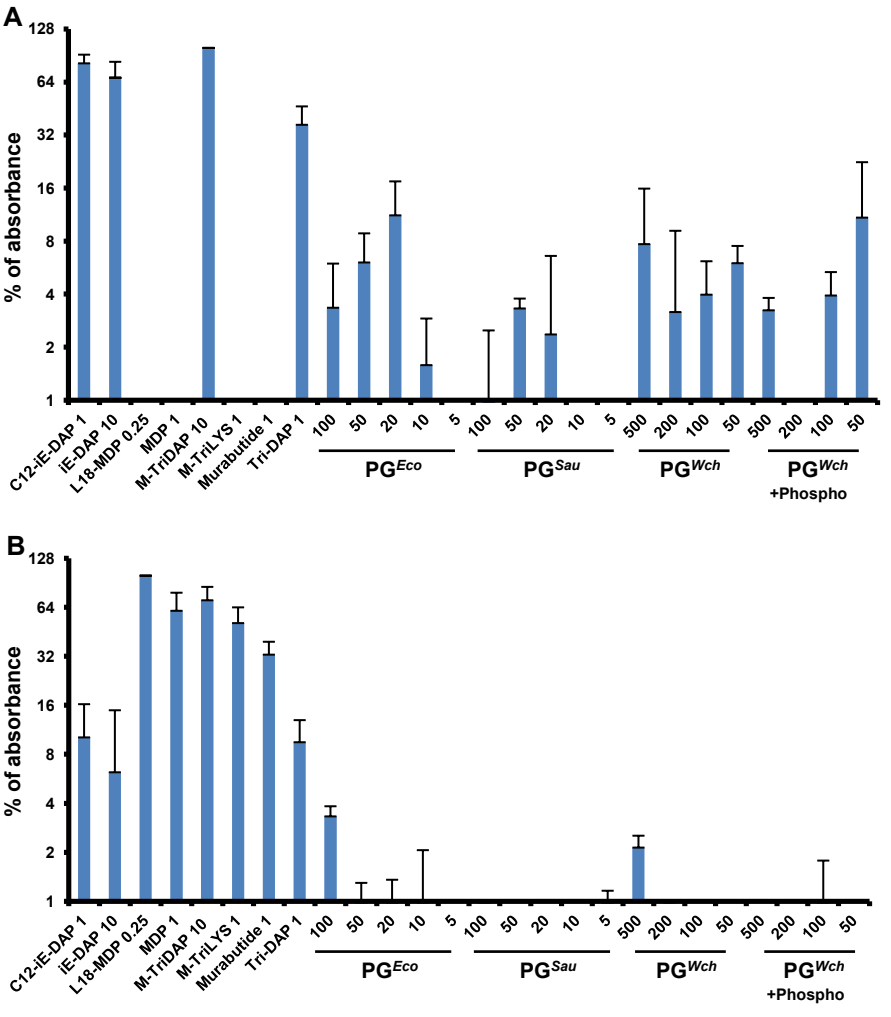

A
Mutanolysin
B
MltA
C
CwlT
a
b c
d
e
f
D
CwlT Phosphomycin
40%
42%
31%
46%
38%
41%
E
CwlT Penicillin
57%
98%
67%
44%
57%
35%
A206
[min]

Figure 2


A
% of absorbance
128
64
32
16
8
4
2
1
C12-iE-DAP 1
iE-DAP 10
L18-MDP 0.25
MDP 1
M-TriDAP 10
M-TriLYS 1
Murabutide 1
Tri-DAP 1
100 50 20 10 5
100 50 20 10 5
500 200 100 50
500 200 100 50
PG Eco
PG Sau
PG Wch
PG Wch +Phospho
B
% of absorbance
128
64
32
16
8
4
2
1
C12-iE-DAP 1
iE-DAP 10
L18-MDP 0.25
MDP 1
M-TriDAP 10
M-TriLYS 1
Murabutide 1
Tri-DAP 1
100 50 20 10 5
100 50 20 10 5
500 200 100 50
500 200 100 50
PG Eco
PG Sau
PG Wch
PG Wch +Phospho

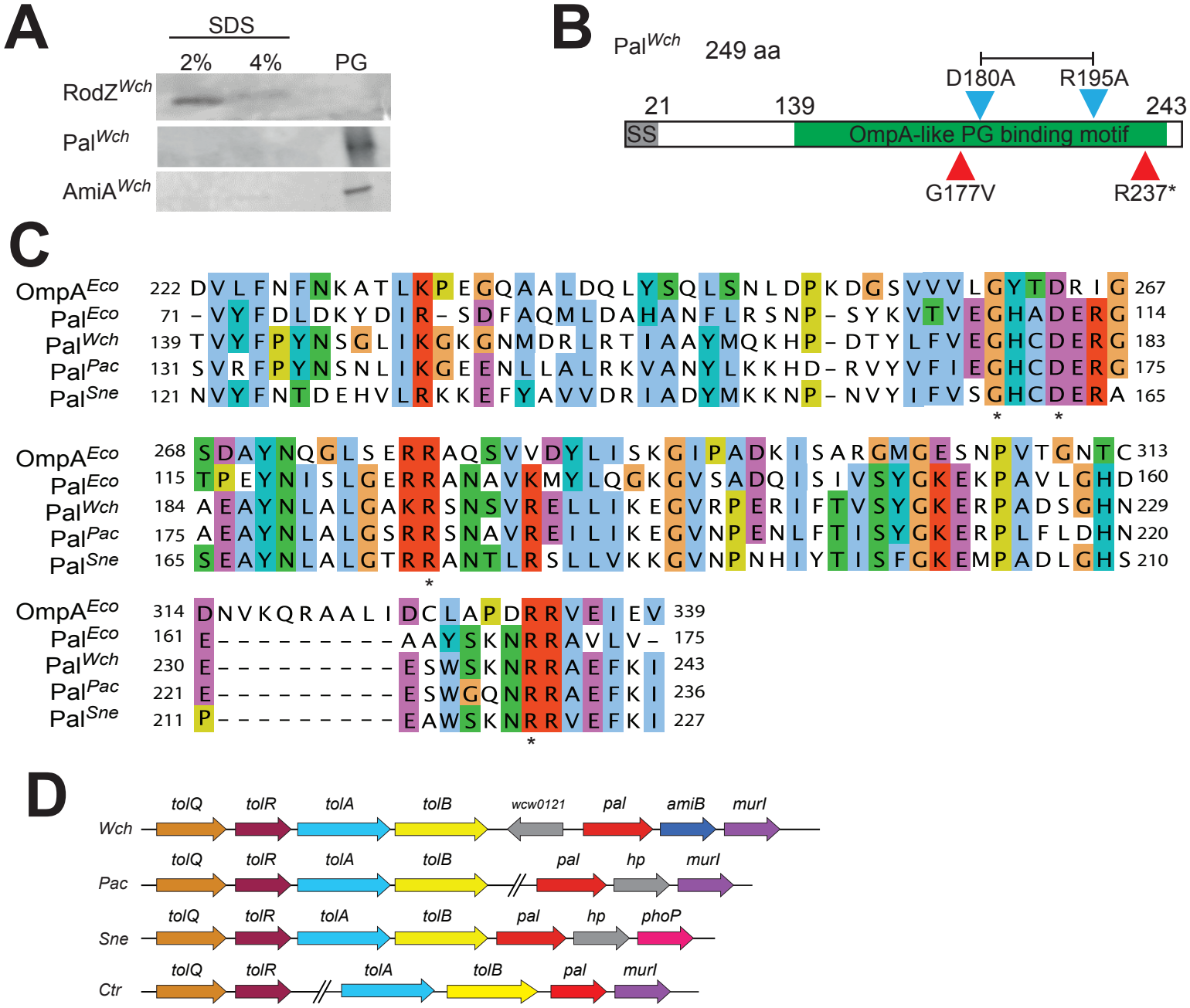

A
SDS
2%
4%
PG
RodZWch
PalWch
AmiAWch
B
PalWch
249 aa
D180A
R195A
21
139
243
SS
OmpA-like PG binding motif
G177V
R237*
C
OmpAEco 222 DVLFNFNKATLKPEGQAALDQLYSQLSNLDPKDGSVVVLGYTDRIG 267
PalEco 71 -VYFDLDKYDIR-SDFAQMLDAHANFLRSNP-SYKVTVEGHADERG 114
PalWch 139 TVYFPYNSGLIKGKGNMDRLRTIAAYMQKHP-DTYLFVEGHCDERG 183
PalPac 131 SVRFPYNSNLIKGEENLLALRKVANYLKKHD-RVYVFIEGHCDERG 175
PalSne 121 NVYFNTDEHVLRKKEFYAVVDRIADYMKKNP-NVYIFVSGHCDERA 165
OmpAEco 268 SDAYNQGLSERRAQSVVDYLISKGIPADKISARGMGESNPVTGNTC 313
PalEco 115 TPEYNISLGERRANAVKMYLQGKGVSADQISIVSYGKEKPAVLGHD 160
PalWch 184 AEAYNLALGAKRSNSVRELLIKEGVRPERIFTVSYGKERPADSGHN 229
PalPac 175 AEAYNLALGSRRSNAVREILIKEGVNPENLFTISYGKERPLFLDHN 220
PalSne 165 SEAYNLALGTRRANTLRSLLVKKGVNPNHIYTISFGKEMPADLGHS 210
OmpAEco 314 DNVKQRAALIDCLAPDRRVEIEV 339
PalEco 161 E---------AAYSKNRRAVLV- 175
PalWch 230 E---------ESWSKNRRAEFKI 243
PalPac 221 E---------ESWGQNRRAEFKI 236
PalSne 211 P---------EAWSKNRRVEFKI 227
D
Wch
tolQ tolR tolA tolB wcw0121 pal amiB murI
Pac
tolQ tolR tolA tolB pal hp murI
Sne
tolQ tolR tolA tolB pal hp phoP
Ctr
tolQ tolR tolA tolB pal murI

Figure 3

Figure 4

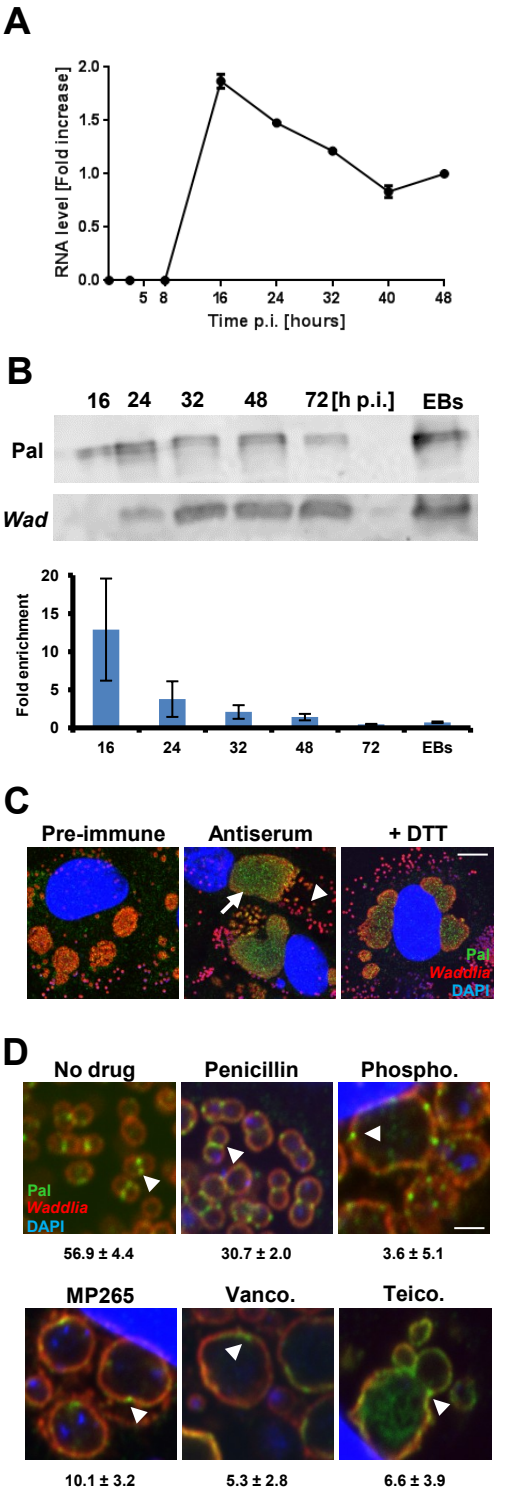

A
RNA level [Fold increase]
2.0
1.5
1.0
0.5
0.0
5 8 16 24 32 40 48
Time p.i. [hours]
B
16 24 32 48 72 [h p.i.] EBs
Pal
Wad
Fold enrichment
20
15
10
5
0
16 24 32 48 72 EBs
C
Pre-immune
Antiserum
+ DTT
Pal
Waddlia
DAPI
D
No drug
Penicillin
Phospho.
Pal
Waddlia
DAPI
56.9 ± 4.4
30.7 ± 2.0
3.6 ± 5.1
MP265
Vanco.
Teico.
10.1 ± 3.2
5.3 ± 2.8
6.6 ± 3.9

Figure 5

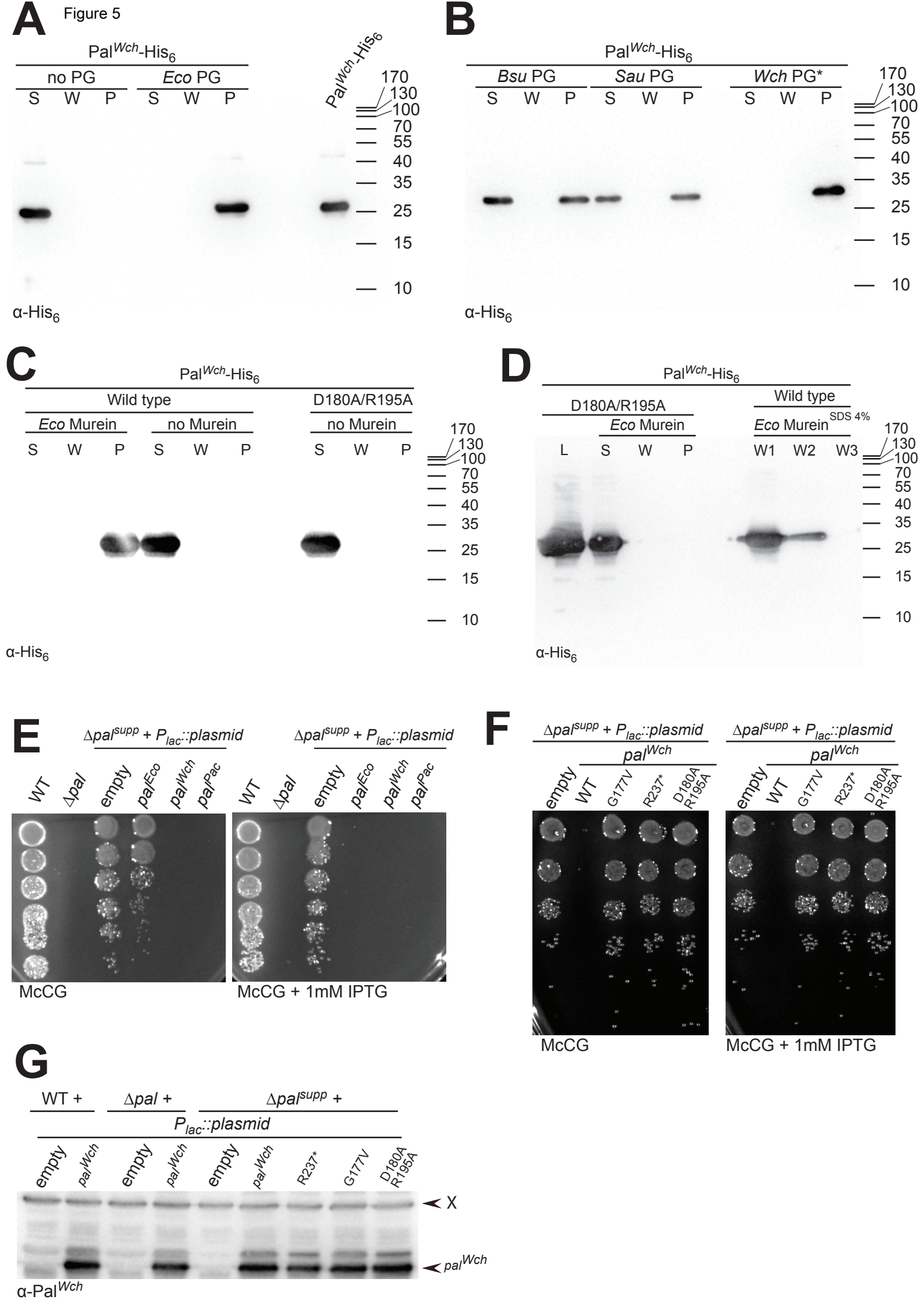

A
Pal^Wch-His6
no PG
Eco PG
S W P S W P
Pal^Wch-His6
170
130
100
70
55
40
35
25
15
10
α-His6
B
Pal^Wch-His6
Bsu PG
Sau PG
Wch PG*
S W P S W P S W P
170
130
100
70
55
40
35
25
15
10
α-His6
C
Pal^Wch-His6
Wild type
D180A/R195A
Eco Murein
no Murein
no Murein
S W P S W P S W P
170
130
100
70
55
40
35
25
15
10
α-His6
D
Pal^Wch-His6
D180A/R195A
Wild type
Eco Murein
Eco Murein SDS 4%
L S W P W1 W2 W3
170
130
100
70
55
40
35
25
15
10
α-His6
E
Δpal^supp + P_lac::plasmid
Δpal^supp + P_lac::plasmid
WT
Δpal
empty
pal^Eco
pal^Wch
pal^Pac
WT
Δpal
empty
pal^Eco
pal^Wch
pal^Pac
McCG
McCG + 1mM IPTG
F
Δpal^supp + P_lac::plasmid
pal^Wch
Δpal^supp + P_lac::plasmid
pal^Wch
empty
WT
G177V
R237*
D180A R195A
empty
WT
G177V
R237*
D180A R195A
McCG
McCG + 1mM IPTG
G
WT +
Δpal +
Δpal^supp +
P_lac::plasmid
empty
pal^Wch
empty
pal^Wch
empty
pal^Wch
R237*
G177V
D180A R195A
X
pal^Wch
α-Pal^Wch

Figure 6

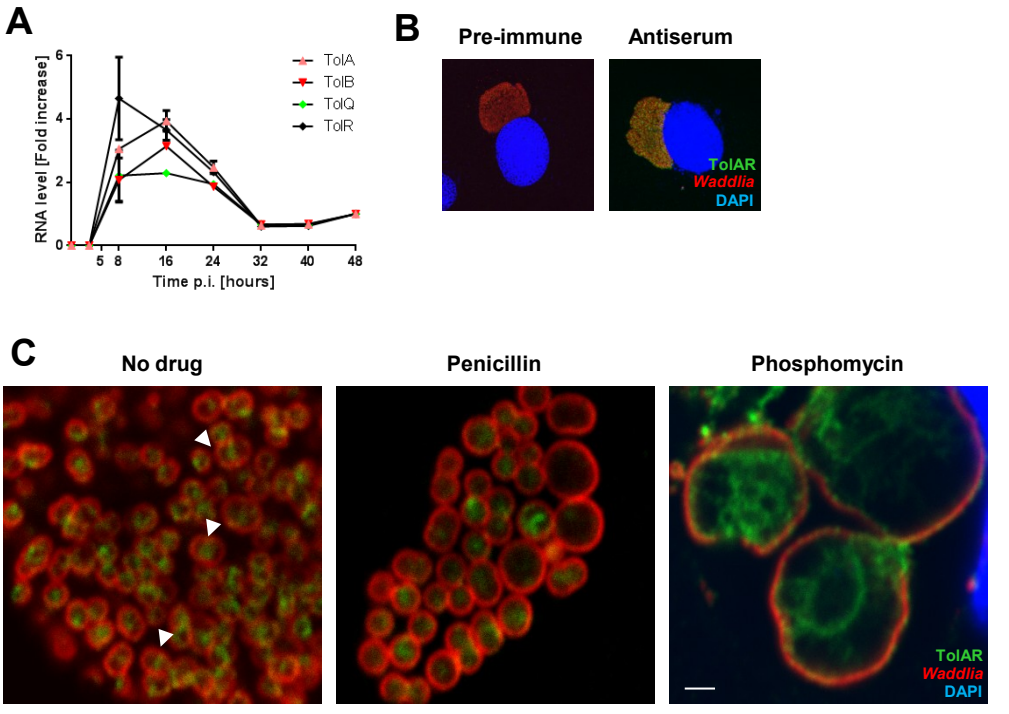
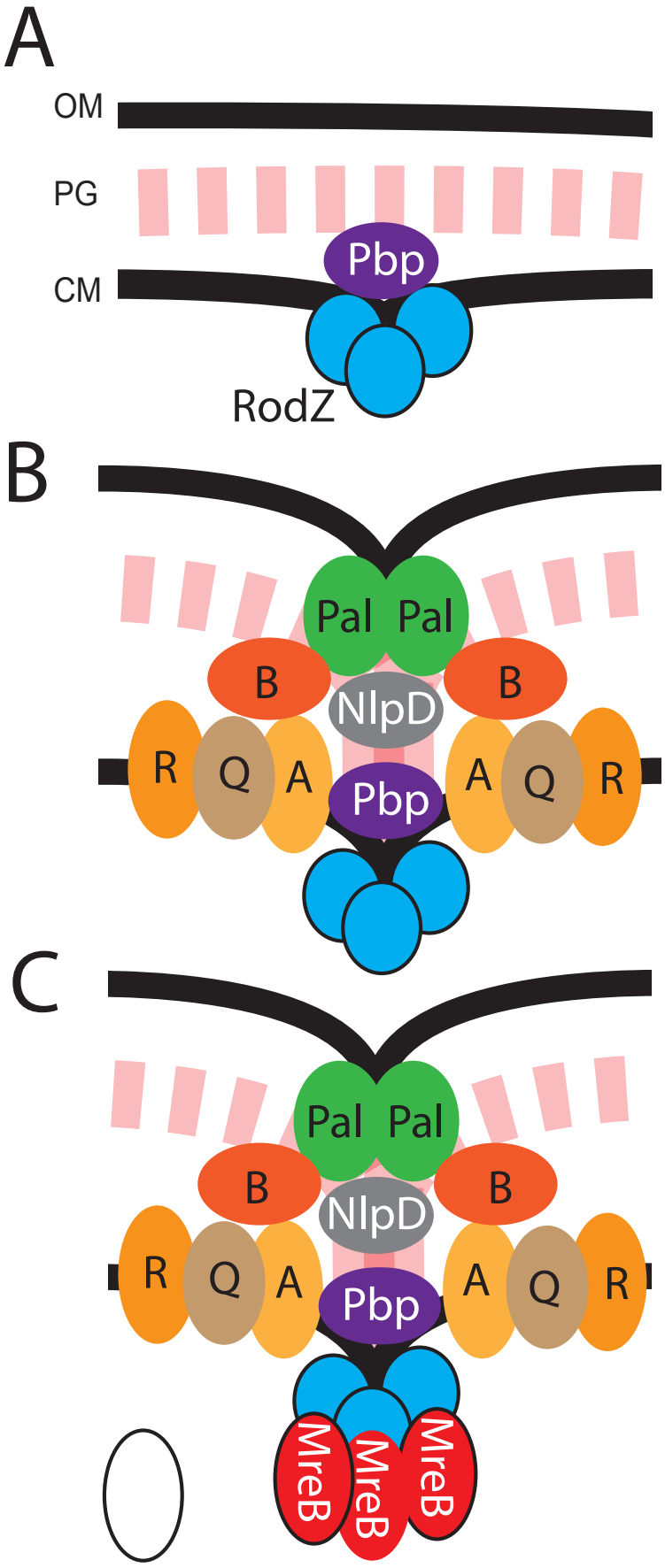

A
RNA level [Fold increase]
Time p.i. [hours]
TolA
TolB
TolQ
TolR
B
Pre-immune
Antiserum
TolAR
Waddlia
DAPI
C
No drug
Penicillin
Phosphomycin
TolAR
Waddlia
DAPI

Figure 7


A
OM
PG
CM
Pbp
RodZ
B
Pal Pal
B
B
NlpD
R Q A
A Q R
Pbp
C
Pal Pal
B
B
NlpD
R Q A
A Q R
Pbp
MreB
MreB
MreB

Highlights

1) We analyzed all proteins binding to chlamydial peptidoglycan (PG), including Pal
2) We confirmed that Pal is binding to PG both *in vivo* and *in vitro*
3) We showed that Pal localizes at the division septum in a PG-dependent process
4) We showed that vancomycin inhibits chlamydial division, dispersing Pal

eToc

Jacquier et al. demonstrated the presence of a functional chlamydial Tol-Pal complex at the division septum, which is bound to chlamydial peptidoglycan and apparently plays an important role in the maintenance of the bacterial membrane integrity.

Figure S1

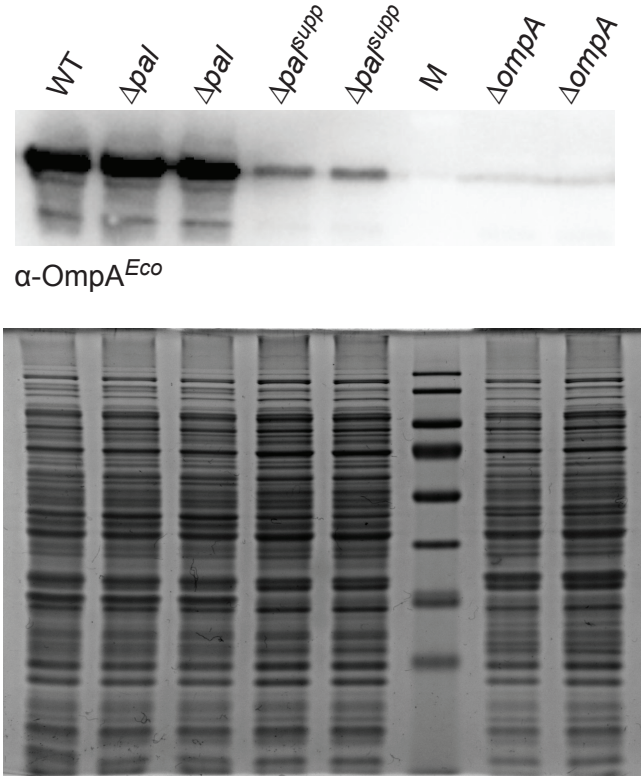
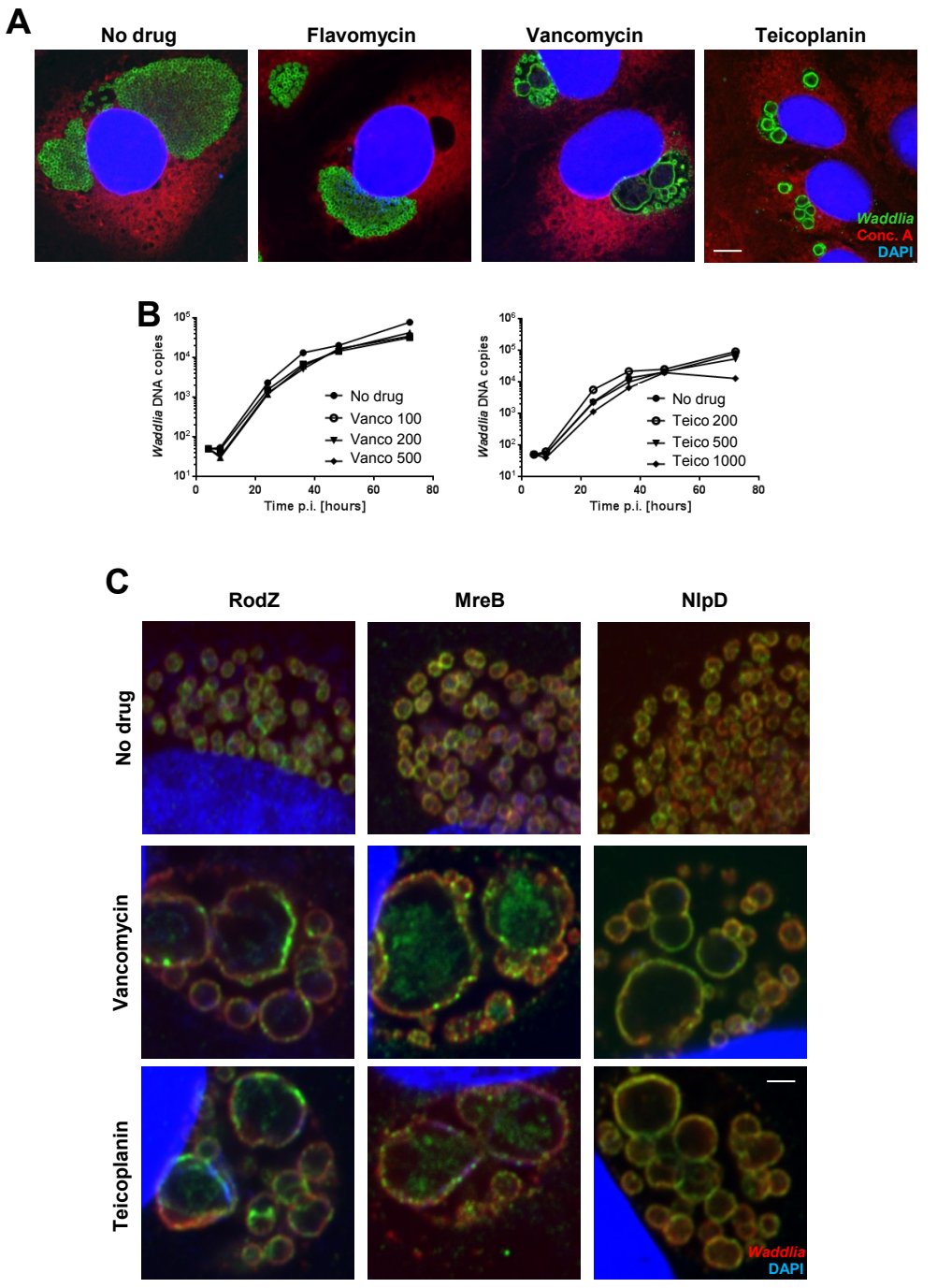

WT
Δpal
Δpal
Δpal^supp
Δpal^supp
M
ΔompA
ΔompA
α-OmpA^Eco
Total Protein

Figure S2


A
No drug
Flavomycin
Vancomycin
Teicoplanin
Waddlia
Conc. A
DAPI
B
Waddlia DNA copies
Time p.i. [hours]
No drug
Vanco 100
Vanco 200
Vanco 500
Teico 200
Teico 500
Teico 1000
C
RodZ
MreB
NlpD
No drug
Vancomycin
Teicoplanin
Waddlia
DAPI

Supplemental experimental procedures

Strain construction

Strain Δ*pal*::*Km*^{R} (MG5) (Gerding et al., 2007) is a derivative of TB28 (Δ*lacZYA <frt>)*. It was kindly provided by Prof. Piet de Boer (Case Western Reserve University, Cleveland, OH).

Plasmids and site directed mutagenesis

Plasmids used in this study were maintained in the *E. coli* cloning strain EC100D (Epicentre, Madison, WI). The expression construct for *pal*^{Wch} (Wcw_0122) (nt: 126916-127662 of the *Waddlia chondrophila* genome, accession number NC_014225.1), *pal*^{Pac} (nt: 119153-119869 of the *Parachlamydia acanthoamoebae* genome, accession number NC_015702.1), and *pal*^{Eco} (nt: 778290-778811 of the *Escherichia coli* MG1655 genome, accession number NC_000913.3) were made by amplification of *pal*^{Wch}, *pal*^{Pac}, and *pal*^{Eco} as 746, 716, and 521 bp fragments, respectively, flanked by an *Nde*I site at the 5' end and an *Eco*RI site at the 3' end. These fragments were ligated into pMT335 that had been restricted with *Nde*I and *Eco*RI. The resulting plasmids were confirmed by sequencing and used to transform *E. coli* strains.

pSRK (Gm^{R}) plasmid constructions were made by liberating *pal*^{Wch}, *pal*^{Pac}, and *pal*^{Eco} from pMT335 recombinant plasmids by digestion with *Nde*I and *Xba*I. The resulting fragments were ligated in pSRK (Gm^{R}) that has been digested with the same restriction endonucleases *Nde*I and *Xba*I. pSRK::*pal*^{Wch}, pSRK::*pal*^{Pac}, and pSRK::*pal*^{Eco} plasmids were confirmed by sequencing and introduced in *E. coli* by chemical transformation.

The overexpression construct for antibody production and murein pull-down assays of *pal*^{Wch} was made by amplification of a shorter fragment of *pal*^{Wch} flanked by *NdeI*/*Eco*RI recognition sequences. The first 22 aminoacids of *pal*^{Wch} were removed as they code for a putative signal sequence. The resulting fragment was then ligated into pET28a (Novagen, Darmstadt,

Germany) restricted with the same endonucleases *Nde*I and *Eco*RI. The resulting plasmids (pET28a::*pal*^{Wch}) was used to produce protein to raise antibodies, and in murein pull-down assays.

Pal^{Wch} synthetic point mutants were purchased as synthetic g-blocks from IDT (Integrated DNA Technology, Coralville, IA), amplified by Hi-Fidelity Taq polymerase and cloned in either in pMT335 or pSRK vectors.

Antibodies, probes and reagents

Polyclonal mouse and rabbit antibodies against *W. chondrophila* were produced locally as described previously (Croxatto and Greub, 2010). The secondary antibodies Alexa Fluor 488 goat anti-rabbit, 488 anti-mouse, 594 anti-rabbit and 594 anti-mouse were purchased from Molecular Probes (Grand Island, NY). Phosphomycin, penicillin, vancomycin and teicoplanin were obtained from Sigma-Aldrich.

His_6-Pal^{Wch} protein was expressed from pET28a::*pal*^{Wch} in *E. coli* Rosetta (DE3)/pLysS (Novagen) and purified under native conditions using Ni^{2+} chelate chromatography. A 5 mL overnight culture was diluted into 1 L of pre-warmed LB at 30°C. OD_{600nm} were monitored until OD_{600nm}=~ 0.3-0.4, then 1mM IPTG were added to the culture and growth continued. After 3 hours cells were pelleted, and resuspended in 25 mL of lysis buffer (10 mM Tris HCl pH8, 0.1 M NaCl, 1 mM ß-mercaptoethanol, 5% glycerol, 0.5 mM imidazole triton 0.02%). Cells were sonicated (Sonifier Cell Disruptor B-30; Branson Sonic power Co., Danbury, CT) on ice using 12 bursts of 20 seconds at output level 5.5. After centrifugation at 6'000 rpm the supernatant was loaded onto a column containing 5 mL of Ni-NTA agarose resin pre-equilibrated with lysis buffer. Column was rinsed with lysis buffer ,400 mM NaCl and 10 mM imidazole, both prepared in lysis buffer. Fractions were collected (in 300 mM Imidazole prepared in lysis buffer) and resolved on a 12.5% SDS polyacrylamide gel. The fractions containing the purified protein were used to

immunize New Zealand white rabbits (Josman LLC, Napa, CA) or mice (Eurogentec, Leuven, Belgium).

Protein extraction, SDS-PAGE, Western Blot

500 µl of infected cell culture were harvested at the indicated time points by centrifugation at 6'000 x *g* for 5 minutes. Proteins were extracted from the cell pellet by resuspension in loading buffer (60 mM Tris pH 6.8, 1% SDS, 1% mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and heated at 95°C for 10 min. 10 µl of the suspension were used to load on a 12.5% polyacrylamide precast gel (BioRad, Hercules, CA). After 45 min of migration at 200V, proteins were electrotransferred onto a nitrocellulose or PVDF membrane (Millipore) at 75V for 1 hour. The membrane was blocked with 5% milk for 2 hours and then incubated for at least 2 hours with the indicated antibody. An incubation of 2 hours with a HRP-conjugated goat anti-mouse or anti-rabbit antibody, depending on the primary antibody used, was then performed. Detection of HRP was performed using 0.03% hydrogen peroxide, 220 µg/ml luminol and 32.5 µg/ml coumaric acid in 0.1 M Tris pH 8.5 or Immobilon Western Blotting Chemoluminescence HRP substrate (Millipore). Chemiluminescence was recorded with the ImageQuant LAS 4000 Mini imager (GE healthcare, Waukesha, WI). Images were then treated using ImageJ.