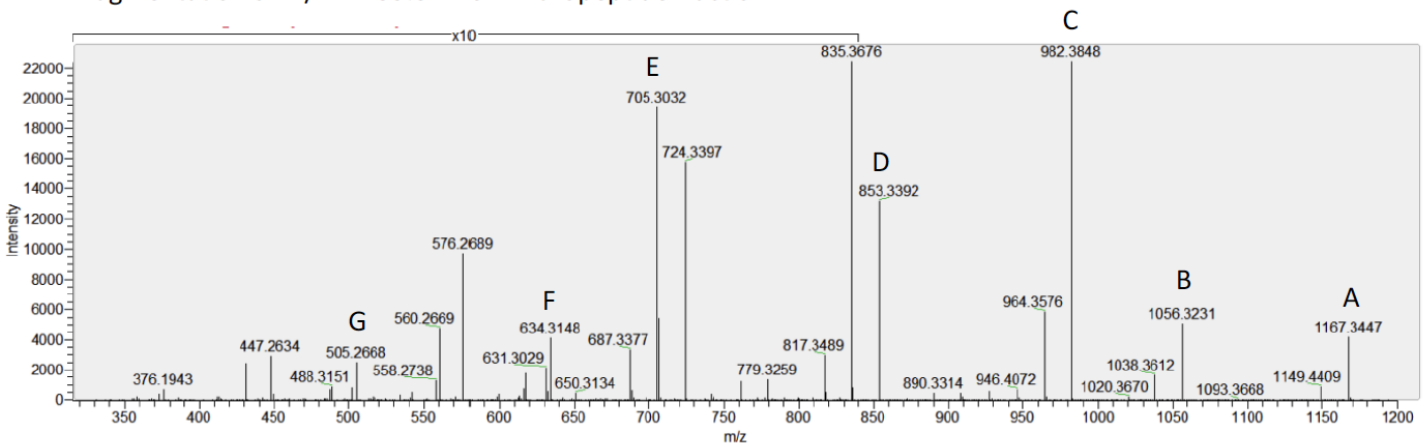
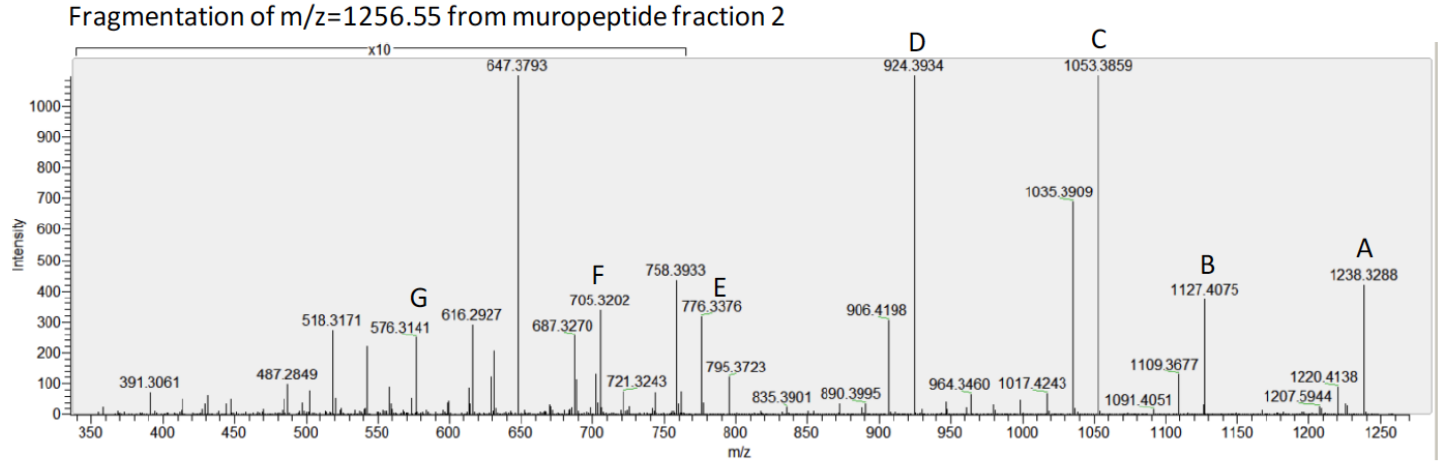
Supplementary Information

Supplementary Figure S1

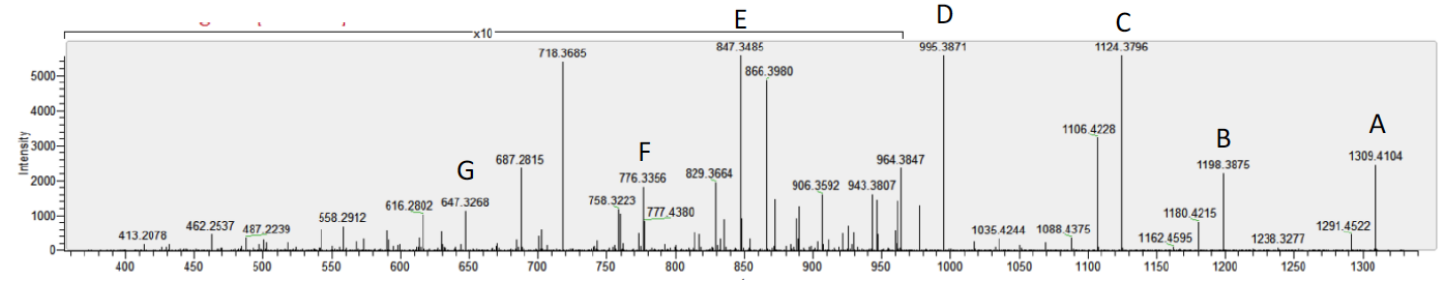
Fragmentation of m/z=1185.51 from muropeptide fraction 1



Fragmentation of m/z=1256.55 from muropeptide fraction 2



Fragmentation of m/z=1327.59 from muropeptide fraction 3

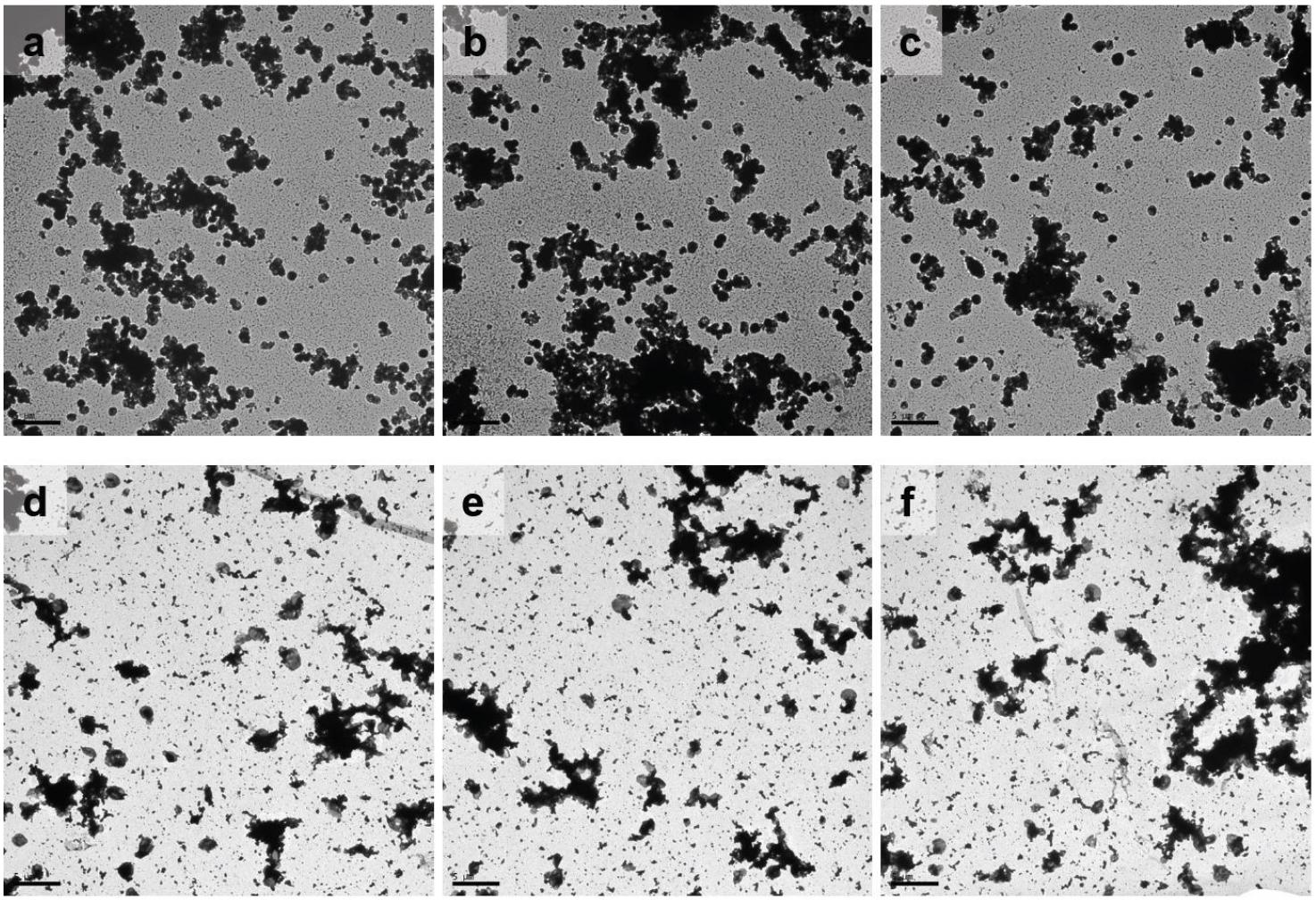


Supplementary Figure S1. *Protochlamydia* HPLC fractions 1-3 contain modified muropeptides.

MS/MS analysis of the three *Protochlamydia* muropeptides (Figure 2 E) show similar fragmentation patterns. The masses of the fragment ions A-G are consistent with modified muropeptides, whereby two modifications add an extra mass of 314 Da compared to the canonical muropeptides with tri-, tetra- and pentapeptide, respectively.

Note: While MS/MS fragments are often referred to with 'y' and 'b' ion designations, according to IUPAC, there is no general nomenclature for mixed compounds like the reduced muropeptides analyzed here. The situation is further complicated by the presence of unknown modifications preventing the clear numbering of the 'y' ions. Therefore we have referred to the fragments as 'A', 'B', 'C', etc.

Supplementary Figure S2

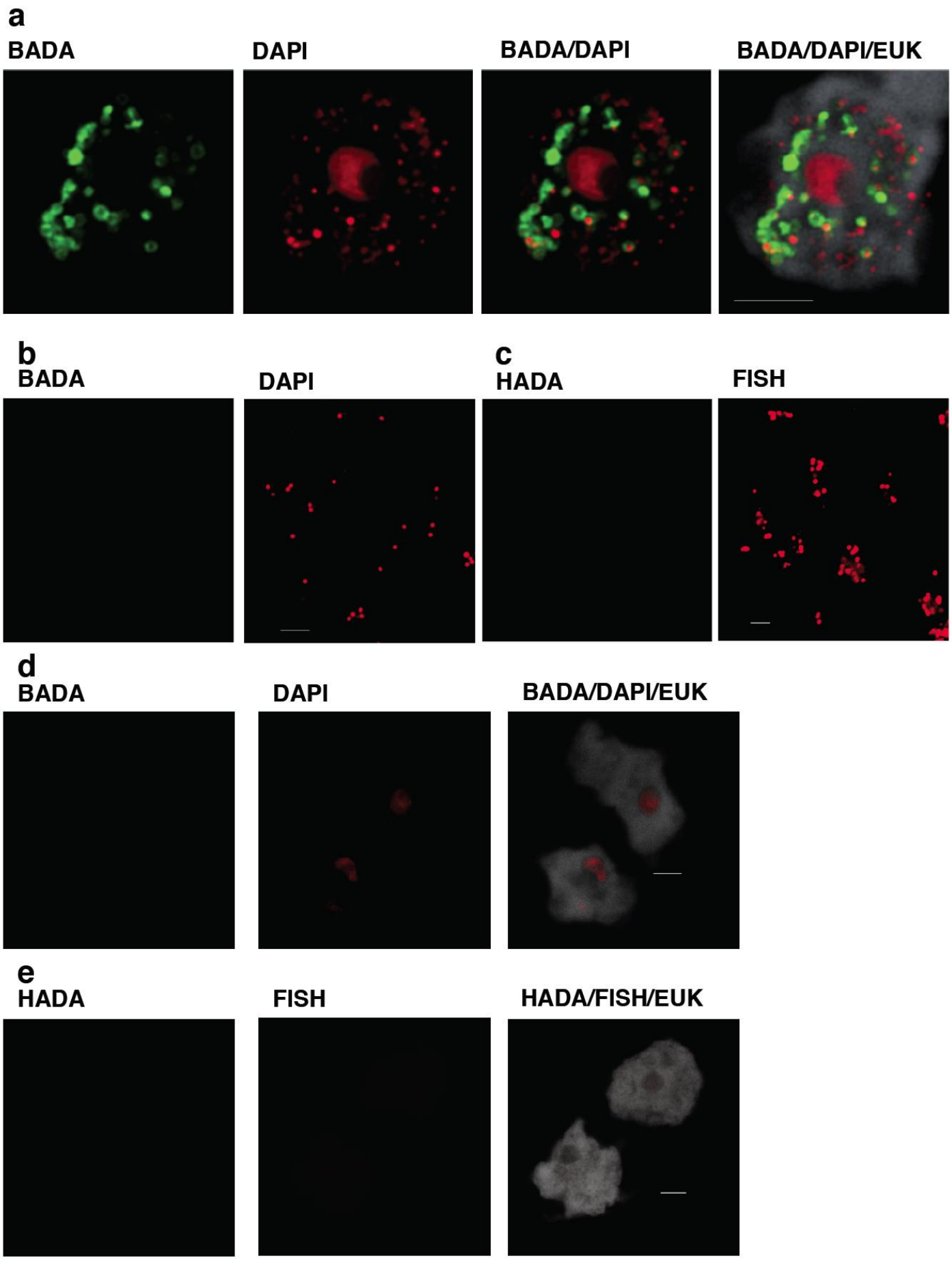


Supplementary Figure S2. *Protochlamydia* sacculi are degraded by lysozyme.

Representative negative stain EM images of *Protochlamydia* sacculi. The undigested control sample (A-C) contained many coccoid sacculi. After digestion with lysozyme (D-F), fewer intact sacculi were present and degraded material was abundant. Bars, 5μm.

Supplementary Figure S3

Protochlamydia

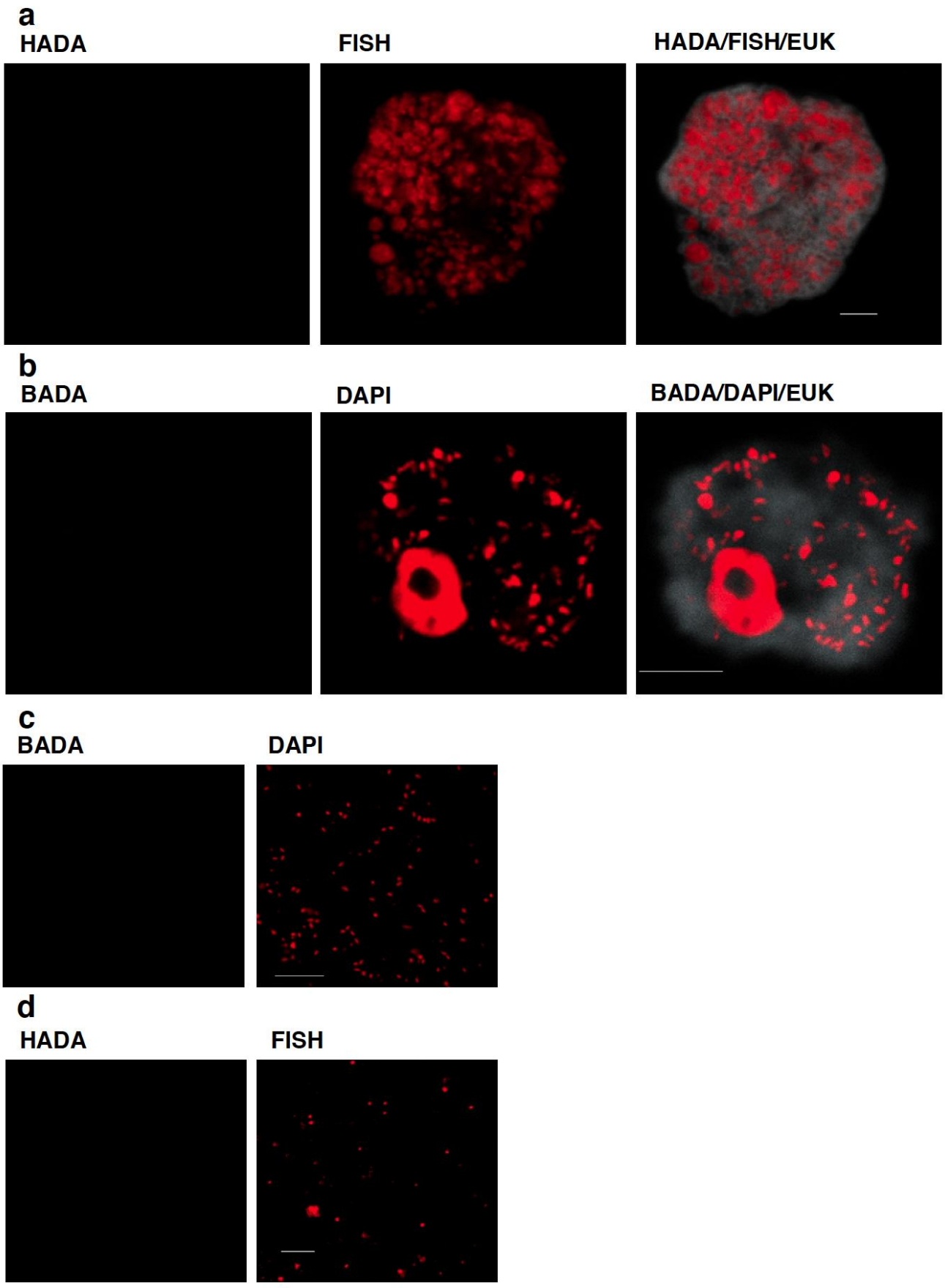


Supplementary Figure S3. Fluorescent D-amino acids label intracellular but not purified *Protochlamydia* cells or uninfected amoebae.

Shown is an amoeba cell infected with *Protochlamydia* and stained with DAPI to detect chlamydial cells and with fluorescently labeled D-alanine [35] (BADA) to detect sites of PG synthesis (A). BADA staining results in multiple strong signals (some of them halo-shaped). Because FISH/DAPI never stains all chlamydial cells inside amoebae, not all FDAA signals have a corresponding signal. Purified chlamydial cells (B, C) or uninfected amoebae (D, E) showed no signal with HADA (C, E) and BADA (B, D). Note that chlamydiae cannot divide in host-free media, and thus would not incorporate FDAA. Amoeba cells are stained (white) with eukaryote-specific FISH in A, D and E. Bars, 5 μm.

Supplementary Figure S4

Simkania



Supplementary Figure S4. Fluorescent D-amino acids label neither intracellular, nor purified *Simkania* cells. Shown are amoeba cells infected with *Simkania* (A, B) and stained by FISH or DAPI to detect chlamydial cells and fluorescently labeled D-alanine [35] (HADA in A, BADA in B). BADA labeling showed no signals. When labeled with HADA, no signals were visible with the image acquisition settings used for *Protochlamydia*; in images recorded at maximum sensitivity few *Simkania* cells showed very weak signals (just above the detection limit) for HADA. Purified *Simkania* elementary bodies (C, D) are not stained by FDAA labeling. Amoeba cells are stained (white) with eukaryote-specific FISH in A, B. Bars, 5 μm.

Supplementary Table S1. PG synthesis genes in chlamydial genomes.

Protein[A]	Function	Comment
MurA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	*Chlamydiaceae* are resistant to fosfomycin due to replacement of Cys115-> Asp in MurA [57]
MurB	UDP-N-acetylmuramate dehydrogenase	
MurC[B]	UDP-N-acetylmuramate alanine ligase	MurC of *C. trachomatis* uses L-alanine, L-serine and glycine with comparable efficiency *in vitro* [22]
MurD	UDP-N-acetylmuramoylalanine D-glutamate ligase	
MurE	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate 2,6-diaminopimelate ligase	MurE of chlamydiae has Arg at position 416, typical of m-DAP specific enzymes [58]
MurF	UDP-N-acetylmuramoyl-tripeptide D-alanyl-D-alanine ligase	
MraY	Phospho-N-acetylmuramoyl pentapeptide transferase	
MurG	N-acetylglucosaminyl transferase	
Class A HMW PBPs	Bifunctional transpeptidase/ transglycosylase	
Class B HMW PBPs	Transpeptidase	In contrast to environmental chlamydiae, members of the *Chlamydiaceae* are susceptible to beta-lactams
PBP2 (PBP1)	Transpeptidase	
PBP3 (PBP2)		
LMW PBP	D-alanyl-D-alanine carboxypeptidase	
PBP6 (PBP3)		
Alr	Alanine racemase	Chlamydiae possess DagA, a D-alanine glycine permease
MurI	Glutamate racemase	
Ddl[B]	D-alanine-D-alanine ligase	Although *Chlamydiaceae* lack Alr, Ddl of *C. trachomatis* uses D-Ala as a substrate [20]
UppP	Undecaprenyl-diphosphate phosphatase	*Chlamydiaceae* lack UppP, but are sensitive to Bacitracin [59]
m-DAP pathway	Synthesis of m-DAP	Like plants and cyanobacteria chlamydiae use the aminotransferase pathway for generation of m-DAP [60]

[A] Genes for proteins highlighted in dark blue are absent from the genomes of all chlamydiae. Proteins highlighted in light blue are encoded in the genomes of *Protochlamydia* and *Parachlamydia*, but not in pathogenic chlamydiae (*Chlamydiaceae*) and *Simkania*. Underlined Proteins were characterized previously [21, 22, 23, 24, 25]. GlcNac, N-acetlyglucosamine; MurNAc, N-acetyl muramic acid; HMW PBP, high molecular weight penicillin binding protein; LMW PBP, low molecular weight penicillin binding protein; UPP, Undecaprenyl-diphosphate; m-DAP, meso-diamino pimelic acid

[B] MurC and Ddl are encoded as a fusion protein in *Chlamydiaceae*