Discovery of chlamydial peptidoglycan reveals bacteria with murein sacculi but without FtsZ

Martin Pilhofer1,2,*, Karin Aistleitner3,*, Jacob Biboy4, Joe Gray5, Erkin Kuru6, Edward Hall6, Yves V. Brun6, Michael S. VanNieuwenhze6, Waldemar Vollmer4, Matthias Horn3 & Grant J. Jensen1,2

Chlamydiae are important pathogens and symbionts with unique cell biological features. They lack the cell-division protein FtsZ, and the existence of peptidoglycan (PG) in their cell wall has been highly controversial. FtsZ and PG together function in orchestrating cell division and maintaining cell shape in almost all other bacteria. Using electron cryotomography, mass spectrometry and fluorescent labelling dyes, here we show that some environmental chlamydiae have cell wall sacculi consisting of a novel PG type. Treatment with fosfomycin (a PG synthesis inhibitor) leads to lower infection rates and aberrant cell shapes, suggesting that PG synthesis is crucial for the chlamydial life cycle. Our findings demonstrate for the first time the presence of PG in a member of the Chlamydiae. They also present a unique example of a bacterium with a PG sacculus but without FtsZ, challenging the current hypothesis that it is the absence of a cell wall that renders FtsZ non-essential.
Chlamydiae are members of the Planctomycetes–Verrucomicrobia–Chlamydiae (PVC) bacterial superphylum. Like most other bacteria, some PVC bacteria are already known to possess peptidoglycan (PG), that is, chains of alternating N-acetylg glucosamine and N-acetylmuramic acid sugars crosslinked by short peptides. PVC bacteria also display striking eukaryote-like and archaea-like cell biological features, which have suggested intriguing hypotheses about their role in cellular evolution.

In Verrucomicrobia and almost all other bacteria, septal PG synthesis is orchestrated by the FtsZ cytoskeleton. In contrast, present in the genomes of all chlamydiae. In addition, several of the genomes, a nearly complete pathway for the synthesis of PG is found in chlamydiae is surprising, however, as despite their highly reduced cellular evolution.

Protochlamydia amoebophila and Simkania negevensis. Through electron cryotomography (ECT), biochemical purification, enzymatic digestion, mass spectrometry (MS), fluorescence microscopy and antibiotic treatment, we show that P. amoebophila are indeed surrounded by sacculi containing a new type of PG. In contrast, no evidence of PG is found in S. negevensis. These results prove that some chlamydiae do in fact synthesize PG sacculi, explaining the presence of PG-synthetic genes, but raising new questions about the identity and purpose of the modifications and the mechanisms of cell division in the absence of FtsZ.

Results
ECT of the chlamydial cell envelope. Two diverse and deeply rooting members of the chlamydial phylum, S. negevensis and P. amoebophila, were imaged by ECT in a near-native state. Bacteria were purified from amoeba cultures, plunge-frozen and 25 and 20 tomograms were collected of intact cells (Fig. 1). Density profiles through the cell envelopes of the two species were quite different. Although four layers were resolved in Simkania envelopes (Fig. 1b,c), five layers were resolved in Protochlamydia (Fig. 1e,f). Because the individual leaflets of lipid bilayers can be resolved in some cryotomograms, especially when the images are taken close to focus, in the case of Simkania, it is unclear whether the four layers represent the two leaflets of the outer and inner membranes (‘O’ and ‘A’ being the two leaflets of the outer membrane, and layers ‘B’ and ‘T’ being the two leaflets of the inner membrane), or whether one or more of these layers are non-membranous. The facts that layers O and A have fairly similar contrast and are consistently spaced even through the undulations are consistent with them being two leaflets of a single (outer) bilayer membrane. Their separation (~ 5 nm), however, is much larger than typical phospholipid bilayer membranes, whose two density peaks (from the phospholipid head groups) are only 3.7–4 nm apart. Similarly, Simkania layers B and I may be the two leaflets of a single (inner) membrane, as they have similar contrast and a consistent spacing, but again they appear too far apart. In contrast to the Simkania envelope, the profile of Protochlamydia surprisingly resembled those of other Gram-negative bacteria with two membranes and a PG cell wall.

Between the Protochlamydia outer and inner membranes (labelled ‘O’ and ‘I’, respectively) there appeared to be three additional layers (labelled ‘C’–’E’). The similar-looking three layers in Treponema pallidum (from the outside in) were identified as proteinaceous (lipoproteins), PG and again proteinaceous. By analogy this suggests that layer C is composed of lipoproteins (perhaps connecting the outer membrane to the cell wall) and other outer-membrane-associated proteins, layer D is a cell wall and layer E is composed of proteinases and inner-membrane-associated proteins (perhaps including the penicillin-binding proteins responsible for cell wall synthesis, the lipoprotein OmCA and the cysteine-rich protein OmCB). Although other interpretations remain possible (cysteine-rich disulphide-crosslinked envelope proteins have been suggested to be the functional equivalent of PG in chlamydiae), the most important and clear observation was that Protochlamydia exhibit a distinct periplasmic layer (D).

Purification and imaging of sacculi. In order to explore whether any of the observed periplasmic layers consisted of PG, we attempted to purify sacculi by boiling chlamydial cells (obtained from asynchronously infected amoeba cultures) in 4% sodium dodecyl sulphate. Strikingly, in three independent experiments, we observed sacculus-like structures in preparations from Protochlamydia (Fig. 2a–d), but not from Simkania (two experiments). Protochlamydia sacculi diameters (679 nm ± 34 s.d. n = 10) and morphologies matched the size and shape of intact cells. Protochlamydia sacculi had one or two 5–7 nm thick layers (arrowheads in Fig. 2e), plus mesh-like (up to 30 nm long) high-density aggregates attached to the outside (arrows in Fig. 2d).

Digestion and biochemical analyses of sacculi. To check for the presence of PG in the purified sacculi, we digested the samples with cellosyl, a glycacin strand-clearing PG muramidase. Cellosyl released soluble material from insoluble sacculi, which was reduced with sodium borohydride and analysed by high-pressure liquid chromatography (HPLC) using conditions for separating muropeptides. The chromatogram (Fig. 2f) showed three main peaks in the monomeric region (20–50 min) and many peaks after 60 min that are poorly separated at higher retention time (> 75 min) forming a ‘hump’, which is typical for highly crosslinked and/or incompletely digested PG material. The retention times and overall pattern of cellosyl digestion products were different, however, from those of muropeptide mixtures obtained from other Gram-positive and Gram-negative bacteria.

To characterize this material, the three main cellosyl products in the monomeric region and one well-separated main product at the beginning of the ‘hump’ region were analysed by MS. The determined neutral masses of the earlier three products were higher than what would be expected for monomeric muropeptides, but the masses of products 1 and 2 and of products 2 and 3 both differed by 71 Da, a typical feature of monomeric muropeptides with a tri-, tetra- and pentapeptide, respectively, due to the presence of none, one or two D-alanine residues (Fig. 2f). In MS/MS analysis, the three products were fragmented in a similar way, showing that they are related (Supplementary Fig. S1, Table 1). For all three peaks, we observed mass differences to the parent ion corresponding to the loss of GlcNAc, GlcNAcMurNAc(O) (r, indicates reduction to N-acetylmuramitol),
GlcNAcMurNAc(r)-l-alanine and GlcNAcMurNAc(r)-l-alanine-D-glutamate, confirming that products 1–3 are all muropeptides. The neutral masses of the Protochlamydia products 1, 2 and 3 were all 314.12 Da larger than the masses of the reduced monomeric muropeptides (with tri-, tetra- or pentapeptide) from Gram-negative bacteria\(^3\), however, suggesting the presence of a common modification in the Protochlamydia muropeptides. The neutral mass of product 4 was consistent with a peptide crosslinked dimer of product 2. Additional mass differences that occurred in all fragmentation spectra indicated the presence of the same and as yet unknown modifications with 129 and 203 Da, respectively, explaining the higher mass of Protochlamydia muropeptides 1–3 compared to the monomeric muropeptides of Escherichia coli.

Figures 1 and 2 describe the multi-layered cell envelopes of Simkania and Protochlamydia, respectively.

**Figure 1** | Chlamydial cell envelopes are multi-layered. Simkania (a–c) and Protochlamydia (d–f) cells were purified from asynchronously infected amoeba cultures, plunge-frozen and imaged by ECT. Shown are tomographic slices through reticulate bodies (a,d and b,e enlarged) and corresponding density profiles (c,f) of the cell envelopes. Profiles are enlarged, aligned and cropped relative to the outer membrane. Distances between peaks (in nm) are indicated. In contrast to the Simkania profile, the Protochlamydia profile resembles those of other bacteria with PG cell walls (see text for a full discussion of each profile and layer). Scale bar, 100 nm in a,d and 20 nm in b,c,e,f.

**Figure 2** | Protochlamydia synthesize purifiable sacculi that contain PG. Cryoprojections (a) and tomographic slices (b–e) through sacculi-like structures purified from Protochlamydia cells. We were unable to obtain similar structures from Simkania. Sacculi had one or two layers (arrowheads) plus short high-density filaments (arrows) on the outside (c enlarged in d,e). Sacculi were digested, reduced and separated by HPLC (f). MS analysis of peaks 1–3 (neutral masses indicated in Da) indicated the presence of modified PG (see also Table 1 and Supplementary Fig. S1). Scale bars, 100 nm.
To confirm that PG was a major component of *Protochlamydia* sacculi and check for the presence of disulphide-crosslinked protein components, purified sacculi were subjected to lysozyme and dithiothreitol treatment, respectively, and imaged with negative stain EM. Only the incubation with lysozyme degraded the sacculi (Supplementary Fig. S2).

**Fluorescence imaging of d-alanine incorporation in vivo.** To further confirm the presence of PG in *Protochlamydia* sacculi, we tested whether fluorescently labelled amino acids (fluorescent d-alanine (FDAA); labelled ψ-alanine in our experiments) would be incorporated into chlamydial cells in *vivo*. Incubation of amoeba cultures continuously infected with *Protochlamydia* (including reticulate bodies, elementary bodies and transitional stages) with FDAA (HADA (hydroxy coumarin-carbonyl-amino- d-alanine) and BADA (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diace-s-indacene-3-propionic acid-3-amino-ψ-alanine)) resulted in multiple strong and chlamydial cell-sized signals inside amoeba cells (Fig. 3a–d, Supplementary Fig. S3). In many cases, the FDAA labelling in infected amoebae overlapped with staining of the chlamydial cells by 4,6-diamidino-2-phenylindole (DAPI) or chlamydial-specific fluorescence in *situ* hybridization (FISH). Not all cells stained by FISH/DAPI showed a corresponding FDAA signal, at least in part because chlamydial cells were in different developmental stages, including non-replicating elementary bodies. No signals were detected when uninfected amoebae were incubated with FDAA (Supplementary Fig. S3D, E), or when infected amoebae were incubated with dimethylsulphoxide only. Interestingly, purified *Protochlamydia* elementary bodies (which cannot undergo cell division and are therefore probably not actively synthesizing new PG) also did not show labelling upon incubation with FDAA (Supplementary Fig. S3B,C), indicating that PG synthesis takes place during *Protochlamydia* replication inside the host. Amoeba cultures infected with *Simkania*, on the other hand, showed either no signals (using BADA) or signals similar to the background level (using HADA) upon labelling with FDAA (Supplementary Fig. S4A,B), consistent with the absence of purifiable sacculi. Purified *Simkania* cells were not labelled by either dye (Supplementary Fig. S4C,D).

**Protochlamydia sensitivity to cell-wall-targeting antibiotics.** Owing to the high conservation of PG throughout the bacterial domain of life, many antibacterial drugs target PG synthesis. The so-called chlamydial anomaly is that despite the fact that PG has not been detected in pathogenic chlamydiae, these organisms are sensitive to cell-wall-targeting β-lactam antibiotics. Penicillin, for instance, leads to the formation of enlarged aberrant cells and blocks the conversion between developmental stages. Environmental chlamydiae, in contrast, are resistant to β-lactams – possibly due to putative β-lactamases encoded in their genomes. To explore the role of the PG sacculus in the *Protochlamydia* life cycle, we used an alternative PG synthesis-targeting antibiotic (fosfomycin) to treat infected amoeba cells. The addition of 500 μg mL⁻¹ fosfomycin to *Protochlamydia*-infected amoeba cultures led to a significant decrease in infection rate (20.2% ± 8 infected amoebae for fosfomycin-treated cultures versus 95.8% ± 2.2 infected amoebae for untreated cultures; P < 0.0001, unpaired t-test). *Protochlamydia* cells within treated cultures were also up to eight-times larger than normal.
We conclude that *Protochlamydia* synthesize sacculi containing PG that can be hydrolysed by cellosyl, contains monomeric and crosslinked muropeptides, and carries yet unknown modifications at virtually every subunit. No evidence of PG in *Simkania* was found. Fluorescence imaging of d-Ala incorporation in vivo and monitoring of cell wall antibiotic sensitivity further suggested that the *Protochlamydia* PG sacculus has an important role in cell cycle and shape. This challenges previous speculations that chlamydiae synthesize a small ring of PG only during cell division. Because this might still be true for *Simkania* and pathogenic chlamydiae, however, our data prompts a reconsideration of whether these organisms lack PG entirely (and the effects of β-lactams are pleiotropic) or if they synthesize novel PG structures that are not purified by standard sacculus preparation protocols.

The presence of sacculi in *Protochlamydia* but not in *Simkania* matches the less complete set of synthetic genes in the latter: *Simkania*, as well as pathogenic chlamydiae, lack an undecaprenyl-diphosphate phosphatase (UppP) and alanine/glutamine racemases (Alr, MurI) (Supplementary Table S1). Interestingly, transglycosylases have not been found in any chlamydial genomes (Supplementary Table S1) or in the genomes of a few other PG-possessing bacteria, so some other enzyme(s) must be capable of synthesizing glycan strands.

The presence of PG sacculi in *Protochlamydia* and in the *Chlamydiae*’s sister phylum *Verrucomicrobia*, together with the fact that the more basal chlamydial lineages have more complete PG synthesis pathways make it likely that the last common chlamydial ancestor synthesized a PG sacculus. The detection of a PG-containing sacculus in *Protochlamydia* challenges the view that FtsZ is essential in PG-possessing bacteria, however, because to our knowledge, *Protochlamydia* is the first example of a bacterium with a PG cell wall, but without FtsZ. Studying cell division and septal development in this organism could help clarify the role of FtsZ and the evolutionary transition to PG- and FtsZ-independency.

**Methods**

**Cultivation of organisms.** *Acanthamoeba castellanii* Neff infected with *P. amoebaphila* UWE25, or *A. castellanii* UWCI infected with *S. negevensis*, were cultivated in TSY medium (30 g l\(^{-1}\) tryptase soy broth, 10 g l\(^{-1}\) yeast extract, pH 7.3) at 20 °C. Amoebal growth was monitored by light microscopy and medium was exchanged every 3–6 days. The presence and identity of the chlamydial symbionts was verified by isolation of DNA from cultures followed by amplification and sequencing of the 16S rRNA genes. In addition, FISH using specific probes combined with DAPI staining of infected cultures was performed using specific probes for the respective symbiont. Amoebae infected with chlamydiae were allowed to attach on slides and were fixed with 4% formaldehyde at 20 °C. Cells were hybridized for 1.5 h at 46 °C at a formamide concentration of 25% with the *Protochlamydia*-specific probe E25-454 (5′-GGA TGT TAG CCA GCT CTC-3′) and the *Simkania*-specific probe Simneg-183 (5′-CAG GCT ACC CCA GCT CTC-3′) and the probe EB338 (ref. 48). Subsequently, cells were stained with DAPI (0.5 μg ml\(^{-1}\) in PBS) for 5 min, and slides were analysed using an epifluorescence microscope.

**Purification of chlamydiae.** Infected *A. castellanii* cultures were harvested by centrifugation (7,197 × g, 10 min), washed in Page’s Amoebic Saline (PAS) centrifuged and resuspended in PAS. Amoeba cells were disrupted by vortexing with an equal volume of glass beads for 3 min. Glass beads and cell debris were removed by centrifugation (5 min, 300 × g). The supernatant was filtered through a 1.2 μm filter and centrifuged at maximum speed for 10 min, 18,000 × g. The obtained pellet was resuspended in PAS.
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