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

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Abstract

Chlamydiae are important pathogens and symbionts, with unique cell biology features. They lack the cell-division protein FtsZ, which functions in maintaining cell shape and orchestrating cell division in almost all other bacteria. In addition, the existence of peptidoglycan (PG) in chlamydial cell envelopes has been highly controversial. Using electron cryotomography, mass spectrometry and fluorescent labeling dyes, here we show that some environmental chlamydiae have cell-wall sacculi consisting of an unusual 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.

Introduction

Chlamydiae are members of the *Planctomycetes-Verrucomicrobia-Chlamydiae* (PVC) bacterial superphylum[1]. Like most other bacteria, some PVC bacteria are already known to possess peptidoglycan, i.e. chains of alternating N-acetylglucosamine and N-acetylmuramic acid sugars crosslinked by short peptides. PVC bacteria also display striking eukaryote-like

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Author contributions

MP initiated the study. MP and KA performed all experiments except HPLC/MS analyses of sacculi, which were done by JB, JG, and WV. EK, EH, YVB, and MSV provided the FDAA dyes and advice on the FDAA labeling experiments. MP, KA, WV, MH, and GJJ designed the experiments and wrote the manuscript.

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and archaea-like cell biological features, which have suggested intriguing hypotheses about their role in cellular evolution[2, 3].

In *Verrucomicrobia* and almost all other bacteria, septal PG synthesis is orchestrated by the FtsZ cytoskeleton[4, 5]. In contrast, *Planctomycetes* lack both PG and FtsZ[5]. This fits to the notion that PG-loss renders FtsZ dispensable, like in mycoplasmas[6] or L-form bacilli[7]. While from genome sequences it is clear that chlamydiae do not possess FtsZ, the presence or absence of chlamydial PG has been highly controversial[8, 9, 10, 11, 12, 13, 14, 15, 16]. One early study reported the colorimetric detection of muramic acid in chlamydiae[14], but more reliable chromatographic methods subsequently failed to confirm this result[11, 15]. All attempts to purify chlamydial sacculi have failed[10, 12] and no periplasmic density layers have been detected between the inner and outer membranes of chlamydiae by electron microscopy (including for instance[17, 18, 19]). The apparent absence of PG in chlamydiae is surprising, however, since despite their highly reduced genomes, a nearly complete pathway for the synthesis of PG is present in the genomes of all chlamydiae[13]. In addition, several of the chlamydial PG biosynthetic enzymes have been characterized and shown to be functional *in vitro* and in complementation assays[20, 21, 22, 23, 24, 25].

Here we look for evidence of peptidoglycan cell walls in two diverse and deeply rooting chlamydiae[26], *Protochlamydia amoebophila* and *Simkania negevensis*. Through electron cryotomography (ECT), biochemical purification, enzymatic digestion, mass spectrometry, 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

Electron cryotomography of the chlamydial cell envelope

Two diverse and deeply rooting members of the chlamydial phylum, *Simkania negevensis* and *Protochlamydia amoebophila*, were imaged by electron cryotomography (ECT) in a near-native state. Bacteria were purified from amoeba cultures, plunge-frozen, and 25 and 20 tomograms were collected of intact cells (Figure 1). Density profiles through the cell envelopes of the two species were quite different. While four layers were resolved in *Simkania* envelopes (Figure 1 B, C), five layers were resolved in *Protochlamydia* (Figure 1 E, 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 “I” 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[27]. Similarly, *Simkania* layers B and I may be the two leaflets of a single (inner) membrane, since 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 peptidoglycan cell wall[28, 29, 30]. Between the *Protochlamydia* outer and inner membranes (labeled “O” and “I”, respectively) there appeared to be three additional layers (labeled “C”-“E”). The similar-looking three layers in *Treponema pallidum* (from the outside in) were identified as proteinaceous (lipoproteins), peptidoglycan, and

again proteinaceous[29]. 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 lipoproteins 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). While other interpretations remain possible (cysteine-rich disulphide-cross-linked envelope proteins have been suggested to be the functional equivalent of PG in chlamydiae[31]), 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 peptidoglycan (PG), we attempted to purify sacculi by boiling chlamydial cells (obtained from asynchronously infected amoeba cultures) in 4% sodium dodecyl sulfate. Strikingly, in three independent experiments, we observed sacculus-like structures in preparations from *Protochlamydia* (Figure 2, A-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 Figure 2 E), plus mesh-like (up to 30 nm long) high-density aggregates attached to the outside (arrows in Figure 2 D).

Digestion and biochemical analyses of sacculi

To check for the presence of peptidoglycan in the purified sacculi, we digested the samples with cellosyl, a glycan strand-cleaving peptidoglycan muramidase. Cellosyl released soluble material from insoluble sacculi, which was reduced with sodium borohydride and analyzed by high pressure liquid chromatography (HPLC) using conditions for separating muropeptides[32]. The chromatogram (Figure 2 F) 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 cross-linked and/or incompletely digested PG material[33]. 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 analyzed by mass spectrometry (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 (Figure 2 F). In MS/MS analysis the three products 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 Glc*N*Ac, Glc*N*AcMur*N*Ac(r) (r, indicates reduction to *N*-acetylmuramitol), Glc*N*AcMur*N*Ac(r)-L-alanine and Glc*N*AcMur*N*Ac(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[34], however, suggesting the presence of a common modification in the *Protochlamydia* muropeptides. The neutral mass of product 4 was consistent with a peptide cross-linked 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 *E. coli*.

To confirm that PG was a major component of *Protochlamydia* sacculi and check for the presence of disulfide cross-linked protein components, purified sacculi were subjected to lysozyme and DTT treatment, respectively, and imaged with negative stain electron microscopy. 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 labeled amino acids (FDAA; labeled D-alanine in our experiments)[35] 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 and BADA) resulted in multiple strong and chlamydial cell-sized signals inside amoeba cells (Figure 3 A-D, Supplementary Fig. S3). In many cases the FDAA labeling in infected amoebae overlapped with staining of the chlamydial cells by DAPI or chlamydiae-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. S3 D, E), or when infected amoebae were incubated with DMSO only. Interestingly, purified *Protochlamydia* elementary bodies (which cannot undergo cell division and are therefore probably not actively synthesizing new PG) also did not show labeling upon incubation with FDAA (Supplementary Fig. S3 B, 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 labeling with FDAAs (Supplementary Fig. S4 A, B), consistent with the absence of purifiable sacculi. Purified *Simkania* cells were not labeled by either dye (Supplementary Fig. S4 C, D).

Protochlamydia sensitivity to cell-wall-targeting antibiotics

Due to the high conservation of PG throughout the bacterial domain of life, many antibacterial drugs target PG synthesis. The so-called “chlamydial anomaly”[16] 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[36, 37] and blocks the conversion between developmental stages[38]. Environmental chlamydiae, in contrast, are resistant to β-lactams[39, 40] - 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 fosfomycin to *Protochlamydia*-infected amoeba cultures led to a significant decrease in infection rate (20.2% ± 8 infected amoebae for fosfomycin-treated cultures vs. 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 (diameters of up to 6 μm) (Figure 3 G, H). Lower fosfomycin concentrations (25 μg/ml and 100 μg/ml) induced the formation of fewer aberrant forms and did not affect the infection rate (not shown). Fosfomycin-treatment of *Simkania*-infected amoeba cultures led to only a slight decrease in infection rate (59.9% ± 5.6 infected amoebae for fosfomycin-treated cultures vs. 67% ± 1.7 infected amoebae for untreated cultures) and no differences in cell size were detected (Figure 3 E, F).

Discussion

We conclude that *Protochlamydia* synthesize sacculi containing peptidoglycan that can be hydrolyzed by cellosyl, contains monomeric and cross-linked muropeptides, and carries yet unknown modifications at virtually every subunit. No evidence of peptidoglycan 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 plays an important role in cell cycle and shape. This challenges previous speculations that chlamydiae synthesize a small ring of PG only during cell division[41]. 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)[13, 42, 43]. Interestingly, transglycosylases have not been found in any chlamydial genomes (Supplementary Table S1)[13] or in the genomes of a few other PG-possessing bacteria[44, 45], 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*[46], 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[4, 6, 7], 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 *Protochlamydia amoebophila* UWE25, or *A. castellanii* UWC1 infected with *Simkania negevensis*, were cultivated in TSY medium (30 g/L trypticase soy broth, 10 g/L 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, fluorescence *in situ* hybridization (FISH) using specific probes combined with 4′,6-diamidino-2-phenylindole (DAPI) staining of infected cultures was performed using specific probes for the respective symbiont[47]. 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 hours at 46°C at a formamide concentration of 25% with the *Protochlamydia*-specific probe E25-454 (5′ -GGATGT TAG CCA GCT CAT-3′) and the probe EUB338[48]. Subsequently, cells were stained with DAPI (0.5 μg/ml in PBS) for 5 minutes, and slides were analyzed 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)[49], centrifuged and resuspended in PAS. Amoeba cells were disrupted by vortexing with an equal volume of glass beads for 3 minutes. 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. The obtained pellet was resuspended in PAS.

Plunge-freezing

For plunge-freezing, copper/rhodium electron microscopy (EM) grids (R2/2 or R2/1, Quantifoil) were glow-discharged for 1 min. A 20×-concentrated bovine serum albumin-treated solution of 10 nm colloidal gold (Sigma) was added to purified chlamydiae or sacculi (1:4 v/v) immediately before plunge freezing. A 4-μl droplet of the mixture was applied to the EM grid, then automatically blotted and plunge-frozen into a liquid ethane-propane mixture[50] using a Vitrobot (FEI Company)[51].

Electron cryotomography

Images were collected using a Polara 300 kV FEG transmission electron microscope (FEI Company) equipped with an energy filter (slit width 20 eV; Gatan) on a lens-coupled 4 k×4 k UltraCam CCD (Gatan). Pixels on the CCD represented 0.95 nm (22,500×) or 0.63 nm (34,000×) at the specimen level. Typically, tilt series were recorded from −60° to +60° with an increment of 1° at 10 μm under-focus. The cumulative dose of a tilt-series was 180-220 e $^{-}$/Å^{2}. UCSF Tomo[52] was used for automatic acquisition of tilt-series and 2D projection images. Three-dimensional reconstructions were calculated using the IMOD software package[53] or Raptor[54]. Tomograms were visualized using 3dMOD[53]. Density profiles were generated using ImageJ.

Fluorescent labeling of peptidoglycan

Newly synthesized peptidoglycan was labeled using fluorescent D-amino acids[35]. *A. castellanii* cells continuously infected with *Protochlamydia* or *Simkania* were harvested and resuspended in a mixture of TSY and PAS (1:1). Cells were incubated with 1.5 mM HADA (hydroxy coumarin-carbonyl-amino-D-alanine)[35] or BADA (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Propionic Acid-3-amino-D-alanine) for 6 h with gentle shaking. Cells were pelleted, washed three times and fixed with 4% formaldehyde followed by FISH using the chlamydia-specific probe Chls-0523 or DAPI-staining. As a control, uninfected amoebae and purified chlamydiae were treated in the same way.

Antibiotic treatment of infected amoebae cultures

A. castellanii were seeded into the wells of a multi-well dish and infected with purified *Simkania* and *Protochlamydia* elementary bodies[55]. After centrifugation at 600 × g for 10 min, the medium was exchanged for TSY supplemented with fosfomycin (0, 25, 100 or 500 μg/ml, respectively). Medium was exchanged once at 48 hours post infection. Cells were fixed with methanol at 96 hours post infection followed by immunofluorescence analysis using either anti-PomS antibodies[55] or anti-*Simkania* antibodies raised against purified chlamydiae. The number of infected amoebae was counted for each treatment.

Preparation and composition analysis of sacculi

Chlamydial cells were purified from 1.8 L (*Protochlamydia*) and 3 L (*Simkania*) of infected amoeba culture and, depending on the amount of harvested cells, resuspended in 2.6-5.2 ml 4% SDS (w/v). After shipping (overnight, room temperature), the suspensions were dripped

into 4% SDS (preheated to 90°C, 6 ml final volume) and stirred for 2.5 h at 90°C. Sacculi were pelleted (30 min, 135,000 × g) in a TLA-100.3 rotor (Beckman Coulter), washed 4× in 3 ml water, resuspended in 150 μl water and supplemented with 0.02% (w/v) sodium azide. Muropeptides were released from the PG by an overnight incubation with the muramidase Cellosyl (Hoechst, Frankfurt am Main, Germany) on a thermal shaker at 37°C and 800 rpm. The sample was boiled for 10 min and centrifuged for 10 min at 13,500 × g. The muropeptides present in the supernatant were reduced with sodium borohydride and separated on a 250×4.6 mm 3-μm Prontosil 120-3-C18 AQ reversed phase column (Bischoff, Germany) at 55°C using a 135 min gradient from 50 mM sodium phosphate pH 4.31 to 75 mM sodium phosphate pH 4.95, 15% methanol and a flow rate of 0.5 ml/min[32]. Muropeptides were detected at 205 nm. Fractions were collected, concentrated in a SpeedVac, acidified by 1% trifluoroacetic acid, and analyzed by offline electrospray mass spectrometry on a Finnigan LTQ-FT mass spectrometer (ThermoElectron, Bremen, Germany) in positive ion mode using mass scans over the mass range from m/z = 300 to m/z = 1900 at a typical spray voltage of 1.1-1.5 kV[34]. Parent ion scans were acquired with an FT MS resolution setting of 100,000 (at m/z = 400) with a typical mass accuracy of 3 ppm. MS/MS scans were performed in the ion trap, which has a typical mass accuracy for the fragment ion of ± 0.3 Da. MS spectra were deconvoluted to generate uncharged masses using the QualBrowser program (ThermoElectron, Bremen, Germany)[34].

Negative-stain electron microscopy of treated sacculi preparations

Purified sacculi were incubated at 37°C for 12 h in 20 μl total volume with either lysozyme (10 mg/ml), dithiothreitol (5 mM) or phosphate buffered saline. Samples were applied to a Formvar-coated, carbon-coated, glow-discharged copper EM grid (Electron Microscopy Sciences). Samples were aspirated, stained with 1.5% uranylacetate and imaged on an FEI Tecnai T12 electron microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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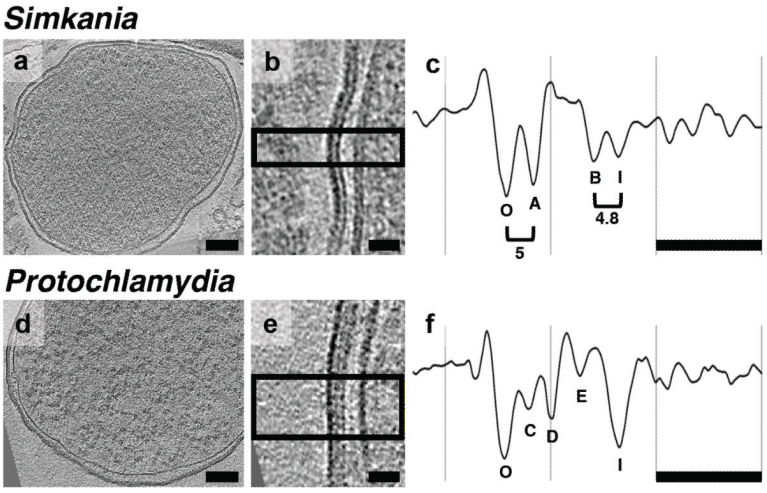

Figure 1. Chlamydial cell envelopes are multi-layered

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 peptidoglycan cell walls (see text for a full discussion of each profile and layer). Bar, 100 nm in A, D and 20 nm in B, C, E, F.

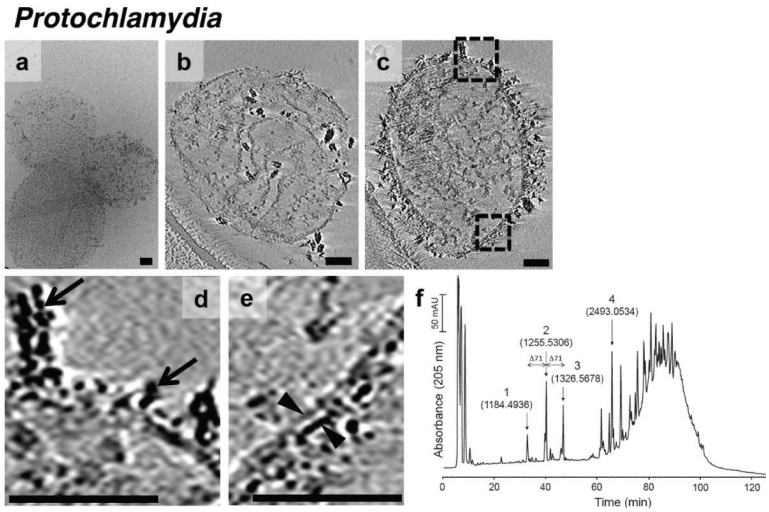

Figure 2. ***Protochlamydia*** **synthesize purifiable sacculi that contain peptidoglycan**

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 high-pressure liquid chromatography (F). Mass spectrometry analysis of peaks 1-3 (neutral masses indicated in Da) indicated the presence of modified peptidoglycan (see also Table 1 and Supplementary Fig. S1). Bars, 100 nm.

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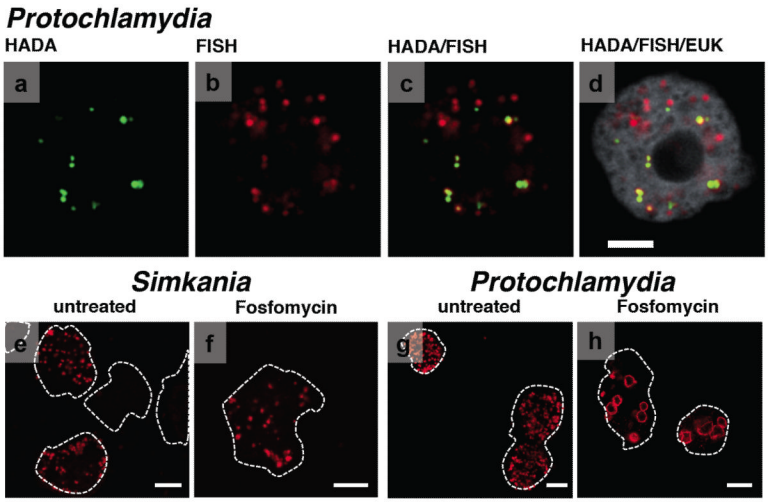

Figure 3. *Protochlamydia* incorporate D-alanine *in vivo* and are sensitive to fosfomycin

Protochlamydia-infected amoebae (A-D), but not purified *Protochlamydia* cells (Supplementary Fig. S2) or uninfected amoeba cells (Supplementary Fig. S2), stained positively for new peptidoglycan synthesis with fluorescently labeled D-alanine dyes HADA (A) and BADA (Supplementary Fig. S2), confirming the synthesis of peptidoglycan in actively-growing *Protochlamydia*. Specific FISH staining of chlamydial cells (B, overlay with A shown in C) and the eukaryotic host (D, shows overlay with C) are shown. The treatment of *Protochlamydia*-infected amoeba cultures (amoeba cell outlines in white) with cell wall synthesis-targeting fosfomycin (H, control shown in G) resulted in a dramatic decrease in infection rate and aberrant or larger chlamydial cell shapes (shown are immunofluorescent stainings of chlamydial outer membrane proteins), suggesting a crucial role of PG in the *Protochlamydia* life cycle. Fosfomycin did not affect *Simkania* (F, control shown in E). Bars, 5 μm in D, 10 μm in E-H.

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Table 1

Fragmentation masses from fractions of reduced *P. amoebophila* muropeptides.

Fraction number or muro-peptide	Fragment	Mass of fragments, H^+ form (m/z)		Proposed structure
		determined	calculated	
1	A	1167.3447	1167.4908	$[M+H]^+$-H_2O
	B	1056.3231	1056.5014	$[M+H]^+$-129.2
	C	982.3848	982.4220	$[M+H]^+$-GlcNAc
	D	853.3392	853.4220	$[M+H]^+$-129.0-GlcNAc
	E	705.3032	705.3059	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)
	F	634.3148	634.2688	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)Ala
	G	505.2668	505.2262	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)AlaGlu
2	A	1238.3288	1238.5255	$[M+H]^+$-H_2O
	B	1127.4075	1127.5361	$[M+H]^+$-129.1
	C	1053.3859	1053.4567	$[M+H]^+$-GlcNAc
	D	924.3934	924.4567	$[M+H]^+$-129.0-GlcNAc
	E	776.3376	776.3406	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)
	F	705.3202	705.3035	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)Ala
	G	576.3141	576.2609	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)AlaGlu
3	A	1309.4104	1309.5684	$[M+H]^+$-H_2O
	B	1198.3875	1198.5790	$[M+H]^+$-129.2
	C	1124.3796	1124.4996	$[M+H]^+$-GlcNAc
	D	995.3871	995.4996	$[M+H]^+$-129.0-GlcNAc
	E	847.3485	847.3835	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)
	F	776.3356	776.3464	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)Ala
	G	647.3268	647.3038	$[M+H]^+$-Glc*N*AcMur*N*Ac(r)AlaGlu
Tetra from *C. jejuni*	A	946.4128	946.3869	$[M^+Na]^+$-H_2O
	B	n.d.	835.4202	$[M^+Na]^+$-129
	C	761.3908	761.3181	$[M^+Na]^+$-GlcNAc
	D	n.d.	632.3181	$[M^+Na]^+$-129-GlcNAc
	E	484.3317	484.2019	$[M^+Na]^+$-Glc*N*AcMur*N*Ac(r)
	F	413.2619	413.1648	$[M^+Na]^+$-Glc*N*AcMur*N*Ac(r)Ala
	G	284.3200	284.1222	$[M^+Na]^+$-Glc*N*AcMur*N*Ac(r)AlaGlu

Fraction numbers correspond to peak numbers in Figure 2 F. Fragments correspond to fragments in Supplementary Fig. S1. The parental ion ($[M+H]^+$ or $[M^+Na]^+$) of each muropeptide fragments to ions lacking either water, an unknown modification with ~129 Da, a Glc*N*Ac residue, ~129 Da and Glc*N*Ac, a Glc*N*AcMur*N*Ac(r) disaccharide (r, indicates reduction to muramitol), Glc*N*AcMur*N*Ac(r)Ala or Glc*N*AcMur*N*Ac(r)AlaGlu (see column 'Proposed structure'). Tetra, reduced disaccharide tetrapeptide muropeptide fraction from *Campylobacter jejuni*[56]. The Na^+ containing ion with m/z 964.4202 was fragmented. n.d., not detected.