Evidence for a peptidoglycan-like structure in Orientia tsutsugamushi

Sharanjeet Atwal,1,2† Suparat Giengkam,2† Suwittra Chaemchuen,2 Jack Dorling,3 Nont Kosaisawe,4 Michael VanNieuwenhze,5 Somponnat Sampattavanich,4 Peter Schumann6 and Jeanne Salje1,2*
1Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK.
2Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.
3Department of Biochemistry, University of Oxford, Oxford, UK.
4Siriraj Laboratory for Systems Pharmacology, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand.
5Indiana University, Bloomington, IN, USA.
6Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

Summary

Bacterial cell walls are composed of the large cross-linked macromolecule peptidoglycan, which maintains cell shape and is responsible for resisting osmotic stresses. This is a highly conserved structure and the target of numerous antibiotics. Obligate intracellular bacteria are an unusual group of organisms that have evolved to replicate exclusively within the cytoplasm or vacuole of a eukaryotic cell. They tend to have reduced amounts of peptidoglycan, likely due to the fact that their growth and division takes place within an osmotically protected environment, and also due to a drive to reduce activation of the host immune response. Of the two major groups of obligate intracellular bacteria, the cell wall has been much more extensively studied in the Chlamydiales than the Rickettsiales. Here, we present the first detailed analysis of the cell envelope of an important but neglected member of the Rickettsiales, Orientia tsutsugamushi. This bacterium was previously reported to completely lack peptidoglycan, but here we present evidence supporting the existence of a peptidoglycan-like structure in Orientia, as well as an outer membrane containing a network of cross-linked proteins, which together confer cell envelope stability. We find striking similarities to the unrelated Chlamydiales, suggesting convergent adaptation to an obligate intracellular lifestyle.

Introduction

In order to maintain correct cell shape, and the capacity to withstand the high internal turgor pressure, most bacteria synthesise a cell wall comprised largely of the macromolecule peptidoglycan. Peptidoglycan is composed of an alternating N-acetylglucosamine-N-acetylmuramic acid polysaccharide backbone, cross-linked via short peptide side chains. The sequence of the peptides, the degree of cross-linking, and the length of glycan chains vary between species and also stages of growth (Vollmer et al., 2008). However, some form of peptidoglycan is present in almost all known bacterial species with the notable exception of the mycoplasmas. The essentiality and conservation of peptidoglycan has made this an attractive antibiotic target (Schneider and Sahl, 2010), from the early discovery of penicillin to the recent identification of teixobactin (Ling et al., 2015). Peptidoglycan elongation and remodelling are highly complex processes, as they need to occur without compromising the integrity of the cell wall. This is achieved through a coordinated process involving the bacterial cytoskeleton and cell division machinery (Haeusser and Margolin, 2016). A detailed knowledge of the structure and composition of peptidoglycan is thus a prerequisite for understanding the molecular mechanisms of growth and division in bacterial cells.

The vast majority of bacteria grow and divide independently, living in a range of environments including soil, water, and the gut or skin of a mammalian host. Some bacteria, such as Salmonella, Shigella and Listeria, have evolved the means to invade and replicate within mammalian cells during the course of an infection.

Accepted 14 May, 2017. *For correspondence. E-mail jeanne.salje@ndm.ox.ac.uk; Tel. +19175625174; Fax +19175625174.
†These authors contributed equally.
(Pizarro-Cerda and Cossart, 2006). A small group of bacteria, however, have evolved this ability to the extreme that they are no longer able to grow and divide outside their host cells. This unusual and fascinating group of organisms are predominantly comprised of two orders of Gram-negative bacteria: the Chlamydiales and the Rickettsiales. The Chlamydiales include human and animal pathogens, as well as a number of environmental strains that are found in soil-dwelling amoeba (Elwell et al., 2016). The Rickettsiales are a large and diverse group of vector-borne bacteria, that include the promiscuous insect symbiont Wolbachia, a number of human and animal pathogens in the Anaplasmataceae, and a group of typhus-causing pathogens in the Rickettsiaceae. The Rickettsiaceae are also thought to include the closest relatives of the precursor of modern mitochondria (Andersson et al., 1998). Although the Rickettsiales and Chlamydiales are unrelated, they exhibit similarities in their obligate intracellular life cycles. The bacterial cell wall has been extensively studied in the Chlamydiales and the long-standing ‘chlamydial anomaly’ describes the paradox that these bacteria are sensitive to the cell wall targeting drug penicillin, but that peptidoglycan could never be directly detected by chemical analysis (Moulder, 1993; McCoy and Maurelli, 2006; Mohammadi and Breukink, 2014). This was recently resolved using sensitive mass spectrometry techniques (Packiam et al., 2015) (Pilhofer et al., 2013; Jacquier et al., 2015) whilst a combination of novel labelling methods and cryoelectron tomography techniques have shed light on the arrangement of the cell wall of these organisms (Pilhofer et al., 2013; Liechti et al., 2014). In comparison, very little is known about the cell wall biology of the Rickettsiales. Comparative analyses have revealed the presence of peptidoglycan biosynthesis genes in many Rickettsiales (Gillespie et al., 2012), and the presence of the peptidoglycan precursor lipid II has been demonstrated in Wolbachia (Henrichfreise et al., 2009; Vollmer et al., 2013). However, extensive investigations into the composition and structure of peptidoglycan in these organisms have not been performed.

The genus Orientia is a divergent member of the order Rickettsiales, within the family Rickettsiaceae (Tamura et al., 1991; Ohashi et al., 1995). It is the causative agent of the severe mite-borne human disease scrub typhus, which is endemic across large parts of Asia (Phongmany et al., 2006; Mayfong Mayxay et al., 2013; Capeding et al., 2013; Cosson et al., 2015; Dittrich et al., 2015; ) and which can be life threatening in the absence of effective antibiotic treatment. It is thought to affect at least 1 million people per year (Watt and Parola, 2003). Despite its high incidence and severity, it is less well studied than other Rickettsias such as R. prowazekii, R. conorii and R. rickettsii. Orientia is able to infect a range of cell types, including endothelial, fibroblast, monocyte/macrophage and dendritic cells (Moron et al., 2001; Paris et al., 2012; Keller et al., 2014). Similar to the other Rickettsiaceae, but in contrast to the Chlamydia and Anaplasmataceae that live within remodelled vacuoles (Bastidas et al., 2013; Moumène and Meyer, 2016), Orientia escapes from the endo-lysosomal pathway shortly after infection and replicates freely in the host cell cytoplasm (Chu et al., 2006).

Orientia has previously been reported to completely lack both peptidoglycan and LPS (Amano et al., 1987). This conclusion was based on an insensitivity to penicillin (Wiseman et al., 1982), an inability to detect peptidoglycan fragments by chemical analysis (Amano et al., 1987), and an absence of electron-dense material in the periplasmic inter-membrane space by electron microscopy (Silverman and Wiseman, 1978). However, whilst the sequencing of the Orientia genome confirmed an absence of genes required for biosynthesis of LPS, an almost complete complement of peptidoglycan biosynthesis genes were identified (Cho et al., 2007; Min et al., 2008; Nakayama et al., 2008; Fig. 1). Furthermore, it has been shown that the intracellular host immune receptor Nod1, which recognises fragments of

---

Fig. 1. The peptidoglycan biosynthesis pathway in Orientia tsutsugamushi. Genes shown in green are found in the genome of O. tsutsugamushi (Boryong and Ikeda strains), genes shown in red have no identifiable homolog.
peptidoglycan, is activated in response to infection by Orientia (Cho et al., 2010c). Here, we provide evidence for a peptidoglycan-like structure in the cell envelope of Orientia, and show that cell rigidity is additionally conferred by a highly cross-linked outer membrane protein. This is the first detailed analysis of peptidoglycan in an obligate intracellular bacterium outside the Chlamydiales, and will inform our understanding of rickettsial cell biology as well as enable comparative studies across different bacterial lineages engaging in obligate intracellular life styles.

Results and discussion

The genome of Orientia tsutsugamushi contains a nearly complete set of genes required for peptidoglycan biosynthesis (Fig. 1; Cho et al., 2007; Min et al., 2008; Nakayama et al., 2008). However, homologues of three important groups of enzymes are notably absent: (i) amino acid racemases (ii) glycosyltransferases and (iii) some genes in the meso-diaminopimelic acid (meso-DAP) biosynthesis pathway. Intriguingly, these are the same three pathways that are absent or incomplete in the chlamydial genomes (McCoy and Maurelli, 2006). The peptide side-chains of peptidoglycan are characterised by the presence of unusual amino acids, including D-isomer amino acids (usually D-Glu and D-Ala in Gram-negative bacteria) and the diamino acid meso-diaminopimelic acid (meso-DAP) (Vollmer and Bertsche, 2008; Vollmer et al., 2008; Turner et al., 2014). D-amino acids are not usually present in proteins, and are synthesised by the specific activity of amino acid racemases. Although O. tsutsugamushi does not encode the major L-alanine racemase, Alr, it does possess the gene ddl, which encodes a protein catalysing the ligation of the two D-Ala residues found in peptidoglycan side-chains. One possible alternative pathway for generation of D-Ala is through the GlyA enzyme, which was demonstrated to be a plausible candidate for D-Ala biosynthesis in C. pneumonia (Ghosh and Schirch, 1988; De Benedetti et al., 2014) and for which a homolog is present in the genome of Orientia (OTBS_0253).

We used a highly sensitive mass spectrometric method (GC/EIMS) to search for the peptidoglycan-specific amino acid DAP in purified Orientia biomass. This method is based on GC/EIMS of amino acids after their transformation into volatile N-heptafluorobutyryl-2,6-diaminopimelic acid isobutylester derivatives (Schumann, 2011), and has previously been used to detect low amounts of peptidoglycan in planctomycetes (Jeske et al., 2015) and Verrucomicrobia (Spring et al., 2016). Here, we were able to detect DAP in acid hydrolysates of O. tsutsugamushi cells (Fig. 2). The identification was based on a gas chromatographic peak matching that of the DAP standard derivative, with a retention time of 22.2 min and a set of fragment ions at 380, 324, 306 and 278 m/z (Jeske et al., 2015). The predicted structures corresponding to these fragment ions are shown in Supporting Information Fig. S1. The detection of DAP is highly suggestive of a peptidoglycan-like structure in O. tsutsugamushi and suggests that an alternative pathway may be used for synthesis of this amino acid. It is important to note, however, that the DAP was detected from isolated bacterial biomass rather than a purified sacculus, and that final proof for DAP-containing peptidoglycan in Orientia will require isolation and chemical analysis of this structure.

In order to determine whether the peptidoglycan biosynthesis pathway is active in O. tsutsugamushi we measured the gene expression levels of several peptidoglycan biosynthesis genes throughout the 7-day bacterial infection cycle using qRT-PCR analysis (Fig. 3). We
found that genes murA, murD, murF, ddl and pbp2 were all expressed to a detectable level at some or all time points, when normalised to the housekeeping gene mipZ (Cho et al., 2010a). The gene pbp2, and to a lesser extent murD and murF, showed increased expression levels at early stages of the infection cycle in which bacteria are not yet actively dividing (days 1–3) compared with later stages of the infection cycle (days 5 and 7) potentially reflecting a higher requirement for peptidoglycan precursors during early stages of bacterial cell growth and preparation for division.

Next, we tested whether *O. tsutsugamushi* was sensitive to drugs that target the cell wall, in order to determine whether intact peptidoglycan was required for bacterial viability. *O. tsutsugamushi* is known to be insensitive to penicillin (Wisseman et al., 1982), and this is one of the reasons why it was previously reported to lack peptidoglycan. First, we measured the growth of *O. tsutsugamushi* in the presence of penicillin and also chloramphenicol, the latter of which is used clinically against this organism (Watt et al., 1996). We compared this with bacterial growth in the presence of d-cycloserine and phosphomycin, that target the cell wall biosynthesis proteins Alr/Ddl and MurA respectively (Fig. 4B and F, and Supporting Information Figs. S2 and S3). We found that chloramphenicol inhibited growth.

**Fig. 3.** Expression of genes in the peptidoglycan biosynthesis pathway. Bacterial genome copy number or relative gene expression of *O. tsutsugamushi* grown in mouse fibroblast L929 cells over 7 days. Relative expression level was determined by qRT-PCR and normalised to the housekeeping gene mipZ. Graph shows each individual data point, as well as the mean and standard deviation.
and penicillin did not, as expected, but that both D-cycloserine and phosphomycin inhibited the growth of *O. tsutsugamushi* to the same extent as chloramphenicol. Immunofluorescence microscopy images showed that *Orientia* cells became large and round in the presence of phosphomycin, and displayed reduced structural integrity in the presence of D-cycloserine (Fig. 4F and Supporting Information Fig. S3). This experiment showed, for the first time, a requirement for peptidoglycan precursor biosynthesis for the replication of *O. tsutsugamushi*. Next, we attempted to rescue the growth of *O. tsutsugamushi* treated with D-cycloserine by the addition of exogenous D-Ala. We found that the addition of D-Ala could not completely restore growth, even when added at 10x the molar ratio of D-cycloserine (Fig. 4C). This was in contrast to results from equivalent experiments performed in *Chlamydia* (Moulder et al., 1963) and may reflect a lack of uptake of D-Ala into *O. tsutsugamushi* cells or a complete inhibition of Ddl by D-cycloserine.

The absence of Class A bifunctional PBPs as well as monofunctional glycosyltransferases raises the question of whether *Orientia* has the enzymatic ability to generate peptidoglycan with polymerised glycan chains. In the case of *Wolbachia*, which also lacks these key glycosyltransferase genes, it has been postulated that an unpolymerised lipid II precursor may play an essential role in localisation and coordination of the cell division machinery, but that typical a classical peptidoglycan sacculus is absent (Henrichfreise et al., 2009; Vollmer et al., 2013). Recent reports have shown that the SEDS family protein RodA is able to perform previously undescribed glycosyltransferase activity in *Bacillus subtilis*, and they predict that this may be a general activity of this protein family (Meeske et al., 2016; Emami et al., 2017). Both *Orientia* and *Chlamydia* possess homologs of SEDS.
proteins RodA and FtsW, and it is possible that these could account for the missing glycosyltransferase activity in these organisms. In order to test for the presence of polymerised glycan chains in peptidoglycan of Orientia we measured the sensitivity to the enzymes lysozyme and mutanolysin. These enzymes cleave the glycan backbone of peptidoglycan at the β-1,4-linkage between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). We found that both enzymes caused bacterial cell lysis and resulted in a reduction in bacterial copy number after 7 days (Fig. 4D, Movie 1 and Supporting Information Fig. S2). These results suggest that the peptidoglycan of O. tsutsugamushi may contain at least some degree of MurNAc-GlcNAc disaccharide polymerisation. This could be achieved through the activity of FtsW/RodA SEDS proteins or other unidentified glycosyltransferase genes.

In order to understand why O. tsutsugamushi is insensitive to penicillin, we measured bacterial growth in the presence of the alternative β-lactam antibiotics imipenem and meropenem, which also target penicillin binding proteins (PBPs) but which have improved bacterial permeability properties (Yang et al., 1995), as well as the β-lactam antibiotic mecillinam that specifically targets PBP2. These were also ineffective against O. tsutsugamushi (Fig. 4E). We then tested growth in the presence of penicillin together with a β-lactamase inhibitor, tazobactam, but this was unable to modulate the activity of penicillin (Fig. 4E). One possible explanation is that both PBPs in Orientia, PBP2 and PBP3, are intrinsically resistant to β-lactam antibiotics. In order to test this hypothesis we performed an extensive protein alignment of PBPs 2 and 3 from a diverse set of bacterial species. We analysed known active site amino acid residues (Sauvage et al., 2014) and found that one, K310 (E. coli PBP3 numbering), was strictly conserved across all PBP2/3s tested, but mutated to histidine (PBP3) and glutamine (PBP2) in Orientia (Supporting Information Fig. S4). This mutation could potentially confer the observed β-lactam insensitivity, but this has not been experimentally tested.

A number of groups have recently developed fluorescent α-amino acid derivatives that can be incorporated into bacterial peptidoglycan and directly visualised by fluorescence microscopy (Kuru et al., 2012, 2015; Siegrist et al., 2013, 2015; Liechti et al., 2014; Shieh et al., 2014). We used one of these, the ‘clickable’ alkyne modified α-alala-ala dipeptide EDA-DA (ethynyl-α-alanyl-α-alanine) to visualise peptidoglycan in O. tsutsugamushi cells (Liechti et al., 2014). We found that this labelled the entire bacterial surface, suggesting the existence of a large structure that extends throughout the periplasm of O. tsutsugamushi (Fig. 5A and more examples in Supporting Information Fig. S5). This labelling was abolished in the presence of the cell wall-targeting drugs d-cycloserine and phosphomycin, suggesting that this probe is being incorporated specifically into a peptidoglycan-related structure. We observed a similar pattern of labelling using an alternative peptidoglycan probe, 7-hydroxycoumarin-3-carboxylic acid (HCC)-amino-α-alanine (HALA) (Fig. 5B).

To our surprise, we found that the L-isomer of this probe, HCC-amino-L-alanine (HALA), produced similar labelling to that observed with HALA (Fig. 5B) although this labelling was weaker and was not observed every time. In order to study this further we also labelled the model Gram-negative bacteria E. coli with both probes and found that while HALA labelled all cells, as expected, a subpopulation of cells were also clearly labelled with the HALA probe (Fig. 5B). This was not shown in previous reports and may reflect differences in bacterial strains or exact experimental conditions. This result is unlikely to be due to contamination of the HALA probe with α-amino acids, as this would presumably lead to all cells being weakly labelled, rather than a small number being strongly labelled as observed. The exact mechanism of HALA incorporation into peptidoglycan has not been determined, and may vary between different bacterial species and growth stages. Possible routes may involve substitution into existing peptidoglycan by L,D-transpeptidase activity (we could identify no L,D-transpeptidase homolog in O. tsutsugamushi), or uptake into the cytoplasm and incorporation into peptidoglycan precursors by Ddl and MurF (Cava et al., 2011). Our observation of HALA labelling in both E. coli and O. tsutsugamushi may indicate a pathway involving entry into the cytoplasm and racemisation prior to incorporation into peptidoglycan precursors. Alternatively, it is possible that periplasmic peptidoglycan cross-linking or remodelling enzymes mediate the incorporation of α-amino acid probes, and that these are not strictly isomer-specific, especially at the high concentrations of fluorescent amino acids used in these studies (1 mM). In the case of both EDA-DA and HALA we found that the labelling was not positive every time, and was sensitive to parameters such as host cell confluence, multiplicity of infection, and time after initial infection. This suggests that the peptidoglycan biosynthesis machinery may be regulated in response to unknown signals.

The EDA-DA and HALA labelling patterns in O. tsutsugamushi are suggestive of an extended sacculus-like structure encasing the bacterial cell, although definitive evidence for this will require isolation of this material and subsequent mass spectrometry and microscopy analysis. Our attempts at biochemical isolation of the peptidoglycan-like structure of Orientia were unsuccessful, and this may reflect low levels of this material as well as unknown regulation of its biosynthesis at different stages of the infection and replication cycle. Our HADA/EDA-DA labelling pattern is in
contrast to that observed in pathogenic chlamydiae, in which peptidoglycan labelling localises only to the division septum (Liechti et al., 2016). We never observed septum labelling of Orientia in the experiments reported here. It is worth noting that Orientia possesses both MreB and FtsZ, unlike the Chlamydiales and Planctomycetes that have lost the bacterial cell division cytoskeletal filament FtsZ and have evolved a non-canonical cell division mechanism thought to be driven by the interplay between the bacterial actin homolog MreB and a septal peptidoglycan ring (Liechti et al., 2014, 2016).

A major role of peptidoglycan is in protecting bacterial cells from osmotic stresses. One hypothesis to explain the lack or reduction of peptidoglycan in obligate intracellular bacteria is that they predominantly occupy an osmotically protected cellular environment and therefore have a reduced need for this structure. We performed fluorescence microscopy on Orientia bacteria that had been isolated from host cells and resuspended in sucrose-phosphate-glutamate buffer (SPG), PBS or water, and found that bacteria did not lyse in the presence of PBS or pure water, indicating the presence of a mechanical stress-bearing structure such as would be provided by a peptidoglycan sacculus (Fig. 6A). We performed quantitative image analysis using both a supervised machine learning approach and a principal component analysis approach (PCA), and this showed that bacteria resuspended in SPG were more ‘smooth’ than those resuspended in water or PBS.

Fig. 5. Orientia labelled with peptidoglycan-reactive probes. A. Fluorescence microscopy of Orientia labelled with EDA-DA probe reacted with an azide modified Alexa Fluor 488 via click chemistry (green), antibody against the bacterial surface protein TSA56 (red) and the DNA stain DAPI (blue). Bacteria were grown in the presence or absence of drugs for 24 h. Drug concentrations were as follows: 40 μg/ml phosphomycin; 250 μg/ml D-cycloserine.

B. Fluorescence microscopy of Orientia and E. coli in the presence of the fluorescent D-alanine derivative HADA (blue), the fluorescent L-alanine derivative HALA (blue), antibody against the bacterial surface protein TSA56 (red, Orientia) or the bacterial cytoplasm (Evans blue, red, E. coli). Scale bar = 10 μm.
This morphology may reflect a disruption in the cell envelope structure, which would be consistent with our previous report that the viability of isolated *O. tsutsugamushi* was higher when stored in SPG compared with PBS or water (Giengkam et al., 2015).

The most abundant protein in the outer membrane of *O. tsutsugamushi* is an integral membrane protein called the 56 kDa type-specific antigen (TSA56). This highly immunostimulatory protein has no known homolog in any other species, and contains regions of high diversity between *Orientia* strains. This variability has enabled the use of this gene to classify *Orientia* strains, and corresponds closely to serotype classification of different strains. In spite of the high sequence variability, we observed that this protein has three conserved cysteine residues (C33, C190 and C416 in strain UT76) and therefore wondered whether this highly abundant protein might form a cross-linked network across the surface of the outer membrane, similar to that formed by the major outer-membrane proteins (MOMP) of the *Chlamydiales* (Bavoil et al., 1984; Hatch et al., 1986) and in place of LPS which usually covers the surface of other Gram negative bacteria. The TSA56 protein has previously been shown to form higher order intermolecular aggregates in the absence of reducing agent, using western blot analysis, (Urakami et al., 1986) and we were able to reproduce this result using the Karp-like UT76 strain of *O. tsutsugamushi* used in the current report (Fig. 6C).

We used fluorescence microscopy to monitor the effect of exposing purified bacteria to the reducing agent DTT and found that this had little additional structural effect when cells were resuspended in SPG or PBS buffer (Fig. 6A and B). However, when isolated bacteria were incubated with water, the addition of DTT resulted in more patchy membrane labelling and a higher background, which may indicate greater bacterial fragility (Fig. 6A). Quantitative image analysis confirmed a
greater difference in cell morphology upon DTT treatment in the presence of water compared with PBS or SPG (Fig. 6B and Supporting Information Fig. S7). Control experiments showed that *E. coli* did not undergo morphological changes under the same conditions (Supporting Information Fig. S6). This result supports the hypothesis that a highly cross-linked outer membrane confers some degree of osmotic protection to bacterial cells.

We then tested the importance of this cross-linked surface for bacterial infectivity and found that bacteria pre-exposed to DTT had a dramatically reduced ability to invade host cells compared with untreated bacteria (Fig. 6D and E). The TSA56 protein is known to be required for bacterial entry into host cells through interaction with extracellular fibronectin, and it is possible that the addition of DTT results in the depletion of higher-order structures of TSA56 required for efficient interaction with fibronectin (Lee et al., 2008; Cho et al., 2010b). Taken together, these results support the hypothesis that the *O. tsutsugamushi* outer membrane is stabilised by a cross-linked network of proteins including the TSA56 protein and potentially other partners, and that this structure is important for bacterial cell integrity and infectivity.

**Conclusion**

In this study, we have analysed the structure of the cell envelope of the obligate intracellular human pathogen *O. tsutsugamushi*. We present evidence for a peptidoglycan-like molecule as well as disulphide cross-linked outer membrane proteins, and demonstrate that these components are required for bacterial growth, cell integrity and host cell invasion. Important outstanding questions include the identity of the hypothesised glycosyl transferase, amino acid racemase and meso-DAP biosynthesis enzymes and the exact chemical composition and structure of the *O. tsutsugamushi* peptidoglycan-like network. This first study paves the way for a detailed analysis of the molecular mechanisms of growth and division in this important human pathogen and model intracellular organism. We also show that both the D-isomer and L-isomer of the fluorescent alanine amino acid probe are able to label at least some *O. tsutsugamushi* and *E. coli* cells, and this raises further questions about the mechanism of incorporation of these probes. The reduced level of peptidoglycan found in this organism, the absence of key peptidoglycan biosynthesis enzymes, and the presence of a cross-linked protein network embedded in the outer membrane of these bacteria are all highly reminiscent of the unrelated *Chlamydiales* and suggest convergent adaptation to an obligate intracellular life style.

**Experimental procedures**

**Bacterial strains, cell lines and growth conditions**

All experiments were performed using the mouse fibroblast cell line L929 (ATCC CCL-1) which was a kind gift from Dr. Blacksell at the Mahidol Oxford Tropical Medicine Research Unit, Bangkok, Thailand. The Thai Karp-like clinical isolate *Orientia tsutsugamushi* strain UT76 (Giengkam et al., 2015), and *E. coli* strain NEM 5 alpha (New England Biolabs, USA, catalog number C2987I) was used throughout. Cell culture was performed using DMEM media with 10% FBS at 37°C (uninfected cells) or 35°C (infected cells) and 5% CO₂. For routine propagation bacteria were grown in 25 or 75 cm² culture flasks as described previously (Giengkam et al., 2015). All experiments were performed using bacteria harvested from the intracellular fraction of infected cells, 7 days after infection.

**Mass spectrometry analysis**

Mass spectrometry analysis was performed on biomass extracted from between 15 and 45 ml infected cells, which had been infected at an MOI of 10:1 to 1000:1. Bacteria were grown in L929 cells for 7 days, then purified using mechanical lysis of host cells and filtering with a 2 μm filter (Giengkam et al., 2015). The sample was pelleted and washed in 600 μl of 300 mM ice-cold sucrose two times. The supernatant was removed, and the pellet was autoclaved prior to extraction. The pellet was hydrolysed using 4M HCl at 100°C for 16 h, then amino acids were transformed into volatile N-heptafluorobutyryl-isobutylester derivatives using Protocol 10 of Schumann (2011). The samples were analysed by GC/MS (320 Singlequad, Varian) and diaminopimelic acid was identified as a gas chromatographic peak with a retention time of 22.2 min and a set of fragment ions at 380, 324, 306 and 278 m/z (Jeske et al., 2015).

**Bacterial quantification by qPCR**

Bacterial quantification was performed as described previously (Giengkam et al., 2015). Briefly, the supernatant and/or cellular fraction of infected L929 cells were isolated and DNA extraction was performed using the alkaline lysis method. Quantitative PCR was performed using a primer/probe set (Supporting Information Table S1) within the TSA47 gene (OTT_1319) and the bacterial genome copy number was calculated using a standard curve.

**RNA extraction and gene expression analysis by qRT-PCR**

At each time point, infected cells were placed on ice and rapidly resuspended in RNAProtect Bacteria Reagent (Qiagen,
Drug sensitivity assays

Bacteria were grown in L929 cells in 24 well plates in the presence of different drugs, and the bacterial genomic copy number after 7 days was determined by qPCR. All drugs were added directly to the wells shortly before adding isolated bacteria, with the exception of lysozyme and mutanolysin (which cannot enter mammalian cells). In the case of lysozyme, purified bacteria were resuspended in SPG buffer ± lysozyme and incubated at 37°C for 15 mins before being added to L929 cells for infection. In the case of mutanolysin, purified bacteria were resuspended in DMEM ± mutanolysin and incubated at 37°C for 15 mins before being added to L929 cells for infection. For microscopy analysis, bacteria were grown in glass chamber slides (Ibidi, USA) in the presence of drug (phosphomycin, D-cycloserine) for 24 hours. Concentrations of enzymes, drugs and chemicals used were as follows: chloramphenicol (cam) 100 µg/ml; penicillin G (pen) 150 µg/ml; D-cycloserine (dcs) 250 µg/ml; phosphomycin (pho) 40 µg/ml; D-alanine (D-ala) 250 µg/ml (1:1), 1,000 µg/ml (1:4), 2,500 µg/ml (1:10); EDTA 1 mM, lysozyme (lys) 5 mg/ml; mutanolysin (mut) 80 µg/ml; imipenem (imi) 10 µg/ml; meropenem (mer) 10 µg/ml; mecillinam (mec) 2 mg/ml; tazobactam (taz) 100 µg/ml.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software using a parametric, unpaired t test. P values are illustrated as follows: ns (P > 0.05); * (P ≤ 0.05); ** (P ≤ 0.01); *** (P ≤ 0.001).

Immunofluorescence microscopy

Bacteria were grown in host cells directly in glass chamber slides (Ibidi, USA) or dried onto a regular glass slide, then fixed with 4% paraformaldehyde. Cells were permeabised with 0.5% triton X at 4°C for 5 min, and then labelled using primary (Supporting Information Table S2) and fluorescently conjugated secondary antibodies. For labelling with the probes HADA and HALA, bacteria were grown in host cells on glass chamber slides, and the probe was added directly to the growth media to a final concentration of 1 mM (a dilution of 1/200). The cells were incubated at 35°C with 5% CO2 for 3 h, then washed with PBS and fixed as above. For labelling with EDA-DA, the probe was added to growing bacteria (3 days post infection) at 1 mM concentration and incubated for 24 h at 35°C with 5% CO2. When labelling was performed in the presence of drugs these were added at the same time as EDA-DA and incubated for 24 h at 35°C with 5% CO2. Cells were fixed and permeabilised as above, and the probe was reacted with azide-derived Alexa fluor 488 using the Click-IT cell reaction buffer kit from Invitrogen (catalog number C10269) following the manufacturers instructions.

Quantitative image analysis and classification

We applied quantitative imaging techniques to extract morphometric parameters for the individual O. tsutsugamushi using Cell Profiler (Broad Institute). The extracted 155 features (shown in Supporting Information Table S3) describe size, shape, intensity, granularity, texture and radial distribution of each bacterial cell.

To perform phenotypic classification, we used the Cell Profiler Analyst (Broad Institute) to apply Random Forest classification algorithm to classify O. tsutsugamushi into 3 main phenotypes, (i) 'rough' phenotype from cells in water with DTT, (ii) 'Intermediate' phenotype from cells in PBS and (iii) 'Smooth' phenotype from cells in SPG. These training image sets were taken separately from the images to be used for final prediction. The classification algorithm identified 18 features (Supporting Information Table S4) that can best distinguish training images from the three buffer conditions with 85% accuracy (Supporting Information Fig. S7). We then applied these classification rules to determine the morphological phenotypes for the rest of the images (Fig. 6B).

Principal component analysis was performed on MATLAB (MathWorks, USA, 2015b), using the 18 morphometric parameters previously identified from the Random Forest algorithm. The first three principal components explain over 95% of the observed variance, representing excellent performance for PCA model (Supporting Information Tables S6 and S7). To compare phenotypic differences in different buffer solutions, we performed a pair-wise comparison of PCA loadings using three-dimensional Kolmogorov-Smirnov Test, with the significance level of 0.05.

Western blot analysis

Infected or uninfected L929 cells were mechanically lysed in a Bullet Blender (Next Advance, USA), at power 8 for 1 min and pelleted, washed with PBS 1–2 times, and resuspended in a small volume of PBS. The protein concentration was measured by nanodrop analysis and the sample volume was adjusted to ensure equivalent loading. Samples were mixed with SDS-PAGE buffer with or without 20% β-mercaptoethanol and samples were then loaded on 12% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad,
USA, catalog number 4561043). Western blots were probed using a monoclonal antibody against the major surface protein TSA56 (Rat, Supporting Information Table S2), and then probed with a secondary antibody conjugated with alkaline phosphatase (Promega, USA, catalog number S3831) and developed using an alkaline phosphatase detection kit (Promega, USA, catalog number S3841).

Quantification of bacterial entry into host cells

Bacteria were isolated and resuspended in growth media with or without 1 mM DTT. Samples were incubated for 20 min at room temperature, washed by pelleting two times, and resuspended in fresh media before being added to L929 cells grown in glass chamber slides. Cells were fixed 24 h after infection and the number of bacteria inside host cells counted manually for 100 cells.

Acknowledgements

We are grateful for the support of colleagues at the Microbiology Department of MORU, especially Dr. Blacksell, Prof. Paris, Dr. Limmathurotsakul and Prof. Day. We thank Prof. Waldemar Vollmer of Newcastle University for helpful discussions and comments on this manuscript. This work was funded by a Royal Society Dorothy Hodgkin Fellowship (JS), the Wellcome Trust (JS, SA, SC, SG) and NIH grant AI113172 (MVN).

Author contributions

Conception and design of study: J.D., J.S., S.A., S.G.

Acquisition, analysis or interpretation of data: J.D., J.S., M.V.N., N.K., P.S., S.A., S.C., S.G., S.S.

Writing of the manuscript: J.D., J.S., S.S.

References

Amano, K., Tamura, A., Ohashi, N., Urakami, H., Kaya, S., and Fukushi, K. (1987) Deficiency of peptidoglycan and lipopolysaccharide components in Rickettsia tsutsugamushi. Infect Immun 55: 2290.

Andersson, S.G., Zomorodipour, A., Andersson, J.O., Sicheritz-Ponten, T., AlsMark, U.C., Podowski, R.M., et al. (1998) The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396: 133–140.

Atwal, S., Giengkam, S., VanNieuwenhze, M., and Salje, J. (2016) Live imaging of the genetically intractable obligate intracellular bacteria Orientia tsutsugamushi using a panel of fluorescent dyes. J Microbiol Methods 130: 169–176.

Bastidas, R.J., Elwell, C.A., Engel, J.N., and Valdivia, R.H. (2013) Chlamydial intracellular survival strategies. Cold Spring Harb Perspect Med 3: a010256.

Bavoil, P., Ohlin, A., and Schachter, J. (1984) Role of disulfide bonding in outer membrane structure and permeability in Chlamydia trachomatis. Infect Immun 44: 479–485.

Capeding, M.R., Chua, M.N., Hadinegoro, S.R., Hussain, I.I., Nallusamy, R., Pitsutitthum, P., et al. (2013) Dengue and other common causes of acute febrile illness in Asia: an active surveillance study in children. PLoS Negl Trop Dis 7: e2331.

Cava, F., de Pedro, M.A., Lam, H., Davis, B.M., and Waldor, M.K. (2011) Distinct pathways for modification of the bacterial cell wall by non-canonical D-amino acids. EMBO J 30: 3442–3453.

Cho, B.A., Cho, N.H., Min, C.K., Kim, S.Y., Yang, J.S., Lee, J.R., et al. (2010a) Global gene expression profile of Orientia tsutsugamushi. Proteomics 10: 1699–1715.

Cho, B.A., Cho, N.H., Seong, S.Y., Choi, M.S., and Kim, I.S. (2010b) Intracellular invasion by Orientia tsutsugamushi is mediated by integrin signaling and actin cytoskeleton rearrangements. Infect Immun 78: 1915–1923.

Cho, K.A., Jun, Y.H., Suh, J.W., Kang, J.S., Choi, H.J., and Woo, S.Y. (2010c) Orientia tsutsugamushi induced endothelial cell activation via the NOD1-IL-32 pathway. Microb Pathog 49: 95–104.

Cho, N.H., Kim, H.R., Lee, J.H., and Kim, I.S. (2007) The Orientia tsutsugamushi genome reveals massive proliferation of conjugative type IV secretion system and host–cell interaction genes. Proc Natl Acad Sci USA 104: 17212–17217.

Criado, S., Bastidas, R.J., Elwell, C.A., and Valdivia, R.H. (2016) Live imaging of the genetically intractable obligate intracellular human pathogen Orientia tsutsugamushi. PLoS Negl Trop Dis 9: e0004009.

© 2017 The Authors. Molecular Microbiology Published by John Wiley & Sons Ltd, Molecular Microbiology, 105, 440–452
II: Rickettsiales, 1st ed. Palmer, G.H., and Azad, A. (eds). Washington, DC: ASM Press, pp. 84–141.

Haeusser, D.P., and Margolin, W. (2016) Splittsville: structural and functional insights into the dynamic bacterial Z ring. Nat Rev Microbiol 14: 305–319.

Hatch, T.P., Miceli, M., and Sublett, J.e (1986) Synthesis of disulfide-bonded outer membrane proteins during the developmental cycle of Chlamydia psittaci and Chlamydia trachomatis. J Bacteriol 165: 379–385.

Henrichfreise, B., Schiefer, A., Schneider, T., Nzukou, E., Poellinger, C., Hoffmann, T.j., et al. (2009) Functional conservation of the lipid II biosynthesis pathway in the cell wall-less bacteria Chlamydia and Wolbachia: why is lipid II needed?. Mol Microbiol 73: 913–923.

Jacquier, N., Viollier, P.H., and Greub, G. (2015) The role of Henrichfreise, B., Schiefer, A., Schneider, T., Nzukou, E., Poellinger, C., Hoffmann, T.j., et al. (2009) Functional conservation of the lipid II biosynthesis pathway in the cell wall-less bacteria Chlamydia and Wolbachia: why is lipid II needed?. Mol Microbiol 73: 913–923.

Jeske, O., Schuler, M., Schumann, P., Schneider, A., Boedeker, C., Jogler, M., et al. (2015) Planctomycetes do possess a peptidoglycan cell wall. Nat Commun 6: 7116.

Keller, C.A., Hauptmann, M., Kolbaum, J., Gharabeih, M., Neumann, M., Glatzel, M., and Fleischer, B. (2014) Dissemination of Orientia tsutsugamushi and inflammatory responses in a murine model of scrub typhus. PLoS Negl Trop Dis 8: e3064.

Kuru, E., Hughes, H.V., Brown, P.J., Hall, E., Tekkam, S., Cava, F., et al. (2012) In situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent D-amino acids. Angew Chem Int Ed Engl 51: 12519–12523.

Kuru, E., Tekkam, S., Hall, E., Brun, Y.V., and Van Nieuwenhze, M.S. (2015) Synthesis of fluorescent D-amino acids and their use for probing peptidoglycan synthesis and bacterial growth in situ. Nat Protoc 10: 33–52.

Lee, J.H., Cho, N.H., Kim, S.Y., Bang, S.Y., Chu, H., Choi, M.S., and Kim, I.S. (2008) Fibronectin facilitates the invasion of Orientia tsutsugamushi into host cells through interaction with a 56-kDa type-specific antigen. J Infect Dis 198: 250–257.

Liechti, G., Kuru, E., Packiam, M., Hsu, Y.P., Tekkam, S., Hall, E., et al. (2016) Pathogenic Chlamydia lack a classical sacculus but synthesize a narrow, mid-cell peptidoglycan ring, regulated by MreB, for cell division. PLoS Pathog 12: e1005590.

Liechti, G.W., Kuru, E., Hall, E., Kalinda, A., Brun, Y.V., VanNieuwenhze, M., and Maurelli, A.T. (2014) A new metabolic cell-wall labelling method reveals peptidoglycan in Chlamydia trachomatis. Nature 506: 507–510.

Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., et al. (2015) A new antibiotic kills pathogens without detectable resistance. Nature 517: 455–459.

McCoy, A.J., and Maurelli, A.T. (2006) Building the invisible wall: updating the chlamydial peptidoglycan anomaly. Trends Microbiol 14: 70–77.

Mayfong Mayxay, M., Castonguay-Vanier, J., Chansamouth, V., Dubot-Peres, A., Paris, D.H., Phetsouvanh, R., et al. (2013) Causes of non-malarial fever in Laos: a prospective study. Lancet Glob Health 1: e46–e54.

Meeske, A.J., Riley, E.P., Robins, W.P., Uehara, T., Mekalanos, J.J., Kahne, D., et al. (2016) SEDS proteins are a widespread family of bacterial cell wall polymers. Nature 537: 634–638.

Min, C.K., Yang, J.S., Kim, S., Choi, M.S., Kim, I.S., and Cho, N.H. (2008) Genome-based construction of the metabolic pathways of Orientia tsutsugamushi and comparative analysis within the Rickettsiales order. Comp. Funct Genomics 2008: 1–14.

Mohammadi, T., and Breukink, E. (2014) The chlamydial anomaly clarified? ChemBiochem 15: 1391–1392.

Moron, C.G., Popov, V.L., Feng, H.M., Wear, D., and Walker, D.H. (2001) Identification of the target cells of Orientia tsutsugamushi in human cases of scrub typhus. Mod Pathol 14: 752–759.

Moulder, J.W. (1993) Why is Chlamydia sensitive to penicillin in the absence of peptidoglycan? Infect Agents Dis 2: 87–99.

Moulder, J.W., Novosel, D.L., and Officer, J.E. (1963) Inhibition of the growth of agents of the psittacosis group by D-cycloserine and its specific reversal by D-alanine. J Bacteriol 85: 707–711.

Moumène, A., and Meyer, D.F. (2016) Ehrlichia’s molecular tricks to manipulate their host cells. Microbes Infect 18: 172–179.

Nakayama, K., Yamashita, A., Kurokawa, K., Morimoto, T., Ogawa, M., Fukuhara, M., et al. (2008) The Whole-genome sequencing of the obligate intracellular bacterium Orientia tsutsugamushi revealed massive gene amplification during reductive genome evolution. DNA Res 15: 185–199.

Ohashi, N., Fukuhara, M., Shimada, M., and Tamura, A. (1995) Phylogenetic position of Rickettsia tsutsugamushi and the relationship among its antigenic variants by analyses of 16S rRNA gene sequences. FEMS Microbiol Lett 125: 299–304.

Packiam, M., Weinrick, B., Jacobs, W.R.J., and Maurelli, A.T. (2015) Structural characterization of muropeptides from Chlamydia trachomatis peptidoglycan by mass spectrometry resolves “chlamydial anomaly”. Proc Natl Acad Sci USA 112: 11660–11665.

Paris, D.H., Phetsouvanh, R., Tanganuchitcharnchai, A., Jones, M., Jenjaroen, K., Vongsouvath, M., et al. (2012) Orientia tsutsugamushi in human scrub typhus eschars shows tropism for dendritic cells and monocytes rather than endothelium. PLoS Negl Trop Dis 6: e1466.

Phongmany, S., Rolain, J.M., Phetsouvanh, R., Blacksell, S.D., Soukhaseum, V., Rasachack, B., et al. (2006) Rickettsial infections and fever, Vientiane, Laos. Emerg Infect Dis 12: 256–262.

Plhofner, M., Aistleitner, K., Biboy, J., Gray, J., Kuru, E., Hall, E., et al. (2013) Discovery of chlamydial peptidoglycan reveals bacteria with murein sacculi but without FtsZ. Nat Commun 4: 2856.

Pizarro-Cerda, J., and Cossart, P. (2006) Bacterial adhesion and entry into host cells. Cell 124: 715–727.

Sauvage, E., Derouaux, A., Fraipont, C., Joris, M., Herman, R., Rocaboy, M., et al. (2014) Crystal structure of penicillin-binding protein 3 (PBPs) from Escherichia coli. PLoS One 9: e98042.

Schneider, T., and Sahl, H.G. (2010) An oldie but a goodie - cell wall biosynthesis as antibiotic target pathway. Int J Med Microbiol 300: 161–169.

Schumann, P. (2011) Peptidoglycan structure. Methods Microbiol 38: 101–129.

© 2017 The Authors. Molecular Microbiology Published by John Wiley & Sons Ltd, Molecular Microbiology, 105, 440–452.
Shieh, P., Siegrist, M.S., Cullen, A.J., and Bertozzi, C.R. (2014) Imaging bacterial peptidoglycan with near-infrared fluorogenic azide probes. *Proc Natl Acad Sci USA* **111**: 5456–5461.

Shostak, K., and Schirch, V. (1988) Serine hydroxymethyltransferase: mechanism of the racemization and transamination of D- and L-alanine. *Biochemistry* **27**: 8007–8014.

Siegrist, M.S., Whiteside, S., Jewett, J.C., Aditham, A., Cava, F., and Bertozzi, C.R. (2013) D-Amino acid chemical reporters reveal peptidoglycan dynamics of an intracellular pathogen. *ACS Chem Biol* **8**: 500–505.

Siegrist, M.S., Swarts, B.M., Fox, D.M., Lim, S.A., and Bertozzi, C.R. (2015) Illumination of growth, division and secretion by metabolic labeling of the bacterial cell surface. *FEMS Microbiol Rev* **39**: 184–202.

Silverman, D.J., and Wisseman, C.L. (1978) Comparative ultrastructural study on the cell envelopes of *Rickettsia prowazekii, Rickettsia rickettsii*, and *Rickettsia tsutsugamushi*. *Infect Immun* **21**: 1020–1023.

Spring, S., Bunk, B., Spröer, C., Schumann, P., Rohde, M., Tindall, B.J., and Hp, K. (2016) Description and complete genome sequence of *Kiritimatiella glycovorans* gen. nov., sp. nov., the first cultured representative of a novel phylum originally designated *Verrucomicrobia* subdivision 5. *ISME J* **10**: 2801–2816.

Tamura, A., Urakami, H., and Ohashi, N. (1991) A comparative view of Rickettsia tsutsugamushi and the other groups of rickettsiae. *Eur J Epidemiol* **7**: 259–269.

Turner, R.D., Vollmer, W., and Foster, S.J. (2014) Different walls for rods and balls: the diversity of peptidoglycan. *Mol Microbiol* **91**: 862–874.

Urakami, H., Ohashi, N., Tsuruhara, T., and Tamura, A. (1986) Characterization of polypeptides in *Rickettsia tsutsugamushi*: effect of preparative conditions on migration of polypeptides in polyacrylamide gel electrophoresis. *Infect Immun* **51**: 948–952.

Vollmer, J., Schiefer, A., Schneider, T., Julicher, K., Johnston, K.L., Taylor, M.J., *et al.* (2013) Requirement of lipid II biosynthesis for cell division in cell wall-less Wolbachia, endobacteria of arthropods and filarial nematodes. *Int J Med Microbiol* **303**: 140–149.

Vollmer, W., and Bertche, U. (2008) Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. *Biochim Biophys Acta* **1778**: 1714–1734.

Watt, G., and Parola, P. (2003) Scrub typhus and tropical rickettsioses. *Curr Opin Infect Dis* **16**: 429–436.

Watt, G., Chouriayagune, C., Ruangweerayud, R., Watcharapichat, P., Phulsuksombat, D., Jongsakul, K., *et al.* (1996) Scrub typhus infections poorly responsive to antibiotics in northern Thailand. *Lancet* **348**: 86–89.

Wisseman, C.L.J., Silverman, D.J., Waddell, A., and Brown, D.T. (1982) Penicillin-induced unstable intracellular formation of spheroplasts by rickettsiae. *J Infect Dis* **146**: 147–158.

Yang, Y., Bhachech, N., and Bush, K. (1995) Biochemical comparison of imipenem, meropenem and biapenem: permeability, binding to penicillin-binding proteins, and stability to hydrolysis by beta-lactamases. *J Antimicrob Chemother* **35**: 75–84.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.