

## REVIEW ARTICLE

# The role of peptidoglycan in chlamydial cell division: towards resolving the chlamydial anomaly

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One sentence summary: This review summarizes recent advances regarding the chlamydial division, including detection of chlamydial peptidoglycan and discovery of mid-cell localized division proteins, which allow a better understanding of the division of *Chlamydiales* in absence of an FtsZ homologue. Editor: Kenn Gerdes

## ABSTRACT

*Chlamydiales* are obligate intracellular bacteria including some important pathogens causing trachoma, genital tract infections and pneumonia, among others. They share an atypical division mechanism, which is independent of an FtsZ homologue. However, they divide by binary fission, in a process inhibited by penicillin derivatives, causing the formation of an aberrant form of the bacteria, which is able to survive in the presence of the antibiotic. The paradox of penicillin sensitivity of chlamydial cells in the absence of detectable peptidoglycan (PG) was dubbed the chlamydial anomaly, since no PG modified by enzymes (Pbps) that are the usual target of penicillin could be detected in *Chlamydiales*. We review here the recent advances in this field with the first direct and indirect evidences of PG-like material in both *Chlamydiaceae* and *Chlamydia*-related bacteria. Moreover, PG biosynthesis is required for proper localization of the newly described septal proteins RodZ and NlpD. Taken together, these new results set the stage for a better understanding of the role of PG and septal proteins in the division mechanism of *Chlamydiales* and illuminate the long-standing chlamydial anomaly. Moreover, understanding the chlamydial division mechanism is critical for the development of new antibiotics for the treatment of chlamydial chronic infections.

**Key words:** *Waddlia chondrophila*; cell division; contractile ring; MreB; RodZ; peptidoglycan; cell wall

## INTRODUCTION

*Chlamydiales* is an order of obligate intracellular bacteria comprising the *Chlamydiaceae* and the *Chlamydia*-related bacteria. Members of the *Chlamydiaceae* family include the well-known pathogens *Chlamydia trachomatis*, which causes genital tract infections and trachoma, and the causative agents of pneumonia, *C. pneumoniae* and *C. psittacci* (Greub 2009b). This family also contains important animal pathogens like *C. abortus*, which causes abortion in cattle (Vretou et al., 2001). *Chlamydia*-related bacteria were described more recently and there is increasing evi-

dence that at least some of them are indeed pathogenic. *Waddlia chondrophila*, for example, is thought to play a role in abortion in animals and miscarriage in humans (Rurangirwa et al., 1999; Baud et al., 2007, 2011; Baud, Regan and Greub 2008; de Barsey and Greub 2013). There are also good indications that *Parachlamydia acanthamoebae*, *Simkania negevensis* and *Protochlamydia naeglerio-phila* are agents of human pneumonia (Corsaro and Greub 2006; Casson et al., 2008; Greub 2009a; Lamoth and Greub 2010).

Members of the *Chlamydiales* order are characterized by a particular infection cycle, involving two developmental stages: the elementary bodies (EBs) that are infectious but have

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reduced metabolic activity (Sixt et al., 2013), and the non-infectious reticulate bodies (RBs) that are able to divide (Friis 1972; Greub and Raoult 2002; Abdelrahman and Belland 2005). EBs enter the cell generally through phagocytosis or endocytosis and are thus engulfed in an endosome. Release of effectors modify the properties of the surrounding membrane by modification of protein and cytoskeleton recruitment and allow escape of the endocytic pathway. The resulting vacuole is called an inclusion (Abdelrahman and Belland 2005). In this environment, they differentiate into RBs, proliferate and finally re-differentiate into EBs that are expelled from the cells either by exocytosis or by cell lysis (Todd and Caldwell 1985; Hybiske and Stephens 2007). In some cases, in the presence of stress or nutrient deprivation, *Chlamydiales* can enter a persistent stage, in which the bacteria becomes enlarged and polyploid (described in the section 'box 2: aberrant bodies and persistence').

*Chlamydiales* rely on an atypical cell division mechanism, which is still not well understood. First, *Chlamydiales* do not possess a homologue of FtsZ, the typical organizer of the cytokinetic machinery in bacteria (Stephens et al., 1998; Margolin 2005; Bertelli et al., 2010). Secondly, *Chlamydiales* do not possess a peptidoglycan (PG) cell wall with a conventional structure (Fox et al., 1990; Moulder 1993; Ghuysen and Goffin 1999). This appears contradictory to the effect of penicillin on chlamydial division (Matsumoto and Manire 1970; Skilton et al., 2009) and with previous findings showing that the enzymes involved in the PG biosynthesis pathway are indeed functional (McCoy and Maurelli 2006; Henrichfreise et al., 2009). This apparent contradiction was called the 'chlamydial anomaly' (Moulder 1993).

In this review, we summarize the recent progress on understanding the division of *Chlamydiales* and the chlamydial anomaly. Indeed, recent studies showed (i) that an intact PG polymer surrounding chlamydial cells and of PG-like chemical composition is indeed extractable from at least one *Chlamydiales* member (Pilhofer et al., 2013), (ii) that typical peptide components of PG accumulate at the division site as in other bacteria and may be incorporated into a PG-like polymer by chlamydial cells (Liechti et al., 2013) and (iii) that PG precursors are required for proper localization of PG- and bacterial actin-homologue-binding proteins at the division septum (Frandi et al., 2014; Jacquier et al., 2014). This new knowledge allows us to draw an updated model of chlamydial division.

## DIVISION IN GRAM-NEGATIVE BACTERIA, A SHORT OVERVIEW

The Gram-negative bacteria possess two lipid bilayers, the inner membrane (IM) and the outer membrane (OM). Between them, a layer of PG maintains the cellular integrity and allows tolerance to osmotic pressure. During division, all these three layers have to be produced and subsequently split between the daughter cells: IM invagination is followed by modification of the PG layer (new synthesis and subsequent hydrolysis), to allow the new formation of PG layers between the two daughter cells. Finally, invagination of the OM is tightly linked to the IM invagination and PG splitting through a transenvelope structure linking all three layers, the Tol-Pal complex (reviewed in Gerding et al., 2007; Egan and Vollmer 2013).

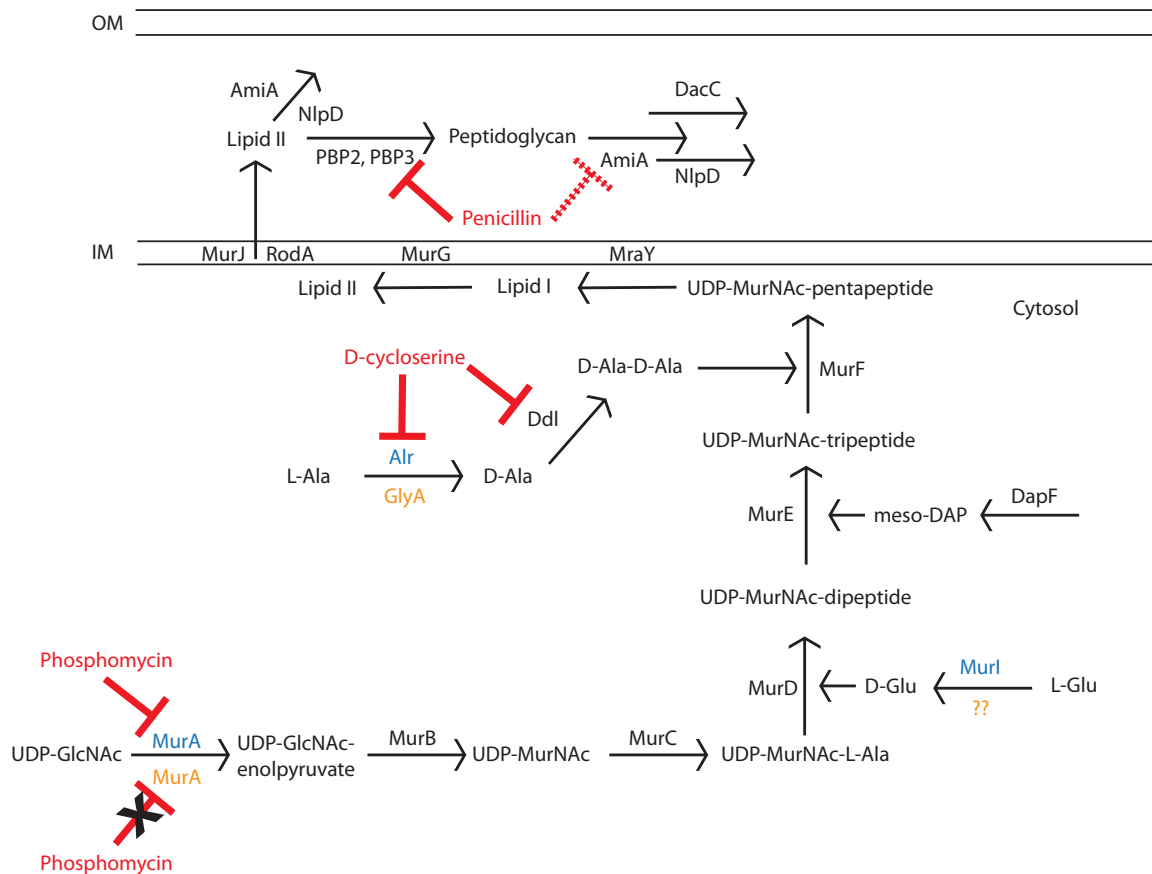
Bacteria usually divide by complex mechanisms organized by the bacterial tubulin homologue FtsZ, which assembles into short protofilaments that encircle the division site in a structure called the Z-ring (Margolin 2005). The driving force for constriction by FtsZ seems to stem primarily from septal PG synthe-

sis (Adams and Errington 2009). In addition, FtsZ recruits more than 10 essential proteins (the Fts proteins) into the divisome complex at mid-cell. More accessory proteins also localize to the division septum during division, but are not essential for invagination or septal PG synthesis, likely regulating the proper placement and/or timing of divisome recruitment (Kirkpatrick and Viollier 2011; Natale, Pazos and Vicente 2013). Z-ring formation requires stabilization of FtsZ polymerization and anchoring of the polymers to the membrane via the action of the proteins ZipA and FtsA, which are essential for bacterial division (Haney et al., 2001; Pichoff and Lutkenhaus 2005). Four Z-ring-associated proteins, ZapA, ZapB, ZapC and ZapD are associated with FtsZ, but not essential for *Escherichia coli* division, but likely support efficient Z-ring formation or maturation (reviewed in Egan and Vollmer 2013). Several proteins are then recruited to this nascent Z-ring. One of them, FtsK, has a bifunctional role in cell division and in chromosome segregation (Grenge et al., 2008). FtsK recruits FtsQ, FtsL and FtsI to the division septum (Chen and Beckwith 2001). This results into further recruitment of late division factors such as FtsW (Mohammadi et al., 2011). The fact that FtsZ not only recruits PG-biosynthetic (PBP3) and -precursor enzymes (MurG, see below Aaron et al., 2007; Mohammadi et al., 2007) but also proteins (FtsEX) that indirectly regulate PG-hydrolyzing enzymes (AmiA/B) highlights the importance of tightly coordinated PG remodelling during division (Yang et al., 2011).

## THE ROLE OF PG IN DIVISION OF GRAM-NEGATIVE BACTERIA

PG is a polymer of glycan chains composed of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) connected by short peptides. These peptides are usually synthesized as pentapeptides containing both L- and D-aminoacids. The first steps of PG biosynthesis occur in the cytosol by the enzymes MurA and MurB. These two enzymes synthesize MurNAc using GlcNAc as a substrate. Amino acids are sequentially added to MurNAc by MurC, D, E and F (Fig. 1, reviewed in Barreteau et al., 2008). The resulting monosaccharide-pentapeptide is then attached to the IM by addition of a lipid by the enzyme MraY to form undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide, also called lipid I. Lipid I is then further modified by MurG, which adds a GlcNAc, forming the disaccharide-pentapeptide unit, lipid II (Bouhss et al., 2008). Lipid II is then flipped across the IM by the MurJ flippase (Sham et al., 2014; Ruiz 2008). Glycosyltransferases (penicillin-binding proteins class A) then accept this functional unit of lipid II, incorporating it into a growing glycan strand. Glycan strands are then cross-linked by pentapeptide bridges introduced by the transpeptidation activity of penicillin-binding proteins class A and B (PBP2/3) (Sauvage et al., 2008).

PG biosynthesis enzymes are recruited to the divisome at a later stage of division, the Z-ring maturation, being followed by the initiation of cell-wall constriction. This starts with the recruitment of FtsK. FtsQ is then recruited in an FtsK-dependent manner. Subsequently, FtsL, FtsB, FtsW and PBP3 are recruited to the divisome (Begg et al., 1990; Aarsman et al., 2005; Mohammadi et al., 2011). The *de novo*-synthesized septal PG separates the daughter compartments, but the cells remain attached until the septal PG is cleaved by amidases (Yang et al., 2011). Amidase activity should be tightly regulated, as uncontrolled activity can lead to cell lysis. Specific activation of amidases at the septum is regulated by EnvC and NlpD (Uehara et al., 2010). These proteins are recruited to the division septum by the ABC-transporter complex FtsEX and the SPOR-domain protein FtsN, respectively (Uehara et al., 2010; Yang et al., 2011).



**Figure 1.** PG biosynthesis pathway comparison between *W. chondrophila* and Chlamydiaceae. Enzymes present only in *W. chondrophila* (Alr and MurI) are depicted in blue, as enzyme replacing Alr in Chlamydiaceae (GlyA) is depicted in orange. Orange question marks highlight the unknown source of D-Glu in Chlamydiaceae. Steps inhibited by D-cycloserin, phosphomycin and penicillin are shown in red. MurA is depicted in blue for *W. chondrophila*, which is sensitive to phosphomycin and in orange for Chlamydiaceae, which are resistant to phosphomycin (illustrated by a black cross).

## OM INVAGINATION DURING CYTOKINESIS

The Tol-Pal complex is composed of five conserved proteins, TolQ, TolR and TolA, which are transmembrane proteins inserted in the IM, TolB, a soluble periplasmic protein and Pal, a lipoprotein localized at the inner leaflet of the OM. Pal directly interacts with PG (Mizuno 1981) and through its transient interaction with TolB is thought to coordinate invagination of the OM-PG-IM layers, drawing the OM inwards once septal PG splitting commences (for review, see Godlewska et al., 2009). Other complexes might also play a role in coordinating OM invagination as the PBP1B-LpoB complex (Typas et al., 2010) or other lipoproteins that can bind PG covalently or non-covalently such as Lpp or OmpA.

## REGULATION OF BACTERIAL DIVISION

Bacterial division has to be tightly regulated to ensure a division resulting in two viable daughter cells in an efficient way. Moreover, PG splitting mediated by potentially lethal hydrolytic enzymes (amidases, endopeptidases) is carefully controlled in time and space to prevent lysis of cells. Several conditions have to be met before division is initiated: (i) control of the energetic status of the bacteria to allow complete replication of the chromosome, (ii) identification of mid-cell for the assembly of the septum and (iii) complete segregation of the chromosome to clear the division site so that no DNA is trapped during invagination of the envelope and does not get bisected at cell separation.

The energetic status of the bacteria is measured mainly by the availability of ATP. DnaA is the replication initiator protein and its active form is bound to an ATP molecule. In contrast, ADP bound DnaA is inactive. Active DnaA binds to the origin of replication OriC and induces the recruitment of the replication machinery (for review, see Leonard and Grimwade 2011). It has also to be mentioned that DNA replication by DNA primase in *Bacillus subtilis* is regulated by ppGpp availability, thus controlled by the nutrient availability (Wang, Sanders and Grossman 2007).

Several mechanisms are involved in the correct positioning of the divisome at mid-cell. The best-known regulatory system is the MinCD(E/J) (Lutkenhaus 2007). MinC inhibits the polymerization of FtsZ and blocks its interaction with other divisomal proteins. MinC is recruited to the membrane and activated by binding to MinD. A polar gradient of MinCD induces polymerization of FtsZ at mid-cell only and allows a correct spatial assembly of the Z-ring (Lutkenhaus 2007; Kirkpatrick and Viollier 2011). Anionic phospholipids like cardiolipins seem to also be important for correct Z-ring assembly (Kawai et al., 2004). They are enriched at the division site and at the poles and may influence localization of the Z-ring (reviewed in Mileykovskaya and Dowhan 2005). During delays in DNA replication, septation is inhibited by a process called nucleoid occlusion. In this process, septation over nucleoid is inhibited by the proteins Noc in *B. subtilis* (Wu and Errington 2004) or SlmA in *E. coli* (Bernhardt and de Boer 2005). This process prevents an uneven partition of the chromosomes between the daughter cells and delays the

**Table 1.** Conservation and percentage of identity of chlamydial division proteins compared to *E. coli*.

	Cpn	Wch	Comments
FtsZ	–	–	Tubulin homologue, bacterial division organizer
ZapA/B/C/D	–	–	
FtsA	–	–	
ZipA	–	–	
FtsE	–	–	
FtsX	–	–	
FtsK	39.29	40.91	Putative chromosome segregation protein
FtsB	–	–	
FtsQ	–	29.82	
FtsL	–	11.40	
FtsW	31.25	36.11	Putative lipid II flippase
FtsN	–	–	
FtsP	–	–	
MreB	57.40	59.17	Actin homologue
RodZ	28.57	23.81	MreB interactor

**Table 2.** Percentage of identity and coverage (in brackets) of *W. chondrophila* division proteins compared to *C. pneumoniae* (Cpn), *C. trachomatis* (Ctr), *P. acanthamoebae* (Pac) and *S. negevensis* (Sne).

	Cpn	Ctr	Pac	Sne
FtsK	44.84 (95)	46.04 (97)	59.24 (96)	65.92 (59)
FtsQ	25.87 (76)	21.85 (90)	28.57 (87)	30.95 (92)
FtsL	49.21 (63)	44.26 (61)	57.83 (83)	43.75 (80)
FtsW	51.40 (95)	51.96 (95)	59.28 (97)	51.58 (94)
MreB	85.55 (96)	86.92 (94)	89.84 (100)	90.90 (93)
RodZ	40.15 (93)	42.11 (93)	60.61 (97)	60.15 (97)

cytokinesis until the chromosomes are properly separated (reviewed in Rothfield, Taghbalout and Shih 2005).

## ROLE OF MreB ACTIN IN DIVISION OF THE CHLAMYDIALES

Chlamydial division is mysterious, since it occurs in the absence of a sequence homologue of FtsZ, the usual organizer of bacterial division (Ghuysen and Goffin 1999). Nevertheless, chlamydial cells divide by binary fission, in a process that highly resembles FtsZ-dependent coccoid division (Brown and Rockey 2000; Greub and Raoult 2002; Abdelrahman and Belland 2005). Other key components of division are also not encoded in chlamydial genomes. Chlamydiaceae only possess annotated homologues of FtsK, FtsW and FtsI (PBP3, Tables 1 and 3). *Waddlia chondrophila* possesses homologues of FtsK, FtsW, FtsI (PBP3), FtsQ and FtsL (Table 1). Interestingly, Chlamydiaceae possess homologues of all these *W. chondrophila* proteins, even if FtsQ and FtsL homologues were not annotated by alignment against the *E. coli* proteins (Tables 1 and 2) (Ouellette et al., 2012; Jacquier et al., 2014). The replacement of the homologue of tubulin FtsZ by MreB, an actin homologue that assembles into filaments that line the cytoplasmic membrane (Dominguez-Escobar et al., 2011; Garner et al., 2011; van Teeffelen et al., 2011; Reimold et al., 2013), was discussed by several groups (Gaballah et al., 2011; Ouellette et al., 2012; Jacquier et al., 2014). An important role of MreB for chlamydial division could be shown, as MreB inhibition by its

known inhibitors A22 and MP265 blocked chlamydial proliferation in *C. trachomatis* (Ouellette et al., 2012), *C. pneumoniae* and *W. chondrophila* (Jacquier et al., 2014). However, MreB localizes at the division septum only at a late stage of division in *W. chondrophila* (Jacquier et al., 2014). This indicates that, even if MreB plays an important role in chlamydial division, it does not seem to be an early cell division protein such as FtsZ. Interestingly, the MreB-binding protein RodZ localizes to the septum earlier than MreB, but the MreB-inhibitor MP265 prevents the localization of RodZ to mid-cell (Jacquier et al., 2014). An unknown organizer of chlamydial division (if not RodZ itself) seems thus to recruit RodZ to the division septum in an MreB-dependent manner. Given that RodZ and MreB interact and co-localize in *E. coli* and at the *C. crescentus* division septum (described in the section 'Box 3: MreB and RodZ'), septal RodZ might subsequently recruit MreB to the chlamydial division septum. This is consistent with the recent identification by two hybrid of RodZ as an MreB interactor in *C. trachomatis* (Ouellette et al., 2014; Kemege et al., 2014). However, it is unclear why MreB should only concentrate at the deeply constricted division furrow and not earlier. It is possible that the direct interaction between chlamydial MreB and RodZ is required for the positioning of RodZ at the future division site, for example by MreB-facilitated movement of the bitopic membrane protein RodZ within the cytoplasmic membrane. Alternatively, the dependence of RodZ positioning on MreB may be indirect, relying instead on the recruitment of MurG and/or MurF (both involved in lipid II synthesis, see below) to the division septum for the production of lipid II. In this model, inhibition of MreB via A22/MP265 would interfere with lipid II biosynthesis. Thus, MurG/F or other biosynthetic enzymes would remain in the cytoplasm and could no longer participate in lipid II biosynthesis at the membrane. A link has been proposed between MreB and PG biosynthesis enzymes through direct interactions between MreB and MurF, and possibly MurG (Gaballah et al., 2011), suggesting that an intact MreB-based cytoskeleton is required for PG biosynthesis in chlamydial cells (see the section 'Box 3: MreB and RodZ').

## SPATIAL CONTROL OF THE CHLAMYDIAL DIVISION SEPTUM BY LIPID II

The important role of lipid II in localization of RodZ to the chlamydial division septum was unearthed by recent experiments using the antibiotic phosphomycin (Frandi et al., 2014; Jacquier et al., 2014). This antibiotic inhibits MurA, the first enzyme in the lipid I and lipid II biosynthesis pathways, and thereby prevents chlamydial division, presumably because lipid II (and/or lipid I) is no longer available for septal PG synthesis. While RodZ is no longer septal in phosphomycin-treated *W. chondrophila* cells, in the presence of penicillin the accumulation of RodZ at the division septum is increased. This suggests that PG biosynthetic enzymes can also affect the dispersion of proteins from the division septum (Jacquier et al., 2014).

Why lipid II (and/or lipid I) is required for septal localization of RodZ is unclear. However, labelling of chlamydial cell with fluorescent PG precursors provides evidence that lipid II (and/or lipid I) is concentrated at the septum. Thus, it is conceivable that lipid II (and/or lipid I) acts as a spatial cue by being itself confined to the division site. Alternatively, spatial control could be governed indirectly for example through stabilization of the septal PG biosynthetic apparatus even if PG is dispersed throughout the envelope. Perhaps, the PG biosynthetic machine along with RodZ disintegrates in the absence of the enzymatic

substrate. Such a reverse-instability effect was reported for FtsZ in *Caulobacter crescentus* where depletion of the later-acting division protein FtsK causes the collapse of the FtsZ-ring (Wang, West and Shapiro 2006). RodZ and MreB are known to localize to the division plane in *Caulobacter*, but in this case this localization is dependent on FtsZ (Alyahya et al., 2009). It has been reported that certain epsilonproteobacteria encode an MreB homologue, but not RodZ (Alyahya et al., 2009). This finding is consistent with the interpretation that RodZ and MreB do not always have to be functionally linked, and thus that RodZ or MreB may adopt functions that are independent of the other. Moreover, the chlamydial RodZ is truncated, lacking a C-terminal periplasmic domain and cannot complement an *E. coli rodZ* mutant (Ouellette et al., 2014). It is therefore plausible that RodZ has acquired a new function in division control of *Chlamydiales* that is independent of MreB.

## ORGANIZATION OF THE CHLAMYDIAL DIVISOME

Other potential components of the septum, such as FtsK, AmiA, FtsW, FtsQ and FtsL, are highly conserved in the *Chlamydiales* order (Tables 1 and 2). Immunolocalization experiments with antibodies to FtsK or AmiA did not provide evidence for overt and pervasive septal localization (Frandi et al., 2014; Jacquier et al., 2014). However, evidence indicates that some PG-biosynthetic and remodelling enzymes are located at the chlamydial division septum (Frandi et al., 2014). FtsI (PBP3), a protein that is localized to the division septum in other bacteria where it promotes septal PG synthesis, is also encoded in *Chlamydiales* (Tables 3 and 4). Localization of FtsI (PBP3) in *C. trachomatis* was punctuate, with no more than one focus per cell (Ouellette et al., 2012), a result consistent with a septal localization. With the recent identification of the LysM-domain (Buist et al., 2008) protein NlpD as a 'late' septal protein, three proteins are now known to reside at the division septum: RodZ and NlpD are early and intermediate septal recruits, while MreB is a late recruit (Frandi et al., 2014; Jacquier et al., 2014). NlpD was indeed shown to localize at mid-cell primarily in constricted cells and this localization was inhibited by prior addition of penicillin or phosphomycin, suggesting that NlpD recognizes a septal PG-like polymer. In support of this idea, NlpD binds *E. coli* PG *in vitro* and in a manner that depends on an intact LysM-domain (Frandi et al., 2014). Cell division can be inhibited in *Chlamydiales* exposed to beta-lactams (targeting PBPs) suggesting that septal PG synthesis drives chlamydial division but a classical PG was not detected in *Chlamydiaceae* (Fox et al., 1990; Moulder 1993; Ghuysen and Goffin 1999). Nevertheless, a non-proteinaceous antigen, which might be part of a PG-like structure, was observed at the chlamydial division septum (Brown and Rockey 2000). In addition, *C. trachomatis* activates the Nod1 receptor, which recognizes PG fragments (muropeptides containing meso-diaminopimelate, see below) during cell infection (Welter-Stahl et al., 2006). These results suggest that the *Chlamydiaceae* either assemble classical PG, but only in trace amounts, and/or that PG is of an atypical (modified) form that at least carries the muramyltripeptide that is recognized by Nod1. As intracellular bacteria and in strong contrast to free-living bacteria that rely on the PG-based cell wall for osmo- and chemical protection, there is less need for osmoprotection for *Chlamydiales* when intracellularly located. Furthermore, PG fragments have the capacity to alert the innate immune system of a bacterial infection. Thus, PG production could translate in a fitness cost to invading bacterial pathogens, a cost that is limited for *Chlamy-*

**Table 3.** Conservation and percentage of identity of chlamydial PG biosynthesis enzymes compared to *E. coli*.

	<i>C. pneumoniae</i>	<i>W. chondrophila</i>	Remarks
Alr	–	29.00	
Ddl	33.05	28.53	Fused to MurC in <i>C. pneumoniae</i>
MurI	–	28.57	
DacC	29.52	28.31	Homologue of PBP6 of <i>E. coli</i>
DapA	28.37	39.64	
DapB	28.10	37.85	
DapL	26.97	26.61	Homologous to <i>E. coli</i> aspartate aminotransferase
DapF	33.33	30.47	
MurA	34.63	35.63	Target of phosphomycin, Cpn MurA is resistant
MurB	27.22	30.79	
MurC	33.26	34.88	
MurD	31.24	33.26	
MurE	36.95	34.52	
MurF	27.46	30.36	Fused to Alr in <i>W. chondrophila</i>
MraY	38.26	43.08	
MurJ	24.56	29.03	
MurG	30.56	31.14	
Pbp1	–	–	
Pbp2	22.55	26.16	Target of penicillin and mecillinam
Pbp3/FtsI	27.72	29.42	Target of penicillin and piperacillin
Pbp4	–	–	
Pbp5	–	–	
Pbp7	–	–	
Pbp8	–	–	
AmiA	28.93	27.13	Target of penicillin
AmiB	18.54	22.05	Contains a LysM domain, which binds PG
AmiC	–	–	
NlpD	25.75	17.24	
EnvC	–	–	

*diales* because of the limited production of a PG-like component. This might explain why *Chlamydiales* may remain nearly undetected (Welter-Stahl et al., 2006). Potentially this pressure for reduced PG material is counterbalanced by the advantage of having PG synthesis at the division plane, which is thought to facilitate constriction of bacterial cells, a process that *Chlamydiales* should also rely on and that can be inhibited by penicillin. Indeed, it has been proposed that chlamydial shape and osmotic pressure resistance can be maintained, at least in EBs, by OM proteins highly cross-linked by disulphide bridges (Hatch 1996).

Recently, a modified form of PG was detected biochemically in the *Chlamydia*-related bacteria *Pr. amoebophila* by cell-wall extraction and HPLC/MS (Pilhofer et al., 2013). Moreover, PG or lipid II was also indirectly detected by fluorescent labelling in a ring-like structure at the division septum in *C. trachomatis* (Liechti et al., 2013). In this study, Liechti et al. (2013) developed a novel metabolic labelling technique using D-amino acids dipeptide probes and showed their integration in replicating *C. trachomatis*.

**Table 4.** Percentage of identity and coverage (in brackets) of *W. chondrophila* PG biosynthesis enzymes compared to *C. pneumoniae* (Cpn), *C. trachomatis* (Ctr), *P. acanthamoebae* (Pac) and *S. negevensis* (Sne).

	Cpn	Ctr	Pac	Sne
Ddl	25.85 (98)	29.41 (87)	28.35 (98)	32.47 (98)
MurI	–	–	47.67 (93)	–
DacC	32.73 (88)	33.05 (82)	43.81 (97)	41.64 (88)
DapA	28.95 (90)	31.62 (86)	49.09 (93)	45.07 (97)
DapB	25.87 (77)	27.35 (93)	49.56 (97)	47.60 (96)
DapL	46.11 (96)	47.30 (96)	55.98 (97)	50.13 (97)
DapF	32.30 (86)	35.23 (95)	47.21 (93)	37.17 (94)
MurA	56.30 (93)	55.40 (92)	76.50 (96)	67.12 (93)
MurB	44.80 (94)	46.74 (97)	56.29 (96)	50.17 (97)
MurC	40.32 (99)	40.32 (99)	52.38 (98)	44.07 (100)
MurD	37.83 (97)	34.38 (98)	45.62 (99)	43.61 (99)
MurE	43.32 (100)	40.85 (99)	54.41 (99)	47.75 (99)
MurF-Alr	30.21 (43)	28.32 (40)	49.22 (99)	28.36 (39)
MraY	40.00 (92)	38.29 (90)	66.50 (100)	62.84 (100)
MurJ	32.78 (94)	34.26 (94)	50.46 (94)	42.76 (93)
MurG	32.66 (96)	30.84 (96)	39.50 (98)	43.90 (94)
Pbp2	39.11 (99)	37.41 (99)	49.78 (99)	40.66 (96)
Pbp3/FtsI	44.68 (99)	46.44 (99)	59.24 (99)	51.67 (99)
AmiA	40.84 (82)	43.09 (78)	48.48 (96)	42.47 (95)
AmiB	30.51 (83)	28.29 (94)	33.65 (98)	30.64 (83)
NlpD	35.00 (100)	37.21 (100)	40.32 (100)	38.17 (100)

These two key articles provided the first evidence for the presence of a PG-like polymer in *Chlamydiales*. This makes *Chlamydiales*, the first bacterial order known to use a functional PG pathway for division without a sequence homologue of FtsZ.

## STRUCTURE OF THE CHLAMYDIAL PG

Following the recent advances in the detection of PG in *Chlamydiales* along with several clues from the predicted coding sequences in chlamydial genomes, we have learned several things about chlamydial PG. Gram-negative bacteria typically have unconventional amino acids, meso-diaminopimelate (m-DAP), D-Alanine (D-Ala) and D-Glutamate (D-Glu). These amino acids should be synthesized by *Chlamydiales* (which are Gram-negative bacteria), because there is no evidence of mDAP, D-Ala or D-Glu in mammalian cells. A specific aminotransferase pathway conserved in *Chlamydiales* and plants is required for m-DAP biosynthesis and Nod1 indeed detects the m-DAP-containing muramyltripeptide. In *Chlamydiales*, a specific conserved enzyme DapL, a L,L-diaminopimelate aminotransferase, bypasses the usual pathway present in other bacteria (McCoy et al., 2006). D-Ala biosynthesis is usually performed by the alanine racemase (Alr). A predicted Alr coding sequence is present in the genomes of *P. acanthamoebae*, *Pr. amoebophila* and *W. chondrophila*, but not in *Chlamydiaceae* and in *S. negevensis* (Tables 3 and 4). Recent evidences involve the serine hydroxymethyltransferase GlyA as an alternative source of D-Ala in *C. pneumoniae*, as chlamydial GlyA can partially complement an *E. coli* racemase double mutant and has a weak racemase activity *in vitro* (De Benedetti et al., 2014). D-Glu biosynthesis involves MurI, a glutamate racemase (Doublet et al., 1993). A *murI*-like gene is indeed encoded in the genomes of *P. acanthamoebae*, *Pr. amoebophila* and *W. chondrophila*, but not in *Chlamydiaceae* and in *S. negevensis* (Tables 3 and 4). There is no clear evidence of presence of D-Glu in *Chlamydiaceae* and no D-Glu biosynthesis pathways encoded by their genomes, suggesting that there is no D-Glu in chlamydial PG. However, absence

of D-Glu in chlamydial PG is highly improbable (see below), because PG biosynthesis enzymes are conserved in all *Chlamydiales* and have a normal *in vitro* activity on PG precursors containing D-Glu. Most importantly, D-Glu was recently identified as a constituent of PG-like building blocks (Pilhofer et al., 2013).

m-DAP, D-Ala and D-Glu are used by enzymes involved in lipid II biosynthesis. Genes encoding MurA, MurB, MurC, MurD, MurE, MurF, MurG, MraY and Ddl orthologues are conserved among the *Chlamydiales* (Henrichfreise et al., 2009) (Fig. 1 and Tables 3 and 4) and present in the genome of the *Chlamydia*-related bacterium *W. chondrophila* (Jacquier et al., 2014). Requirement of MurA for growth of *W. chondrophila* and *Pr. amoebophila* was demonstrated by use of the MurA inhibitor phosphomycin (Pilhofer et al., 2013; Jacquier et al., 2014). Interestingly, *Chlamydiaceae* are naturally resistant to phosphomycin. This resistance is caused by an amino acid exchange in MurA (McCoy, Sandlin and Maurelli 2003). *In vitro* and *in vivo* activity of the *Chlamydiaceae* enzymes was demonstrated for MurA (McCoy, Sandlin and Maurelli 2003), MurC (Hesse et al., 2003), MurE (Patin et al., 2009), MurF (Patin et al., 2012), MurG and MraY (Henrichfreise et al., 2009). Ddl is active and part of a MurC-Ddl fusion protein (McCoy and Maurelli 2005). Activity of MurB and MurD was not demonstrated yet, even if mRNA of MurB was detected in *C. trachomatis* (McCoy, Sandlin and Maurelli 2003). Taken together, these studies strongly indicate that *Chlamydiales* synthesize a classical lipid II (Henrichfreise et al., 2009). *In vitro* studies based on substrate specificities of chlamydial MurC, MurE, MurF and Ddl enzymes indicate that the possible structure of the pentapeptide in *Chlamydiales* might be L-Ala/L-Ser/Gly-D-Glu-m-DAP-D-Ala-D-Ala (Fig. 1) (Patin et al., 2012). Biochemical analysis by HPLC of sacculi extracted from *Pr. amoebophila*, a *Chlamydia*-related bacterium and digested with cellosyl, a glycan strand-cleaving PG muramidase, showed the presence of PG-like fragments, including a canonical disaccharide unit harbouring D-Glu (GlcNAc-MurNAc(r)-L-Ala- D-Glu) along with an undetermined 314 Da modification in the PG unit and fluorescent labelling provided evidence of the incorporation of D-Ala (Liechti et al., 2013; Pilhofer et al., 2013). Intriguingly, no PG-like material could be detected in *S. negevensis*, another *Chlamydia*-related bacterium. Moreover, *S. negevensis* is completely resistant to penicillin and phosphomycin (Pilhofer et al., 2013). This seems to be specific to *S. negevensis* as other *Chlamydia*-related species are at least partially sensitive to penicillin, despite the presence of a beta-lactamase (Bertelli et al., 2010; de Barsy, Bottinelli and Greub 2014; Jacquier et al., 2014). The *Chlamydiales* family is highly diverse and divergent and we thus cannot exclude that PG presence and structure can be different between its members. Nevertheless, the relatively high conservation of the large majority of the PG biosynthesis enzymes indicates that PG biosynthesis plays an important role in the *Chlamydiales* for which genome sequences are available.

*Chlamydiales* possess only few PG cross-linking enzymes. No class A (bifunctional) PBPs are encoded by members of the *Chlamydiales* order. Only class B (monofunctional) PBPs are present, resembling PBP2 and PBP3 of *E. coli* (Ouellette et al., 2012) (Tables 3 and 4). A low molecular weight PBP is also conserved among *Chlamydiales*, with homologies to PBP6 of *E. coli* (McCoy and Maurelli 2006). Nevertheless, these PBPs play an important role, as *Chlamydiales* are sensitive to penicillin, which induces the formation of aberrant bodies (Matsumoto and Manire 1970; Ouellette et al., 2012). They also impede the dispersion of RodZ from the division septum (Jacquier et al., 2014). Interestingly, PBP3/FtsI is involved in transpeptidation of the pentapeptide stem in lipid II at the *E. coli* division septum and is required

for proper assembly of the Z-ring (Pogliano et al., 1997). In contrast, PBP2 is involved in lateral PG biosynthesis and thus required for rod-shape maintenance in *E. coli* (Bendezu and de Boer 2008). Moreover, PBP2 and PBP3 have distinct and non-redundant functions in *Chlamydiales*, because specific inhibition of PBP2 by mecillinam and of PBP3/FtsI by piperacillin caused different morphologies of aberrant bodies (Ouellette et al., 2012; Jacquier et al., 2014). PBP3 inhibition induced the accumulation of RodZ at the division septum and PBP2 inhibition abolished the septum formation and caused the formation of similar aberrant bodies as with MurA inhibition (Jacquier et al., 2014). This might indicate that PBP2 is required for the PG biosynthesis in a process that affects MreB or depends on it and that PBP3 is also required at a later stage for PG modification during septum dispersion, while PBP2 acts very early in division. Alternatively, PBP2 and PBP3 might simply introduce different types of PG modifications.

In bacteria with PG, daughter cell separation involves PG remodelling by lytic transglycosylases, amidases (N-acetylmuramoyl-L-alanine hydrolases) and peptidases (LD-carboxypeptidases and DD-endopeptidases). *Chlamydiales* possess at least a functional amidase (AmiA) (Frandi et al., 2014; Klockner et al., 2014) and a PG peptidase (NlpD) (Frandi et al., 2014), but no lytic transglycosylase homologues. When expressed in *E. coli*, AmiA of *W. chondrophila* causes an increase in cell lysis, apparently due to ectopic amidase activity (Frandi et al., 2014). While *E. coli* amidases are by default inactive enzymes and need to be stimulated, it appears that chlamydial AmiAs are synthesized as active (lytic) variants that are not autoinhibited (Klockner et al., 2014). Moreover, AmiA from *C. pneumoniae* possesses a carboxypeptidase activity, a function generally carried out by separate proteins in other organisms (Klockner et al., 2014). AmiA regulation seems to be less important for *Chlamydiales* compared to other bacteria and might help to reduce the PG thickness to dampen innate immune detection, akin to the role of staphylococcal autolysins that prevent detection by the *Drosophila* innate immune system (Atilano et al., 2014).

## OM INVAGINATION

In *E. coli*, several PG-binding lipoproteins (Lpp, OmpA, Pal) that are localized to the inner leaflet of the OM are used to coordinate PG remodelling with OM invagination. Of these, a Pal-like protein seems to be encoded in the chlamydial genomes. Pal interacts with the *cis*-encoded TolABQR proteins, which together with Pal assembled into the Tol-Pal transenvelope complex at the *E. coli* division plane. Pal, TolQ, TolR, TolA and TolB (Tables 5 and 6) are conserved among *Chlamydiales* suggesting that the Tol-Pal complex is functional in *Chlamydiales*. By analogy to *E. coli*, chlamydial Pal might interact with chlamydial PG to draw in the OM

**Table 5.** Conservation and percentage of identity of chlamydial proteins possibly involved in OM septation compared to *E. coli*.

	<i>C. pneumoniae</i>	<i>W. chondrophila</i>	Comments
Pal	32.80	48.72	PG associated lipoprotein
TolQ	24.88	28.25	
TolR	25.89	24.09	
TolA	17.94	20.18	
TolB	26.76	23.87	
LpoA	–	–	
LpoB	–	–	
Lpp	–	–	

**Table 6.** Conservation and percentage of identity of chlamydial proteins possibly involved in mid-cell determination compared to *E. coli*.

	<i>C. pneumoniae</i>	<i>W. chondrophila</i>	Comments
MinC	–	–	
MinD/ParA	27.10	26.37	Similar homology to MinD and ParA
MinE	–	–	
Noc/ParB	31.73	35.26	Similar homology to Noc and ParB
SlmA	–	–	

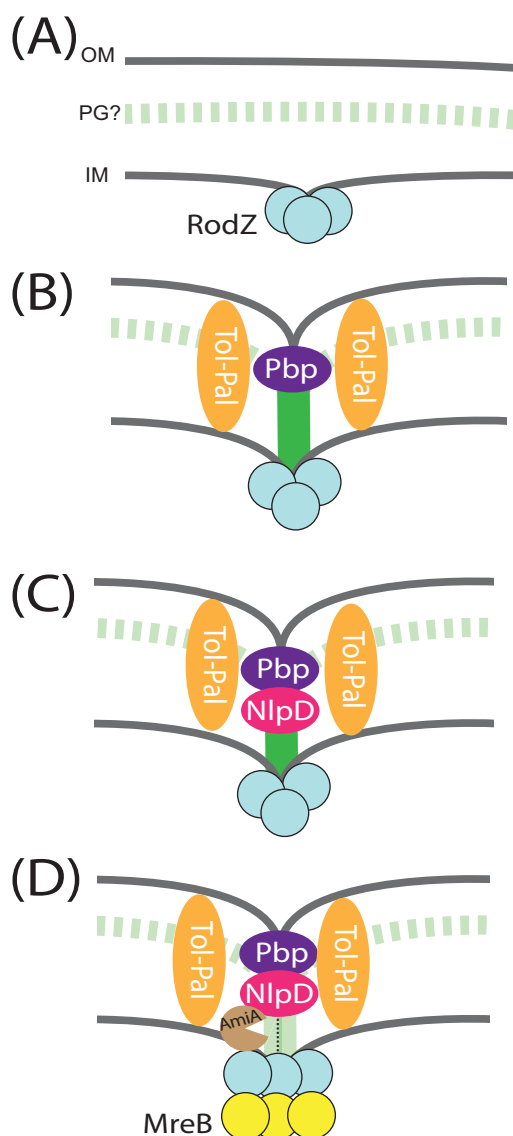
during constriction. This model has yet to be investigated and might help to illuminate how chlamydial divisome extends into the OM.

## DISCUSSION AND PERSPECTIVES

The recent discoveries regarding chlamydial PG synthesis and divisome composition help us to refine the model of chlamydial division. The first detection of PG-related material indicates that, even in absence of an FtsZ homologue, division might occur in a similar way as in other bacteria. *Chlamydiales* thus need to control coordinated invagination of (i) the OM, (ii) the PG layer, which might be present as a sacculus or only at the division septum and (iii) the IM (Fig. 2A). Rod-shape determining proteins MreB and RodZ seem to play an important role in this process. As they are able to control PG biosynthesis in other species, they might act similarly in *Chlamydiales*, by bringing together the PG biosynthesis and remodelling enzymes to the divisome (Fig. 2B).

The structure of chlamydial PG has still to be determined to better understand its exact role in these bacteria. Moreover, a structural difference between the PG of *Chlamydiaceae* and *Chlamydia*-related species cannot be excluded. *Chlamydia*-related bacteria apparently possess a larger amount of PG, which is able to form a sacculus. In contrast, labelling of PG in *Chlamydiaceae* is only observed at the division septum, indicating that new synthesis of PG occurs only at this localization. It has still to be confirmed (i) if PG is really present only at the division septum and (ii) if the observed septal localization of PG is not due to restrained diffusion of the new synthesized PG in *Chlamydiaceae*, even though at least one member of the *Chlamydia*-related bacteria is clearly able to synthesize PG throughout the cell.

Division is tightly regulated in *Chlamydiales*, as only RBs are able to divide. This implies a differentiation program, which allows expression of division genes. Comparison of transcriptional pattern in EBs and RBs was performed in *C. trachomatis* (Albrecht et al., 2010) and *C. pneumoniae* (Albrecht et al., 2011). Expression of several division genes is induced in RBs compared to EBs, including also the PG biosynthesis genes. It thus seems that expression of components of the division machinery is inhibited in EBs and induced during differentiation of EBs into RBs. DnaA is conserved among *Chlamydiales* and was shown to be expressed in *C. pneumoniae* (Byrne et al., 2001). It is thus likely that it has the same function in the regulation of replication initiation as in other bacteria (Ozaki and Katayama 2009). The MinC/D(E/I) system is an important division regulator in many bacteria (see above). A homologue of MinD is conserved in *Chlamydiales*, but it might also be a homologue of ParA, a protein involved in chromosome partitioning (Tables 7 and Box1). Virtually nothing is known about the presence or not of nucleoid occlusion mechanisms in *Chlamydiales*. A potential homologue of the *B. subtilis*



**Figure 2.** Models of chlamydial division. (A) RodZ accumulates at the division septum (OM: outer membrane, PG: putative peptidoglycan layer, IM: inner membrane). (B) PBPs synthesize new PG at the division site and Tol-Pal complex maintains the cohesion between the OM and IM. (C) NlpD is recruited at the septum where it modifies the PG (and/or lipidII). (D) AmiA degrades the PG (and/or lipidII) and MreB is recruited and induces the cytokinesis.

**Table 7.** Percentage of identity and coverage (in brackets) of *W. chondrophila* proteins possibly involved in OM septation and mid-cell determination compared to *C. pneumoniae* (Cpn), *C. trachomatis* (Ctr), *P. acanthamoebae* (Pac) and *S. negevensis* (Sne).

	Cpn	Ctr	Pac	Sne
Pal	47.46 (47)	51.38 (43)	54.51 (97)	39.92 (99)
TolQ	33.61 (95)	29.11 (95)	46.77 (99)	45.53 (93)
TolR	37.59 (94)	35.71 (89)	49.25 (95)	53.66 (87)
TolA	21.80 (35)	23.24 (83)	25.85 (97)	26.54 (68)
TolB	40.37 (96)	39.38 (94)	46.21 (96)	41.80 (86)
MinD/ParA	67.48 (95)	68.70 (95)	78.95 (96)	66.80 (95)
Noc/ParB	43.40 (96)	47.64 (93)	54.83 (97)	22.22 (66)

Noc is conserved among the *Chlamydiales* but is annotated as ParB (Tables 7 and Box 1). Further studies have to be performed to study the division regulation of *Chlamydiales*.

Recent advances in the field of chlamydial transformation might give us in a near future new tools to study the chlamydial division (Binet and Maurelli 2009; Wang et al., 2011; Mishra et al., 2012). For this purpose, we need to genetically modify *Chlamydiales* to place the genes of interest under conditional promoters, allowing their repression. This would help to understand the role of the gene products in the division mechanism. This needs further development of the transformation techniques in *Chlamydiales* to allow insertion of DNA in the chlamydial genome. Another option is the transformation of *Chlamydiales* with antisense DNA, which can modulate the gene expression or to exploit the CRISPR/Cas9 technology for targeted genetic manipulation (Mishra et al., 2012).

In conclusion, recent advances in the field of chlamydial division allow a better understanding of this process, but many questions remain open on the exact composition of the divisome and on the chronology and the regulation of its assembly and disassembly. *Chlamydiales* might have evolved novel mechanisms of division to overcome the absence of an FtsZ homologue. This possibly involves additional unknown proteins, which might interact with components of the divisome as RodZ or PG biosynthetic enzymes. Describing in details these processes is of course of general interest to better understand the particular evolution of these obligate intracellular bacteria. Moreover, this might lead to the discovery of specific antibiotics targeting the chlamydial division, allowing the specific destruction of *Chlamydiales* without side effects on the genital flora, for example and without inducing antibiotic resistance in other bacterial orders. Finally, a better understanding of the persistence mechanisms, which are tightly related to division mechanisms (see the section 'Box 2: aberrant bodies and persistence'), would improve the strategies to treat chronic chlamydial diseases.

## BOX1: PHYLOGENY, ECOLOGY AND BIOLOGY OF CHLAMYDIALES

The *Chlamydiales* order is composed of the Chlamydiaceae and of the *Chlamydia*-related bacteria. Whole genome sequencing was performed for several species of Chlamydiaceae: *C. trachomatis* (Stephens et al., 1998), *C. pneumoniae* (Kalman et al., 1999), *C. muridarum* (Read et al., 2000), *C. caviae* (Read et al., 2003), *C. abortus* (Thomson et al., 2005), *C. felis* (Azuma et al., 2006), *C. pecorum* (Mojica et al., 2011), *C. psittaci* (Voigt et al., 2011), *C. avium*, *C. gallinacea* (Sachse et al., 2014) and *C. suis* (Donati et al., 2014) (Fig. Box1). Their genome size is strongly reduced (between 1.04 and 1.23 Mb, Table Box1). *Chlamydia*-related bacteria were discovered more recently and less genomes are available: *Pr. amoebophila* (Horn et al., 2004), *W. chondrophila* (Bertelli et al., 2010), *P. acanthamoebae* (Greub et al., 2009; Collingro et al., 2011), *S. negevensis* (Collingro et al., 2011) and *Neochlamydia* sp. (Ishida et al., 2014). Genomes of *Chlamydia*-related bacteria are bigger than Chlamydiaceae genomes (between 2.12 and 3.19 Mb, Table Box 1). Divergence between these two groups is estimated to have occurred 1 billion years ago (Fig. Box1) (Greub and Raoult 2003). Nevertheless, genome comparisons indicate that major virulence mechanisms are conserved between these two groups (for review, see Nunes and Gomes 2014). 700 genes have homologues in both groups, but synteny is poorly conserved (Horn et al., 2004). Chlamydiaceae genome reduction seems to be linked to its



**Table Box1.** Genome size, pathogenicity, hosts and references of chlamydial species for which genomes are available (n.d. not determined, \* provisional designations).

Species	Genome size (Mb)	Pathogenicity	Host	Reference
<i>C. trachomatis</i>	1.04	Trachoma, genital infections	Human	Stephens et al. (1998)
<i>C. pneumoniae</i>	1.23	Pneumonia	Human, horse, marsupials, frogs	Kalman et al. (1999)
<i>C. muridarum</i>	1.07	Pneumonitis in mouse	Mouse	Read et al. (2000)
<i>C. caviae</i>	1.17	Conjunctivitis and genital tract infections in guinea pig	Guinea pig	Read et al. (2003)
<i>C. abortus</i>	1.14	Zoonotic infections, miscarriage	Ruminants	Thomson et al. (2005)
<i>C. felis</i>	1.17	Conjunctivitis and respiratory disease in cat	Cat	Azuma et al. (2006)
<i>C. pecorum</i>	1.11	Asymptomatic chronic infections, metritis, arthritis, conjunctivitis and mastitis in animals	Ruminants, swine, birds, koala	Mojica et al. (2011)
<i>C. psittaci</i>	1.17	Zoonotic infections, pneumonia	Human, birds	Voigt et al. (2011)
<i>C. avium</i> *	1.04	n.d.	Pigeon	Sachse et al. (2014)
<i>C. gallinacea</i> *	1.05	n.d.	Poultry	Sachse et al. (2014)
<i>C. suis</i>	1.08	Conjunctivitis, pneumonia, enteritis, reproductive disorders in swine	Swine	Donati et al. (2014)
<i>W. chondrophila</i>	2.12	Miscarriage	Human, ruminants	Bertelli et al. (2010)
<i>S. negevensis</i>	2.50	Respiratory diseases	Human, possibly amoebae	Collingro et al. (2011)
<i>P. acanthamoebae</i>	3.07	Pneumonia	Human, amoebae	Greub et al. (2009), Collingro et al. (2011)
<i>Pr. amoebophila</i>	2.41	n.d.	Amoebae	Horn et al. (2004)
<i>Neochlamydia</i> sp.	3.19	n.d.	Amoebae	Ishida et al. (2014)

evolution within animal hosts. In comparison, *Chlamydia*-related bacteria can infect protists, which may serve as a melting pot for genes exchanges (Greub 2009a; Moliner, Fournier and Raoult 2010) and several of them are agents of human and/or animal diseases (Table Box1). Chlamydiaceae lost several amino acid biosynthesis enzymes, consequence of their parasitic life style. *Chlamydia*-related bacteria lost less enzymes, perhaps because their environment contains a lower concentration of nutrients and they thus need to synthesize more molecules. For example, *W. chondrophila* possesses the complete pathways to synthesize 11 essential amino acids, whereas *C. trachomatis* only 3 (Bertelli et al., 2010). *Chlamydia*-related bacteria are thus able to develop in different niches (from protists to human cells). In contrast, Chlamydiaceae are more specialized and can infect only limited species. Thus, *C. trachomatis* can infect only human (Table Box1).

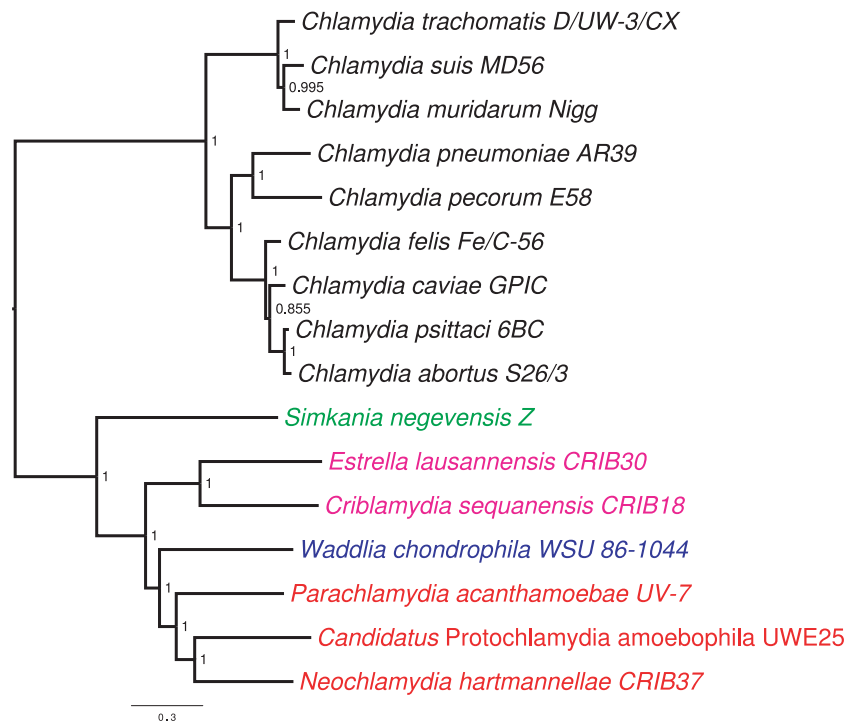
## BOX2: ABERRANT BODIES AND PERSISTENCE

When the proliferation cycle of *Chlamydiales* is blocked, enlarged bacteria called aberrant bodies accumulate (Wyrick 2010). Aberrant bodies can be induced by diverse stimuli including the addition of penicillin (Matsumoto and Manire 1970; Lambden, Pickett and Clarke 2006), activation of interferon-gamma (Shemer and Sarov 1985; Pantoja et al., 2001), starvation of iron or nutrient (Coles et al., 1993) as well as coinfection of the host with herpes or other viruses (Deka et al., 2006; Borel et al., 2010) in a process that is not mediated by any known persistence inducer (Vanover et al., 2008). Aberrant bodies apparently replicate continuously their DNA in absence of division. This is indicated by the expression of DNA replication genes, but not cytokinesis genes during

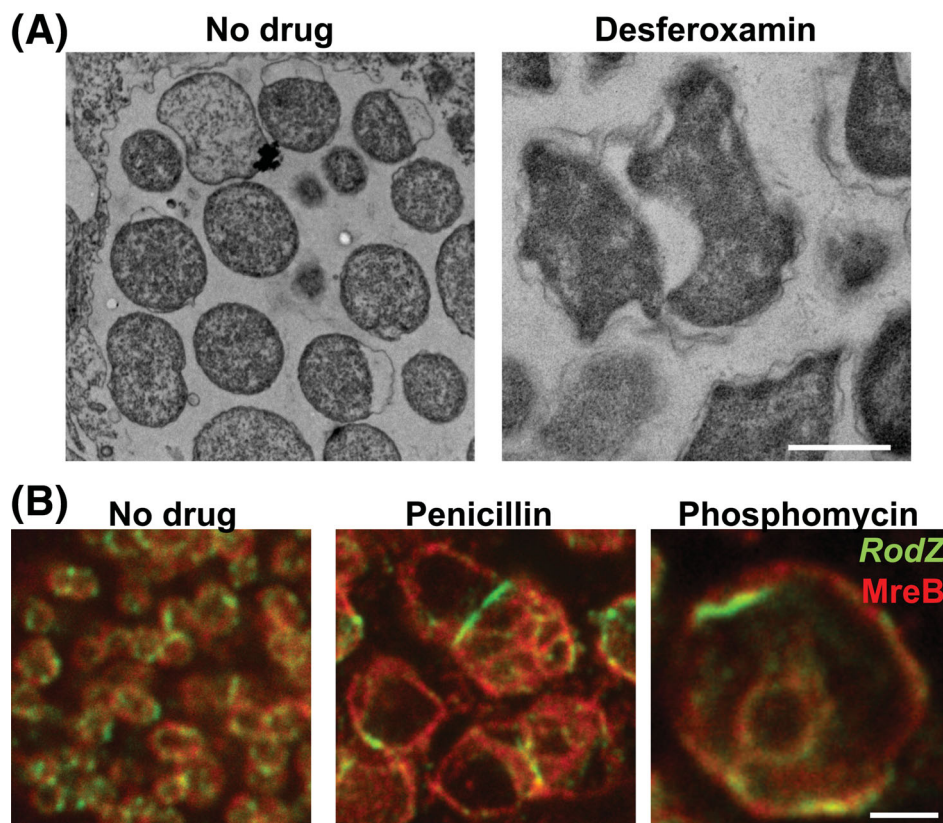
persistence in *C. pneumoniae* (Byrne et al., 2001; Abdelrahman, Rose and Belland 2011). This is consistent with the accumulation of a minimum of 16 copies of the genome in the aberrant bodies in *C. trachomatis* treated with penicillin (Lambden, Pickett and Clarke 2006). Aberrant bodies are considered a persistent stage since they de-differentiate in RBs and subsequently into infectious EBs when the stress is relieved (Matsumoto and Manire 1970). This persistence mechanism allows *Chlamydiales* to survive in presence of penicillin and is believed to account for the observed recurrence of infection when the beta-lactam treatment is stopped. Persistent aberrant bodies formation is a conserved feature among *Chlamydiales* as they are also observed in *Chlamydia*-related *W. chondrophila* (Fig. Box2) (Kebbi-Beghdadi, Cisse and Greub 2011). Persistent aberrant bodies were also observed *in vivo* (Borel et al., 2008; Phillips Campbell et al., 2012) and presence of chlamydial DNA and RNA in patients with chronic chlamydial infections was a strong indication for persistence of viable but not proliferating *Chlamydiae* (Gerard et al., 1998). Aberrant bodies are thus believed to play an important role in the onset of chronic infections by *Chlamydiales* (Mpiga and Ravaoarino 2006). Nevertheless, a direct role of aberrant bodies in the onset of chronic infections has still to be demonstrated. It is thus important to better understand the mechanisms that cause the formation of aberrant bodies to improve the treatment of chronic chlamydial infections.

## BOX3: MreB AND RodZ

MreB is an actin homologue, which, already in the eighties, was demonstrated to be required for the rod-shape maintenance in *E. coli* and which is involved in meccillinam resistance



**Figure Box 1.** Phylogenetic tree of Chlamydiales with available genomes. Mid-point-rooted phylogenetic tree of the Chlamydiales order based on concatenated alignments of 470 core proteins was reconstructed using PhyML with LG + [+I model of substitution. It includes representatives of five main clades: the Chlamydiaceae family (black), the Simkaniaceae family (green), the Criblamydiaceae family (pink), the Waddliaceae family (blue) and the Parachlamydiaceae family (red).



**Figure Box 2.** Aberrant bodies caused by different stimuli. (A) Electron microscopy pictures of *W. chondrophila* infecting Vero cells in absence or in presence of 400  $\mu\text{M}$  of the iron chelator deferoxamine (scale bar 2  $\mu\text{m}$ ). (B) Immunofluorescence micrographs of Vero cells infected by *W. chondrophila* in presence or in absence of 500  $\mu\text{g ml}^{-1}$  penicillin or phosphomycin (scale bar 2  $\mu\text{m}$ ).

(Doi et al., 1988). Only later, in 2001, MreB was shown to polymerize in a filamentous and helical actin-like structure (Jones, Carballido-Lopez and Errington 2001). MreB possess a similar tertiary structure as actin, when compared by crystallography even if the primary structures are divergent (van den Ent, Amos and Lowe 2001). MreB is conserved among most of rod-shape bacteria. Some bacteria, including *B. subtilis*, possess several homologues of MreB producing distinct kinds of filaments (Jones, Carballido-Lopez and Errington 2001). Localization studies of MreB by immunofluorescence or by fluorescent proteins fusion first indicated a helicoidal localization around the cell (Jones, Carballido-Lopez and Errington 2001; Figge, Divakaruni and Gober 2004; Gitai et al., 2005; reviewed in Carballido-Lopez 2006). Recently, use of total internal reflection fluorescence microscopy could determine that MreB forms discontinuous patches (Dominguez-Escobar et al., 2011; Garner et al., 2011; reviewed in White and Gober 2012). These patches are believed to be sites of PG biosynthesis, as MreB interacts with several PG biosynthesis enzymes (MurB, MurC, MurE, MurF and MurG) (Divakaruni et al., 2007; Mohammadi et al., 2007; Varma and Young 2009; White, Kitich and Gober 2010; Gaballah et al., 2011). Moreover, MreB requires the membrane proteins MreC and MreD for the organization of the PG biosynthesis and modification (White, Kitich and Gober 2010). MreB filaments are anchored to the plasma membrane by several interactions: direct hydrophobic interactions with the plasma membrane through an N-terminal amphipathic helix and a membrane insertion loop (Salje et al., 2011), and interactions with integral membrane proteins like RodZ and FtsK (Alyahya et al., 2009; van den Ent et al., 2010; Ouellette et al., 2012). RodZ is required to get a normal assembly of the MreB cytoskeleton (Bendezu et al., 2009). It is hypothesized that RodZ might be the link between the cytoplasmic MreB and periplasmic PG modifying enzymes PBPs. This is consistent with the fact that RodZ interacts with RodA and MreD in a two hybrid assay in *C. crescentus* (White, Kitich and Gober 2010). Moreover, RodZ and MreB localize at the division septum in *C. crescentus* and *W. chondrophila* (Alyahya et al., 2009; Jacquier et al., 2014). Thus, MreB and RodZ likely localize to the places where active PG biosynthesis occurs (reviewed in White and Gober 2012).

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