Peptidoglycan (PG), an essential structure in the cell walls of the vast majority of bacteria, is critical for division and maintaining cell shape and hydrostatic pressure. Bacteria comprising the Chlamydiales were thought to be one of the few exceptions. Chlamydia harbour genes for PG biosynthesis and exhibit susceptibility to ‘anti-PG’ antibiotics, yet attempts to detect PG in any chlamydial species have proven unsuccessful (the ‘chlamydial anomaly’). We used a novel approach to metabolically label chlamydial PG using D-amino acid dipeptide probes and click chemistry. Replicating Chlamydia trachomatis were labelled with these probes through their biphasic developmental life cycle, and the results of differential probe incorporation experiments conducted in the presence of ampicillin are consistent with the presence of chlamydial PG-modifying enzymes. These findings culminate 50 years of speculation and debate concerning the chlamydial anomaly and are the strongest evidence so far that chlamydial species possess functional PG.

Chlamydia trachomatis is the leading cause of infectious blindness and sexually transmitted bacterial infection worldwide. It is a member of the Chlamydiae, a phylum consisting of obligate, intracellular, bacteria that cause a wide variety of infectious diseases in humans and animals. Their obligate intracellular nature and dimorphic life cycle has made studying Chlamydia a challenge and questions remain about even the basic processes of cell division and cell envelope maintenance in these pathogens. The infectious form of the organism, the elementary body (EB), is small (~0.3 μm) and essentially metabolically inert.

After attachment to and infection of a host cell, the EB undergoes a transition to the metabolically active reticulate body (RB), which replicates via binary fission but is incapable of attaching to or infecting new host cells. Thus, RBs must differentiate back to the EB form to complete the developmental cycle. Infected cells then lyse, releasing infectious EBs that infect new host cells.

Peptidoglycan (PG) is a sugar amino acid polymer that forms a mesh-like sheet surrounding the cytoplasmic membrane of bacterial cells. In the vast majority of free-living bacteria, PG aids in cell division, maintenance of osmotic pressure, and provides a stable anchor for transmembrane complexes and integral membrane proteins. Bacteria maintain their cell shape largely due to the presence of this rigid yet modifiable cell wall. A single PG subunit consists of a disaccharide backbone coupled to a pentapeptide chain (Fig. 1a). During cell-wall synthesis, disaccharide pentapeptide monomers are linked together at their corresponding sugars, creating a sugar polymer with polypeptide stems, which are crosslinked by transpeptidation. The pentapeptide chain is assembled sequentially by a series of ligases that specifically incorporate both L- and D-amino acids (D-glutamic acid and D-alanine) (Fig. 1a). These two D-amino acids are unique to bacteria and they are not used by mammalian cells. Thus, the enzymes involved in their synthesis and incorporation into PG are excellent targets for antibiotics such as β-lactams and D-cycloserine.

The existence of PG in Chlamydia has long been debated. Whereas genetic analysis and antibiotic susceptibility indicate that chlamydial PG exists, all attempts to detect or purify PG in Chlamydia have been unsuccessful, resulting in the ‘chlamydial anomaly’. It has been established that the cytosolic receptor for PG, Nod1, is triggered upon infection by various chlamydial species. Chlamydia homologues of PG biosynthetic enzymes have been extensively studied and a growing body of literature supports the functionality of a complete biosynthesis pathway. A functional chlamydial UDP-N-acetylgalactosamine enolpyruvyl transferase (MurA) has been described. The product of the MurA reaction is specifically used for the synthesis of UDP-N-acetylmuramic acid (the sugar unique to the PG disaccharide backbone), suggesting the presence of the sugar component of PG in Chlamydia.

During PG biosynthesis in most bacteria, D-alanine-D-alanine (DA-DA) generated by D-alanine-D-alanine ligase (Ddl) is incorporated directly into growing PG peptide chains through the MurF enzyme (Fig. 1a). Characterization of Ddl and MurF enzymes in Chlamydia coupled with recent advances in the chemical modification of PG through the incorporation of single D-amino acids present an opportunity to covalently label the PG of actively growing Chlamydia.

C. trachomatis can take up both D-alanine and DA-DA dipeptide; however, efforts to successfully label Chlamydia using previously characterized D-amino acid probes were unsuccessful (Extended Data Fig. 1). We reasoned that this result was due to the inability of the chlamydial PG synthesis machinery to incorporate the modified single D-amino acids. Thus, we developed a novel and broadly applicable PG-labelling approach that bypassed the bacterial Ddl enzyme and used DA-DA dipeptide analogues modified with alkyne or azide functional groups (Fig. 1). Initial studies in Escherichia coli and Bacillus subtilis established that the alkyne and azide analogues of DA-DA (EDA-DA) are capable of rescuing the growth of bacteria with depleted DA-DA dipeptide pools, whereas an azide analogue of the enantiomer L-alanine-L-alanine (LA-LA) is not capable of rescuing growth (Extended Data Table 1). In rich medium, bacterial growth is unaffected by the presence of DA-DA analogues (Extended Data Fig. 2). Once incorporated into a macromolecule such as PG, the functional groups of these dipeptides can be selectively captured via a click-chemistry reaction. Labelling studies using DA-DA analogues in conjunction with clickable, modified Alexa Fluor dyes confirmed D-enantiomer-specific incorporation of the modified dipeptides in diverse bacterial species (Extended Data Figs 2 and 3). Polarly growing Streptomyces venezuelae was grown in the presence of the previously characterized, fluorescent D-amino acid HADA (7-hydroxycoumarin-3-carboxylic acid-3-amino-D-alanine, see ref. 19) for a few generations, and upon addition of EDA-DA for a brief period, subsequent polar-labelling confirmed that these dipeptides specifically label areas of new PG synthesis (Extended Data Fig. 3e). The labelling of E. coli and B. subtilis was covalent and cell-wall-specific (Extended Data
Labelling was only present in a distinct, ring-like shape, consistent with a cellular division plane and MOMP-labelled RBs (Extended Data Fig. 5). The labelling was arranged EDA-DA labelling appeared as either a ring or a single line bisecting antibody to the chlamydial major outer membrane protein (MOMP), individual bacteria clearly discernible (Fig. 2). When co-labelled with DA probe. The probe localized within chlamydial inclusions with a terminal azide group to the alkyne group present on the EDA-DA and click chemistry was used to attach an Alexa fluorophore modified infected for 18 h with Chlamydia oration in intracellular that the probes were incorporated in the PG. PG-digesting enzyme, lysozyme (Extended Data Fig. 4b), indicating that the majority of labelled chlamydial PG is localized to the septum of dividing RBs. However, we cannot rule out the possibility that low levels of PG exist elsewhere on the bacterium and are simply below the detection limit of fluorescence microscopy. Similar to our results with B. subtilis, we found that incubation with lysozyme for two hours was sufficient to remove EDA-DA labelling within chlamydial inclusions (Extended Data Fig. 6d, e), supporting our conclusion that the dipeptide probes are incorporating into chlamydial PG.

To further confirm that the modified probes were being taken up and incorporated into chlamydial PG, we performed plaque assays that allow quantification of intracellular bacterial growth and infectivity. D-cycloserine (DCS) is an inhibitor of cell-wall biosynthesis that targets bacterial alanine racemase and D-alanine-D-alanine ligase and previous studies have shown that Chlamydia growth is inhibited by DCS at millimolar concentrations. Growth inhibition is overcome by supplementation with exogenous D-alanine or DA-DA dipeptide, most likely owing to the exogenous single D-amino acids outcompeting DCS for the binding sites of the chlamydial ligase or, in the case of DA-DA, bypassing the need for the ligase altogether. Various D-amino acids, dipeptides and their corresponding alkyne-modified probes were tested to determine the level of DCS rescue they conferred upon B. subtilis label could be removed by treatment with the PG-digesting enzyme, lysozyme (Extended Data Fig. 4b), indicating that the probes were incorporated in the PG.

We next attempted to measure dipeptide probe uptake and incorporation in intracellular Chlamydia. L2 mouse fibroblast cells were infected for 18 h with C. trachomatis serovar L2 strain 434/Bu in the presence of 1 mM EDA-DA. Cells were then fixed and permeabilized, and click chemistry was used to attach an Alexa fluorophore modified with a terminal azide group to the alkyne group present on the EDA-DA probe. The probe localized within chlamydial inclusions with individual bacteria clearly discernible (Fig. 2). When co-labelled with antibody to the chlamydial major outer membrane protein (MOMP), EDA-DA labelling appeared as either a ring or a single line bisecting MOMP-labelled RBs (Extended Data Fig. 5). The labelling was arranged in a distinct, ring-like shape, consistent with a cellular division plane and the labelling bore a striking resemblance to images previously obtained for intracellular C. trachomatis stained with antibody generated with Ribi adjuvant. Labelling was only present in Chlamydia-infected cells and only in the presence of probe (Extended Data Fig. 6a–c). This result indicates that the majority of labelled chlamydial PG is localized to the septum of dividing RBs. However, we cannot rule out the possibility that low levels of PG exist elsewhere on the bacterium and are simply below the detection limit of fluorescence microscopy. Similar to our results with B. subtilis, we found that incubation with lysozyme for two hours was sufficient to remove EDA-DA labelling within chlamydial inclusions (Extended Data Fig. 6d, e), supporting our conclusion that the dipeptide probes are incorporating into chlamydial PG.

To further confirm that the modified probes were being taken up and incorporated into chlamydial PG, we performed plaque assays that allow quantification of intracellular bacterial growth and infectivity. D-cycloserine (DCS) is an inhibitor of cell-wall biosynthesis that targets bacterial alanine racemase and D-alanine-D-alanine ligase and previous studies have shown that Chlamydia growth is inhibited by DCS at millimolar concentrations. Growth inhibition is overcome by supplementation with exogenous D-alanine or DA-DA dipeptide, most likely owing to the exogenous single D-amino acids outcompeting DCS for the binding sites of the chlamydial ligase or, in the case of DA-DA, bypassing the need for the ligase altogether. Various D-amino acids, dipeptides and their corresponding alkyne-modified probes were tested to determine the level of DCS rescue they conferred upon...
Table 1 | DCS Chlamydia trachomatis plaque assay in the presence of natural and modified d-amino acids

| DCS (µM) | Amino acid, amino acid to DCS ratio | Plaque formation |
|----------|------------------------------------|-----------------|
| 0        | No amino acid                      | +++++           |
| 294      | Dipeptides                         |                 |
|          | DA-DA, 1:1                         | ++              |
|          | DA-EDA, 1:1                        | ++              |
|          | DA-EDA, 1:10                       | +++             |
|          | EDA-DA, 1:1                        | −               |
|          | EDA-DA, 10:1                       | +               |
|          | Single amino acids                 |                |
|          | D-Ala, 1:1                         | + + + +         |
|          | D-Ala, 1:10                        | + + + +         |
|          | EDA, 1:1                           | −               |
|          | EDA, 10:1                          | +               |

C. trachomatis serovar L2 strain 434/Bu was grown in the plaque assay as previously described in the presence of d-cycloserine (DCS) and varying molar equivalent concentrations of d-alanine (D-Ala), d-alanine-d-alanine (DA-DA), EDA, DA-EDA, and EDA-DA. +++, complete infection, bacterial growth and lysis of the monolayer; +++, numerous small plaques but less than complete lysis of the monolayer; +, few (10–20) small plaques; −, no plaque formation (no bacterial growth). Data represent the average of three biological replicates and each experiment was conducted with technical duplicates.

growing C. trachomatis. We found that DA-DA dipeptide and the corresponding modified dipeptides (EDA-DA and DA-EDA) were both capable of rescuing chlamydial plaquing (Table 1), indicating their successful uptake and incorporation by Chlamydia. However, whereas unmodified d-alanine was capable of overcoming the growth inhibitory effects of DCS, the corresponding chemically modified, single d-alanine probe (EDA) was not. These results were consistent with our inability to detect fluorescent labelling of C. trachomatis through single EDA probes (Extended Data Fig. 1).

Despite rescue of chlamydial growth by both EDA-DA and DA-EDA in the DCS plaque assay, we initially were not able to label chlamydial PG with DA-EDA (Extended Data Fig. 7). Similarly, we were unable to obtain labelling with DA-EDA in E. coli (Extended Data Fig. 2). We reasoned that the inability to label Chlamydia with DA-EDA was due to the removal of the terminal, modified EDA amino acid from the PG pentapeptide stem during either transpeptidation or carboxypeptidation (Fig. 1a). To test this hypothesis and to further validate that our probes were incorporated into C. trachomatis PG, we conducted EDA-DA and DA-EDA labelling studies in the presence of two antibiotics that block PG biosynthesis: DCS, a competitive inhibitor of both alanine racemase and d-alanine-d-alanine ligase, and ampicillin, an inhibitor of PG transpeptidases/carboxypeptidases. When grown for 18 h in the presence of either antibiotic, inclusions contained enlarged, aberrant RBs. The presence of fewer bacteria per inclusion is indicative of a pre-division block, due to the absence of transpeptidation, and is consistent with the literature. In the presence of DCS and 1 mM EDA-DA, fluorescent PG was discernible within aberrant RBs (Fig. 3a). This result indicates that EDA-DA was capable of partly substituting for DA-DA after depletion of the bacterium’s natural dipeptide pool and confirms the DCS plaquing assay results. EDA-DA labelling intensity seemed unaffected by inhibition of PG transpeptidation/carboxypeptidation with ampicillin (Extended Data Fig. 7), indicating that probe incorporation is not dependent on transpeptidation and does not occur in the periplasm in Chlamydia. When imaged by epifluorescence, labelling of aberrant bodies grown in the presence of ampicillin often appeared punctate, owing to the enlarged PG ring structures that no longer exist within a single focal plane (see Extended Data Fig. 8). z-stacks taken of the ampicillin-treated aberrant RBs clearly revealed labelled PG sequestered to an equatorial region where the bacterial division plane would normally form (Fig. 3b). Fluorescence labelling of Chlamydia with DA-EDA was only observed when transpeptidation/carboxypeptidation was inhibited with ampicillin (Extended Data Fig. 7). DA-ADA labelling of a B. subtilis D,D-carboxypeptidase mutant (ΔdacA) confirmed this finding: labelling was greatly increased compared to the parental, wild-type strain and was not significantly turned over as the labelled cells were allowed to grow (Extended Data Fig. 3). These observations indicate that PG modifications (through transpeptidation and/or carboxypeptidation) occur in vivo in Chlamydia, as inhibition of these modifications would preserve the terminal d-alanine in the stem peptide, thus allowing for labelling of PG with DA-EDA.

Several investigators have examined the transcriptional profile of PG biosynthesis genes throughout the chlamydial life cycle. The current consensus is that PG biosynthesis genes are upregulated during the transition phase between EB and RB, indicating the need for PG before cell division. These results fit well with the knowledge that PG is closely involved in bacterial growth and cytokinesis. To correlate these findings with actual PG production as measured via the incorporation of dipeptide probes into actively forming chlamydial PG, we conducted a time-course study to determine the earliest time at which dipeptide incorporation occurs. We detected EDA-DA-labelled C. trachomatis as early as 8 h post infection (Extended Data Fig. 9), and labelled bacteria were always seen in the context of developing chlamydial inclusions. Pre-incubating L2 cells with EDA-DA for 8 h before infection with C. trachomatis did not result in probe incorporation at earlier time points (data not shown). This suggests that probe incorporation and

Figure 3 | Labelling in the presence of PG synthesis inhibitors. a, b, DIC and fluorescent microscopy of infected L2 cells 18 h post infection in the presence of 1 mM EDA-DA and either d-cycloserine (DCS) (a) or ampicillin (AMP) (b). Labelling was conducted as described in the Fig. 2 legend. A merge of all three fluorescent channels is presented in the panels on the right. Fluorescent images are maximum intensity projections of z-stacks and three-dimensional renderings are provided in Supplementary Videos 3 and 4.
new PG synthesis occur during the early stages of EB to RB transition, consistent with transcriptome data.\(^2\)

In conclusion, we have successfully labelled the peptidoglycan of intracellular Chlamydia trachomatis using modified dipeptide probes. The development and characterization of this versatile, general and non-toxic method for metabolically labelling peptidoglycan provides a unique and powerful technique for studying bacterial peptidoglycan biosynthetic pathways in a myriad of bacterial species. The strength of the click chemistry approach used in this work is in its specificity, but future studies will also exploit its versatility. These probes can be selectively captured by molecules other than conventional dyes, such as specified fluorophores for use in super-resolution microscopy, modified gold nanoparticles for use in electron microscopy, or agarose resins for use in peptidoglycan enrichment and chemical characterization. Although this technique has opened the door to a wide range of studies in Chlamydia, it is readily adaptable to other model systems, thus providing a powerful investigative tool for use in the examination of bacterial peptidoglycan biosynthesis, modification and degradation pathways.

METHODS SUMMARY

EDA was purchased from BiotraSource and dipeptide probes EDA-DA, DA-EDA, ADA-DA, and ADA-DA were synthesized (details in the Supplementary Methods section). Click chemistry was carried out with a ‘clickable’ Alexa Fluor 488 and Click-IT Cell Reaction Buffer Kit, which were purchased from Invitrogen. Wild-type Escherichia coli strain MG1655 and mutants, Bacillus subtilis strain PY79, Streptomyces venezuelae and Streptococcus pneumoniae strain IU1945 were all grown as previously described.\(^4\) L2 mouse fibroblasts and C. trachomatis serovar L2 strain 434/Bu were grown, bacterial and antibiotic titres calculated, and infections/plaque assays were conducted as previously described.\(^5\) For counter-labelling of C. trachomatis, either monoclonal anti-MOMP antibody (LifeSpan Biosciences) or anti-InC antibody (D. Rockey) was used. Detailed protocols for PG labelling of all bacteria described in this work are provided in the Methods section, along with lysozyme treatment protocols, plaquing assays and details concerning image acquisition and analysis for all microscopy.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 3 September; accepted 21 November 2013.

Published online 11 December 2013.

1. Egan, A. J. & Vollmer, W. The physiology of bacterial cell division. Annu. NY Acad. Sci. 1277, 28–38 (2013).
2. McCoy, A. J., Sandlin, R. C. & Maurelli, A. T. In vitro and in vivo functional activity of Chlamydia MurA, a UDP-N-acetylmuramoyl-L-alanine-N-acetylmuramate synthetase involved in peptidoglycan synthesis and fosfomycin resistance. J. Bacteriol. 185, 1218–1228 (2003).
3. Hesse, L. et al. Functional and biochemical analysis of Chlamydia trachomatis MurC, an enzyme displaying UDP-N-acetylmuramaldehyde-5-amino-5-deoxyglucosamine 3-epimerase activity. J. Bacteriol. 185, 6507–6512 (2003).
4. McCoy, A. J. & Maurelli, A. Characterization of Chlamydia MurC-Ddl, a fusion protein exhibiting α- and -alanine ligation activity involved in peptidoglycan synthesis and cell elongation. J. Biol. Chem. 280, 2541–2553 (2005).
5. Patin, D., Bostock, J., Blanot, D., Mengin-Lecreulx, D. & Chopra, I. Functional and biochemical analysis of the Chlamydia trachomatis lipo-MurE J. Bacteriol. 191, 7430–7435 (2009).
6. Patin, D., Bostock, J., Chopra, I., Mengin-Lecreulx, D. & Blanot, D. Biochemical characterisation of the chlamydial MurF ligase, and possible sequence of the chlamydial peptidoglycan pentapeptide stem. Arch. Microbiol. 194, 505–512 (2012).
7. McCoy, A. J. et al. L- and D-diaminopimelate aminotransferase, a trans-kingdom enzyme shared by Chlamydia and plants for synthesis of diaminopimelate lysine. Proc. Natl Acad. Sci. USA 103, 17909–17914 (2006).
8. Moulder, J. W., Novosel, D. L. & Officer, J. E. Inhibition of the growth of agents of the psittacosis group by o-cycloserine and its specific reversal by o-alanine. J. Bacteriol. 85, 707–711 (1963).
METHODS

Reagents. EDA was purchased from BoaoPharma. ‘Clickable’ Alexa Fluor 488 and Click-it® Cell Reaction Buffer Kit (Invitrogen) were purchased from Invitrogen.

Cell culture conditions. L2 mouse fibroblast cells were obtained from S. Weiss cultured in T-175 flasks (BD Falcon) using Dulbecco’s Modified Eagle Medium+ GlutaMAX (Gibco) (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, HyClone) at 37 °C with 5% CO2 and checked monthly for mycoplasma. When conducting chlamydial infections, cell medium was supplemented with 1× MEM Non-Essential Amino Acids Solution (Sigma) and 0.2 μg ml−1 cycloheximide (Sigma).

Cell infection and bacterial growth conditions. C. trachomatis serovar L2 strain 434/Bu was provided by H. Caldwell (Rocky Mountain Laboratories). Chlamydial EBs were purified from L2 cells 40 h post infection and stored at −80 °C.

Growth curves with E. coli and B. subtilis and dipeptide molecules as noted in the text.

Labelling E. coli, B. subtilis and S. pneumoniae PG.

Involving alkyne-containing PG probes and Alexa Fluor 488 Azide (Invitrogen), E. coli, B. subtilis and S. pneumoniae PG was quantified by comparing average attenuance of each condition to a growth control (LB) (tryptone 10 g, yeast extract, 5 g NaCl 10 g) at 37 °C with aeration. For the wild-type strains, cells were collected in a table-top centrifuge at room temperature, washed once with 1× PBS and combined into 3× 1.7 ml tubes. The cells were resuspended in 3× 1.5 ml ice-cold 70% ethanol and kept at −20 °C for 10–12 min, pelleted and washed twice with an equal volume of 1× PBS. After the last wash, click-chemistry was performed using Click-it® Cell Reaction Buffer Kit (Invitrogen) and Alexa Fluor 488–azide (20 μM) with 45 min incubation at room temperature. The cells were washed once more and resuspended in 3× 1.0 ml 0.1 M Tris–HCl, 7.5% (w/v) sucrose, 17 mM Na2HPO4, 200 mM NaCl, and 0.5% glucose added after sterilization), incubated 2 h at 37 °C. The cells were pelleted and combined in 2 ml water and boiled in an equal volume of SDS (2% w/v) with stirring for 45 min. The sacculi were divided into 3× 1.7 ml tubes and excess SDS was removed by washing three times with 1.5 ml water. Sacculi were further stained with wheat germ agglutinin–Alexa Fluor 647 conjugate (WGA-647, 10 μg ml−1, 15 min at room temperature) and imaged.

M9 medium. The minimal media used were M9 (composition per litre, 2 g (NH4)2SO4, 1.4 g K2HPO4·3H2O, 6 g KH2PO4, 1 g Na·citrate·2H2O, 0.2 g MgSO4·7H2O (plus tryptophan, final concentration of 50 μg ml−1, and 0.5% glucose added after sterilization)), respectively. S. pneumoniae IU459 was grown at 37 °C in brain-heart infusion (BHI) broth.

Growth curves with E. coli and B. subtilis. Exponentially growing E. coli and B. subtilis were diluted to D00 nm = 0.3 in media containing t-alanine or dipeptide analogues. Aliquots were taken after 5 min and 60 min. For copper-catalysed click-chemistry involving alkyne-containing PG probes and Alexa Fluor 488 Azide (Invitrogen), cells were fixed in ice-cold 70% ethanol, washed once with 1× PBS (NaCl 8 g l−1, KCl 0.2 g l−1, Na2HPO4·2H2O 1.78 g l−1, KH2PO4·3H2O 1.71 g l−1, pH 7.4) until use. Stocks were titred using an infection forming unit assay (IFU). For infections, treated glass coverslips were placed in 24-well tissue culture plates (Costar) and L2 cells were plated to a concentration of 60–70%. Cells were washed twice with DMEM, and then infected at an MOI of between 1 and 10 with C. trachomatis. Plates were placed on a rocker for 2 h to allow for adherence of bacterial EBs to the L2 cells, and then medium was removed and replaced with infection medium (described previously) supplemented with modified t-alanine or dipeptide probes (additional supplementation with t-cycloserine (DCS, 294 μM) or ampicillin (2.8 μM) where indicated). E. coli MG1655 and B. subtilis PY79 (wild type or ΔaddAΔ) were grown in Luria Broth (LB) (tryptone 10 g, yeast extract, 5 g NaCl 10 g) at 37 °C with aeration. E. coli wild type or ΔaddAΔΔDDbb) and B. subtilis the minimal medium used were M9 + 0.2% glucose ± 1% LB or Spizizen’s minimal medium (composition per litre, 2 g (NH4)2SO4, 14 g K2HPO4, 3H2O, 6 g KH2PO4, 1 g Na·citrate·2H2O, 0.2 g MgSO4·7H2O (plus tryptophan, final concentration of 50 μg ml−1, and 0.5% glucose added after sterilization)), respectively.

Labelling chlamydial PG. At designated time points post infection, infection medium was removed, coverslips were washed three times with PBS, and cells were fixed in methanol at room temperature for five minutes. Cells were again washed in PBS and further permeabilized in 0.5% TritonX for five minutes, and washed again. Cells were then blocked for one hour in 3% BSA before the click chemistry reaction being performed. Click-it® Cell Reaction Buffer Kit (Invitrogen) was used to carry out the click chemistry reaction with Alexa Fluor 488–azide (10 μM). The Click-IT reaction was allowed to proceed for 1 h, after which slides were washed with 3% BSA. For labelling major chlamydial outer membrane protein (MOMP) or inclusion protein A (IncA), coverslips were first blocked in DMEM supplemented with 10% heat-inactivated FBS for one hour. Coverslips were then incubated with monoclonal anti-MOMP antibody (LifeSpan Biosciences) or anti- IncA antibody (D. Rockett®) diluted 1:500 in DMEM (10% FBS) for one hour, washed with DMEM (10% FBS), incubated with a secondary, chicken anti-goat or anti-rabbit IgG (respectively) conjugated to Alexa Fluor 594 (Invitrogen), diluted 1:20 000 in DMEM (10% FBS). Coverslips were washed once in DMEM (10% FBS), incubated with a secondary, chicken anti-goat or anti-rabbit IgG (respectively) conjugated to Alexa Fluor 488 (Invitrogen), diluted 1:20 000 in DMEM (10% FBS). Coverslips were washed once in PBS, then stained with DAPI (Sigma) diluted 1:8 000 in PBS, for five minutes. Coverslips were washed one final time in PBS and mounted to glass slides with SlowFade Gold Antifade reagent (Invitrogen) for imaging.

Chlamydial lysosome treatment assay. L2 cells were infected for 18 h, fixed, permeabilized and washed (as previously described), and blocked with 3% BSA for one hour. The click chemistry reaction was conducted (as described previously) and finally cells were suspended in 250 μl of 25 mM NaPO4 pH 6.0, 0.5 mM MgCl2 in the presence or absence of lysosome (Sigma, 200 μg ml−1).

©2014 Macmillan Publishers Limited. All rights reserved
rocked gently for two hours under tissue culture conditions (37°C 5% CO2). Counter labelling was then conducted as previously described and imaging was conducted via epifluorescence microscopy. The assay was also conducted before running the click chemistry reaction on fixed/permeabilized cells, and as the results were identical, these data were not included. Similarly, 18 h incubation in reaction buffer or lysozyme was also conducted, with results identical to the two-hour incubation.

**Image acquisition and analysis.** Images were acquired via epifluorescence (Olympus BX50 and IX81) or confocal (Zeiss 710) laser-scanning microscopy. Image acquisition was performed with DPController (Olympus Corp.) and Zen 2009 (Carl Zeiss) software, respectively. Settings were fixed at the beginning of image acquisition. Brightness and contrast were adjusted slightly in all channels for images obtained via epifluorescence microscopy. Brightness and contrast were slightly adjusted for differential interference contrast for images taken via confocal microscopy. Image analysis was conducted with ImageJ. Deconvolution was used for generating the fluorescent images in Fig. 2. Due to the limits of detection inherent to fluorescence microscopy using click-chemistry-based probes and the statements made in the text concerning the exact localization of the label within individual bacteria (that is, the label being present only within bisecting rings within normal and aberrant bacteria), we provide maximum intensity projections of unaltered z-stacks for comparison with the deconvoluted images in Extended Data Fig. 5. Deconvolution was conducted with AxioVision (Carl Zeiss) software using the inverse filter setting. Figure 2 is representative of 20 inclusions viewed by confocal microscopy and over 200 inclusions viewed by epifluorescence microscopy at 18 h post infection. Figure 3a is representative of 10 inclusions viewed by confocal microscopy and over 200 inclusions viewed by epifluorescence microscopy at 18 h post infection. Figure 3b is representative of 100/104 (96%) aberrant bodies induced by ampicillin treatment and viewed by confocal microscopy. All conditions were replicated technically twice and encompass at least three biological replicates of each experiment.

Phase and fluorescence microscopy of *E. coli, B. subtilis* and *S. pneumoniae* were performed with a Nikon 90i fluorescence microscope equipped with a Plan Apo ×100 1.40 Oil Ph3 DM objective and a Chroma 83700 triple filter cube. Images were captured using NIS software from Nikon and a Photometrics Cascade 1K cooled charge-coupled device camera, and were processed and analysed using ImageJ.

When a comparison was made, samples were treated the same and the same parameters were applied for collecting and post processing of the microscopy data. **Chlamydial plaque assays.** Plaque assays were adapted from a previously described protocol4. Briefly, confluent monolayers of L2 mouse fibroblast cells in 24-well plates were washed twice with pre-warmed DMEM and then infected with *C. trachomatis* at an MOI of 1. Plates were incubated on a rocker at 37°C with 5% CO2 for two hours after which time, infection medium was removed and cells were overlaid with low-melting point agarose medium (0.25%) containing DMEM (1×), FBS (10%), nonessential amino acids (1×), cycloheximide (200 ng ml⁻¹), and gentamicin (20 µg ml⁻¹). Overlay medium was supplemented as indicated with DCS (30 µg ml⁻¹) and varying concentrations of amino acids and their modified derivatives. At seven days post infection, an additional agarose overlay was added to each well. At 14 days post infection, plaque formation was visualized by staining cells for three hours with 0.5% neutral red.

31. Lambden, P. R., Picknett, M. A. & Clarke, I. N. The effect of penicillin on *Chlamydia trachomatis* DNA replication. *Microbiology* **152**, 2573–2578 (2006).
32. Xu, S., Battaglia, L., Bao, X. & Fan, H. Chloramphenicol acetyltransferase as a selection marker for chlamydial transformation. *BMC Res. Notes* **6**, 377 (2013).
33. Sambrook, J. F. & Russell, D. W. *Molecular Cloning: a Laboratory Manual* 3rd edn (Cold Spring Harbor Laboratory Press, 2001).
34. Sham, L. T., Barendt, S. M., Kopeczy, K. E. & Winkler, M. E. Essential PcsB putative peptidoglycan hydrolase interacts with the essential FtsXSpn cell division protein in *Streptococcus pneumoniae* D39. *Proc. Natl Acad. Sci. USA* **108**, E1061–E1069 (2011).
35. Litzenger, S. et al. Muropeptide rescue in *Bacillus subtilis* involves sequential hydrolysis by beta-N-acetylgalactosaminidase and N-acetylmuramyl-l-alanine amidase. *J. Bacteriol.* **192**, 3132–3143 (2010).
36. De Jonge, B. L., Chang, Y. S., Gage, D. & Tomasz, A. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. *J. Biol. Chem.* **267**, 11248–11254 (1992).
37. Turner, R. D., Hurd, A. F., Cadby, A., Hobbs, J. K. & Foster, S. J. Cell wall elongation mode in Gram-negative bacteria is determined by peptidoglycan architecture. *Nature Commun.* **4**, 1496 (2013).
38. Bannantine, J. P., Stamm, W. E., Suchland, R. J. & Rockey, D. D. *Chlamydia trachomatis* IncA is localized to the inclusion membrane and is recognized by antisera from infected humans and primates. *Infect. Immun.* **66**, 6017–6021 (1998).
Extended Data Figure 1 | Single D-amino acid probe EDA fails to label intracellular Chlamydia despite labelling intracellular Shigella flexneri.

a–c, Phase contrast and epifluorescence microscopy of Chlamydia-infected L2 cells 18 h post infection (a), Shigella flexneri strain 2457T two hour broth cultures (b) and Shigella-infected L2 cells three hours post infection (c). All were grown in the presence of 1 mM EDA. Subsequent tethering of the probe to a modified Alexa Fluor 488 (green) was achieved via click chemistry. Antibody to chlamydial inclusion protein A (IncA, red) was used to visualize chlamydial inclusions. Experiments were conducted in technical duplicates and biological triplicates, with between 4–5 fields examined (with ~3–10 inclusions viewed per field) per technical replicate.
Extended Data Figure 2 | D-enantiomer dipeptide probes do not affect bacterial growth in rich media, but differentially and specifically label PG of E. coli. a. Growth of wild-type E. coli and B. subtilis in the presence of experimental concentrations of EDA-DA or DA-EDA. A representative growth curve from two biological replicates, each with three technical replicates, is shown. b. Phase contrast and epifluorescence microscopy of E. coli grown with 0.5 mM alkyne containing EDA-DA, DA-EDA or as a positive control with EDA at five minutes and 60 min. These samples together with unlabelled controls were ‘clicked’ to Alexa Fluor 488 azide and imaged. When the alkyne is on the C terminus (DA-EDA), the labelling is not apparent. Signal from N-terminally tagged dipeptide (EDA-DA) is significantly higher, but still lower than EDA and the patterns of labelling at the earlier time points are different. This is probably due to periplasmic incorporation of D-amino acids (for example, EDA) by E. coli L,D-transpeptidases, which result in more efficient peripheral labelling in addition to labelling due to lipid II-dependent PG synthesis. Therefore, in bacteria that have active L,D-transpeptidases, the cytoplasmic PG labelling through dipeptide probes provides a better measure of lipid II-dependent PG synthesis than single D-amino acids. The experiment was conducted twice and images are representative of a minimum of five fields viewed per condition/time point per replicate. c. Comparison of the labelling in E. coli grown with 0.5 mM alkyne containing EDA-DA or the L-enantiomer control ethynyl-L-alanine-L-alanine (ELA-LA) for 45 min and clicked as above shows that the labelling is D-enantiomer-specific. Images are representative of a minimum of four fields viewed per replicate and the experiment was conducted twice.
a) Alexa Fluor® 488 Azide + Bacillus subtilis wt

5 min

Phase EDA

60 min

Phase EDA

Adjusted

5 min

Phase EDA-DA

60 min

Phase EDA-DA

Adjusted

5 min

Phase DA-EDA

60 min

Phase DA-EDA

Phase No label Control

b) Alexa Fluor® 488 DIBO Alkyne +

45 min

Phase EDA-DA

45 min

Phase ELA-LA

c) Bacillus subtilis wt

60 min

Phase ADA-DA 0.4 mM

60 min

Phase DA-ADA 1.6 mM

S. pneumoniae

60 min

Phase DA-ADA

60 min

Phase DA-ADA

60 min

Phase DA-ADA + 30 min chase

d) Bacillus subtilis ΔdacA

60 min

Phase DA-ADA 1.6 mM

60 min

Phase DA-ADA 1.6 mM

60 min

Phase DA-ADA

60 min

Phase DA-ADA + 30 min chase

e) Streptomyces venezuelae

Phase HADA

EDA-DA + Alexa Fluor 488

Overlay

LETTER

RESEARCH

©2014 Macmillan Publishers Limited. All rights reserved
Extended Data Figure 3 | Dipeptide probes differentially and specifically label PG of diverse Gram-positive bacteria allowing live-cell experiments.

a–e, Phase contrast and epifluorescence microscopy of *B. subtilis* (a–c), *Streptococcus pneumoniae* (d), and *Streptomyces venezuelae* (e). a, Five minute and 60 min aliquots were taken from wild-type *B. subtilis* grown with 0.5 mM alkyne containing EDA-DA, DA-EDA or as a positive control with EDA. These aliquots together with unlabelled controls were ‘clicked’ to Alexa Fluor 488 azide and imaged. When the alkyne is on the N terminus (EDA-DA), labelling is comparable to EDA. On the other hand, the labelling with carboxy-terminal tag (DA-EDA) is much fainter. b, *B. subtilis* grown with 0.5 mM alkyne containing EDA-DA or the L-enantiomer control ELA-LA for 45 min and clicked as above indicates that the labelling is D-enantiomer specific. The partial lysis of the cells visible in phase contrast is caused by 70% ethanol fixation. c, d, When live *B. subtilis* and *S. pneumoniae* labelled with azide containing ADA-DA and DA-ADA (0.4 mM and 1.6 mM for c and 0.5 mM for d) were clicked to Alexa Fluor 488 DIBO alkyne using a non-toxic procedure, the signals from N-terminally tagged dipeptide ADA-DA were much higher than the signal from DA-ADA labelled cells. c, Interestingly, the signal from DA-ADA can be elevated to the ADA-DA level, if the labelling is performed in a ΔlacA, D,D-carboxypeptidase-null mutant of *B. subtilis*. Since copper-free click-chemistry is not toxic to cells, a pulse-chase experiment was done, which shows the trapping of old PG at the poles of the cells (lower panel). e, When polarly growing *S. venezuelae* cells are grown with the blue fluorescent D-amino acid HADA (2 h, 0.5 mM) for several generations and briefly pulsed with EDA-DA (10 min, 0.5 mM) and clicked, the signal from EDA-DA complements the signal from HADA. This result shows that dipeptide probes label the cell wall at sites of new PG synthesis. Fluorescent images a–d were taken and processed in the same manner for comparison. In ‘Adjusted’ images, signal intensities were lowered for comparison of labelling patterns. All experiments were conducted in biological duplicates, and images are representative of 2–5 fields viewed per condition/time point/replicate.
Extended Data Figure 4 | EDA-DA labelling is specific to the PG of bacteria.

a, Alexa Fluor 488 Azide ‘clicked’ sacculi from *B. subtilis* and *E. coli* cells grown with 0.5 mM EDA-DA for several generations retained the alkyne label. The labelled cells were clicked before sacculi purification in the case of *B. subtilis* and after purification in the case of *E. coli*. Experiment was conducted in biological duplicates and images are representative of five fields viewed per replicate.

b, The EDA-DA signal retained on the isolated PG can be released by PG-digesting enzymes (~10 mg ml$^{-1}$ lysozyme + 200 μg ml$^{-1}$ mutanolysin). The kinetics of signal disappearance from the lysozyme treated sacculi is much faster than the kinetics of the photo-bleaching during the time-course, indicating that the loss of signal is due to hydrolytic activity of lysozyme. Three experimental replicates were performed.
Extended Data Figure 5 | Fluorescent labelling of intracellular *C. trachomatis* PG: maximum intensity projections of confocal z-stacks before and after deconvolution. a–p, Raw data used for generating Fig. 2, showing merged (a–d) and green (e–h) channels compared with the same maximum intensity projections from z-stacks that have undergone deconvolution, (i–l) and (m–p), respectively.
Extended Data Figure 6 | Fluorescence is specific to chlamydial infected cells in the presence of the dipeptide probe EDA-DA, and lysozyme treatment is capable of removing the label from fixed bacteria. 

a–c, Phase contrast and epifluorescence microscopy was conducted on uninfected L2 cells grown in the presence of 1 mM EDA-DA (a), 18 h C. trachomatis-infected cells in the presence of 1 mM EDA-DA (b), and 18 h C. trachomatis-infected cells grown in the absence of probe (c). Subsequent binding of the probe to a modified Alexa Fluor 488 (green) was achieved via a click chemistry reaction.

d, e, For lysozyme treatments, 18 h C. trachomatis-infected cells (fixed and labelled as described above) were suspended in either buffer (25 mM NaPO₄ pH 6.0, 0.5 mM MgCl₂) (d) or buffer and lysozyme (200 µg ml⁻¹) (e) for two hours. Cells were subsequently washed, blocked and counter-labelled with anti-MOMP, as described previously. Images are representative of between 3–5 fields examined (with 1–10 inclusions viewed per field) per technical replicate, each condition conducted in technical duplicates, and experiments represent a total of three biological replicates.
Extended Data Figure 7 | \(\beta\)-cycloserine (DCS) and ampicillin (AMP) influence labelling of *C. trachomatis* PG by dipeptide probes EDA-DA and DA-EDA. Phase contrast and epifluorescence microscopy of L2 cells infected with *C. trachomatis* 18 h post infection. a–c, Cells were grown in the presence of either EDA-DA or DA-EDA (1 mM) and were either untreated (a), or treated with 294 \(\mu\)M DCS (b) or 2.8 \(\mu\)M AMP (c). Subsequent binding of the probe to a modified Alexa Fluor 488 (green) was achieved via click chemistry. The image used for EDA-DA labelling in the absence of antibiotics is the same image from Extended Data Fig. 6b and experiments were all conducted in parallel on the same day. Images showing labelling by EDA-DA and DA-EDA in the presence or absence of DCS are representative of the vast majority of over 100 inclusions measured 18 h post-infection. Labelling by EDA-DA in the presence of ampicillin is representative of 97% (73/75) total aberrant bodies and labelling by DA-EDA in the presence of ampicillin is representative of 95% (73/77) total aberrant bodies, as viewed by epifluorescence microscopy. Experiments were conducted in technical duplicates and represent at least three biological replicates.
Extended Data Figure 8 | Punctate labelling of aberrant bodies due to enlarged bacteria encompassing multiple focal planes. a–i, Phase contrast (a) and epifluorescence microscopy (b–i) of an 18 h, EDA-DA labelled, ampicillin-induced aberrant body. Images were taken through sequential focal planes in order to show how the ring-like, PG structure is maintained in aberrant bodies and can appear punctate when viewed via an epifluorescence microscope. Images are representative of between 3–5 fields viewed per technical replicate, comprising over 20 independent biological replicates, and each experiment was conducted in technical duplicates.
Extended Data Figure 9 | EDA-DA labelling of *C. trachomatis* is apparent as early as eight hours post infection. a–c, L2 cells infected with *C. trachomatis* 6 (a), 8 (b) and 10 h (c) post infection grown in the presence of 1 mM EDA-DA. Time points examined covered 4, 6, 8, 10, 12, 18, 24 and 40 h infected cells. Subsequent binding of the probe to a modified Alexa Fluor 488 (green) was achieved via a click chemistry reaction. Antibody to chlamydial MOMP (red) was used to label chlamydial EBs and RBs. Experiments were conducted in technical duplicates, and the time course was conducted three independent times, with between 3–5 fields viewed per time point per technical replicate.
Extended Data Table 1 | Exogenous dipeptide analogues rescue growth of cells that have been depleted of DA-DA

| Condition | Condition | E. coli ΔaddA, ΔaddB | E. coli Δadd | B. subtilis Δadd |
|-----------|-----------|-----------------------|-------------|-----------------|
| MM$^1$    | -         | MM                    | -           | -               |
| MM + DA-DA (25 μM)$^2$ | ++ | MM + DCS$^3$ (20 μM for E. coli and 50 μM for B. subtilis) | *           | *               |
| MM + DA-DA (200 μM)$^2$ | ++++ | MM + DCS + DA-DA (50 μM for E. coli and 50 μM for B. subtilis) | ++          | +++            |
| MM + DA-EDA (100 μM)$^2$ | ++++ | MM + DCS + DA-ADA (50 μM for E. coli and 100 μM for B. subtilis) | +++         | +++            |
| MM + DA-ADA (50 μM)$^2$ | +++ | MM + DCS + DA-ADA (50 μM for E. coli and 400 μM for B. subtilis) | +++         | +++            |
| MM + ELA-LA (0.8 mM)$^2$ | - | -                     | -           | -               |
| MM + EDA-DA (0.8 mM)$^2$ | - | -                     | -           | -               |
| MM + ADA-DA (0.8 mM)$^2$ | - | -                     | -           | -               |
| MM + DA-DA (25 μM)$^2$ | - | -                     | -           | -               |
| MM + DA-DA (25 μM) + ELA-LA (0.8 mM)$^2$ | - | -                     | -           | -               |
| MM + DA-DA (25 μM) + EDA-DA (0.8 mM)$^2$ | +++ | | | | |
| MM + DA-DA (25 μM) + ADA-DA (0.8 mM)$^2$ | +++ | | | | |

$^1$ MM = minimal medium
$^2$ Initial inoculum was OD$_{600}$ = 0.025
$^3$ Initial inoculum was OD$_{600}$ = 0.0025

Growth rescue of a α-alanine-α-alanine ligase double mutant (ΔaddA ΔaddB) of E. coli was tested with varying concentrations of natural and modified dipeptides. Similarly, E. coli and B. subtilis cells were rendered auxotrophic for DA-DA by inhibiting their respective α-alanine-α-alanine ligases with δ-cycloserine (DCS). Data represent the average of at least two biological replicates conducted with technical duplicates. ++++, highest culture density achieved within a set; -, no bacterial growth; intermediate values, relative fractions of the highest culture density within a set.