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D-Amino Acid Chemical Reporters Reveal Peptidoglycan Dynamics of an Intracellular Pathogen

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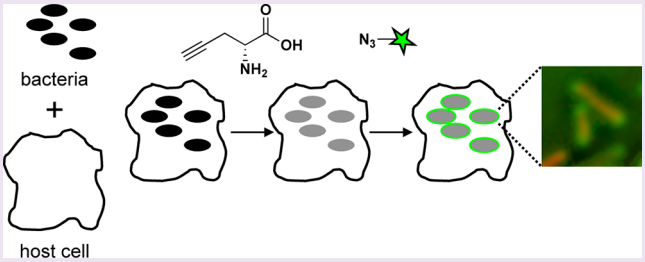
Supporting Information

ABSTRACT: Peptidoglycan (PG) is an essential component of the bacterial cell wall. Although experiments with organisms *in vitro* have yielded a wealth of information on PG synthesis and maturation, it is unclear how these studies translate to bacteria replicating within host cells. We report a chemical approach for probing PG *in vivo* via metabolic labeling and bioorthogonal chemistry. A wide variety of bacterial species incorporated azide and alkyne-functionalized D-alanine into their cell walls, which we visualized by covalent reaction with click chemistry probes. The D-alanine analogues were specifically incorporated into nascent PG of the intracellular pathogen *Listeria monocytogenes* both *in vitro* and during macrophage infection. Metabolic incorporation of D-alanine derivatives and click chemistry detection constitute a facile, modular platform that facilitates unprecedented spatial and temporal resolution of PG dynamics *in vivo*.



Peptidoglycan (PG) is a defining constituent of the bacterial cell wall. Comprising a network of glycan strands cross-linked by short peptides (Figure 1A), PG regulates the cell's physical properties as well as the passage of solutes.[1] Because PG is required for bacterial viability and absent from eukaryotic cells, it has proven an excellent antibiotic target. Despite its medical importance, however, there is little known about PG metabolism in the host environment.[2,3] Investigation of PG in this context has the potential to reveal novel routes of inhibition.

Efforts to track PG synthesis, editing, and turnover span several decades.[4,5] Biochemical methods provide molecular information on PG structure but at the expense of an intact cell wall.[6] More recently, techniques for imaging PG have been reported, including the use of fluorophore-tagged lectins[7] or cell wall-binding antibiotics,[8,9] metabolic labeling strategies based on the detection of free thiols[10] or radioactivity[11] and enzymatic methods utilizing fluorescent substrates.[12−14] Although they have revealed PG dynamics in the context of whole bacterial cells, these techniques suffer from limited species applicability, technical complexity, or low resolution.[15] Among these methods, fluorescent antibiotic conjugates stand out with regard to their relative simplicity and excellent spatial and temporal resolution. However, these attributes are offset by the size and inhibitory activity of the molecules, which in most cases restrict imaging to static portraits of nascent Gram-positive PG.[15] While the collective drawbacks of the current methods for investigating PG are not always significant for studies of model organisms growing in culture, they are a major impediment to probing PG of pathogenic bacteria during infection. Here, we report a strategy for labeling PG in live bacteria growing *in vivo* that exploits PG's unique D-amino acid constituents.

The promiscuity of PG metabolic enzymes toward both natural and unnatural D-amino acid substrates has been established.[16−18] We therefore sought to determine whether D-amino acids bearing bioorthogonal functional groups could be used for metabolic labeling. Azides and alkynes are small chemical reporters that are stable in and absent from biological systems.[19] They undergo selective reaction with each other and, in the case of the azide, with phosphines and strained cyclooctynes as well.[19] To evaluate the ability of unnatural D-alanine derivatives to access the cell wall, we first grew bacteria in media containing *R*-propargylglycine (compound **1**, Figure 1B, abbreviated alkDala) or *R*-2-amino-3-azidopropanoic acid (compound **2**, Figure 1B, abbreviated azDala) for one or more generations. Live or fixed cells were then reacted with a complementary fluorescent dye using strain-promoted cycloaddition or copper-catalyzed azide−alkyne cycloaddition (CuAAC),[19] respectively (Figure 1B). We observed by microscopy clear cell surface labeling of all species tested

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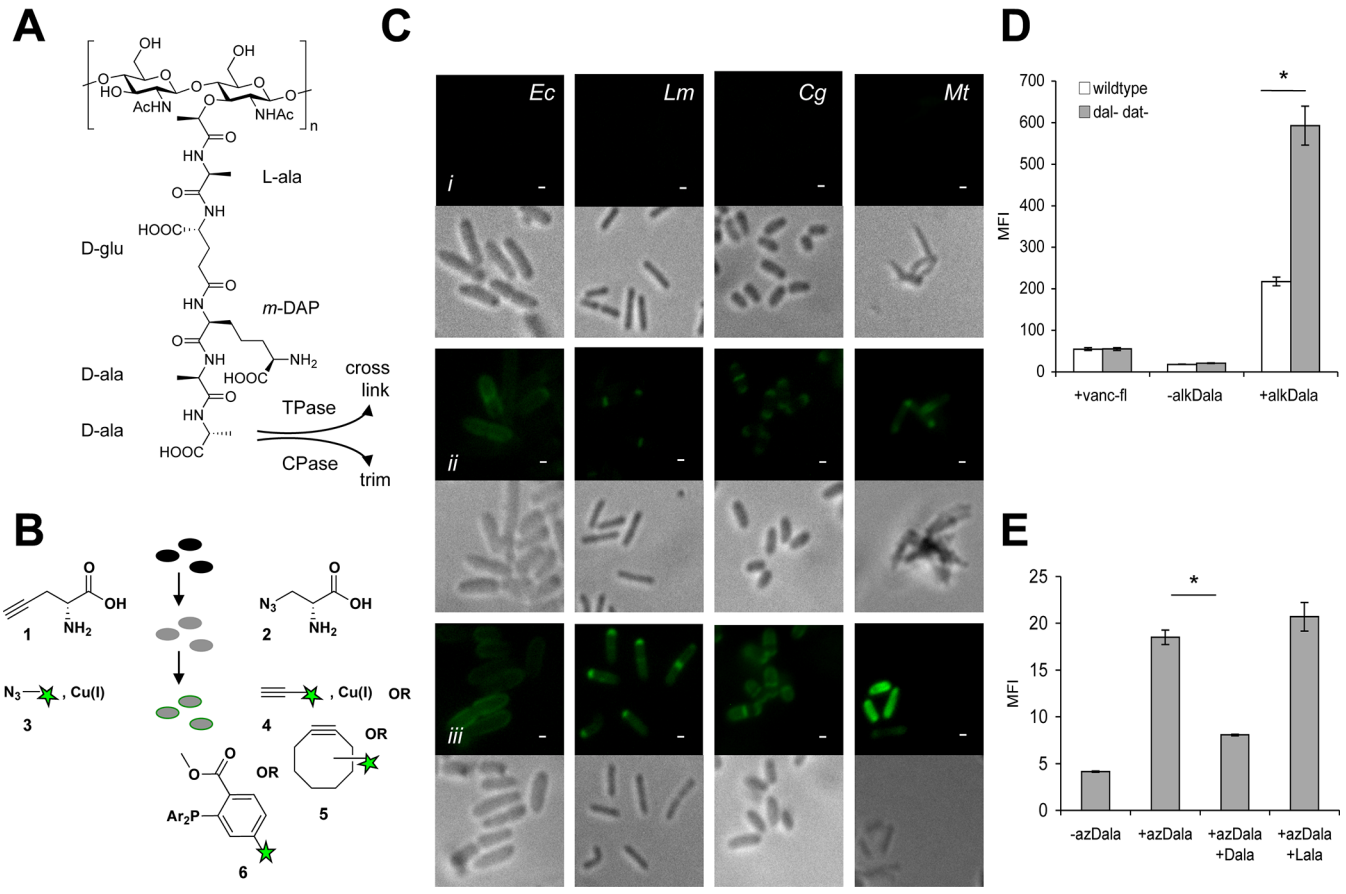

Figure 1. Incubation of bacteria in D-alanine analogues followed by reaction with click chemistry probes results in cell surface fluorescence. (A) Chemical structure of *Escherchia coli* and *Listeria monocytogenes* PG (*m*DAP = *meso*-diaminopimelic acid). Newly synthesized disaccharide pentapeptides are substrates for penicillin-binding protein (PBP) processing, including cross-linking by transpeptidases (TPases) and trimming by carboxypeptidases (CPases). (B) Schematic representation of *in vitro* metabolic labeling with D-alanine analogues (**1**, **2**) followed by click chemistry detection (**3**–**6**). *R*-Propargylglycine (**1**, alkDala), 2-amino-3-azidopropanoic acid (**2**, azDala), azide (**3**), and alkyne (**4**) conjugates for Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), cyclooctyne probe for strain-promoted cycloaddition (**5**), phosphine reagent for Staudinger ligation (**6**). The identity of the green star varies according to application, e.g., fluorophores or affinity handles. (C) From left to right: *E. coli* (*Ec*), *L. monocytogenes* (*Lm*), *Corynebacterium glutamicum* (*Cg*), and *Mycobacterium tuberculosis* (*Mt*). From top to bottom for each species, (i) D-alanine, (ii) short alkDala pulse, and (iii) long alkDala pulse. Details in Supplementary Table 1. Scale bars, 1 μm. The fluorescence intensity of the image of *Cg* labeled with a long pulse of alkDala was lowered to prevent apparent saturation. (D) The *dal*$^-$ *dat*$^-$ D-alanine auxotroph labels better than wildtype *L. monocytogenes* with alkDala, CuAAC. MFI, mean fluorescence intensity. (E) Two millimolar D- but not L-alanine competes with labeling by 5 mM azDala, strain-promoted cycloaddition in *dal*$^-$ *dat*$^-$ *L. monocytogenes*. The mutant was supplemented with an additional 1 mM D-alanine in all conditions for panel E. Error bars for panels D and E, ± SD; $^*P = 0.0002$ for panel D and $^*P = 2 \times 10^{-5}$ for panel E, two-tailed Student's *t* tests. Data are in triplicate and representative of four and two experiments, respectively.

(Figure 1C, compare top and bottom rows, and Supplementary Figure 1), including several Gram-positive bacteria, one Gram-negative, and *Mycobacterium tuberculosis*, a Gram-positive with an unusually complex cell wall. The concentrations of D-alanine analogue used for labeling did not inhibit bacterial growth (Supplementary Figure 2) and, with the exception of *M. tuberculosis*, labeled entire populations in a dose and time-dependent fashion (Figure 1C and Supplementary Figure 3).

We focused on the facultative intracellular pathogen *L. monocytogenes* for further characterization of D-alanine analogue metabolism. We compared alkDala labeling of wild-type *L. monocytogenes* to that of a D-alanine auxotroph (termed *dal*$^-$ *dat*$^-$).[20] The auxotroph, which is unable to synthesize natural D-alanine as an endogenous competitor of the synthetic substrate, labeled more strongly (Figure 1D). Similarly, the addition of exogenous D-alanine suppressed azDala labeling (Figure 1E). These results suggest that alkDala and azDala access the same metabolic pathways as natural D-alanine.

We hypothesized that there are three potential sites of D-alanine analogue incorporation: proteins, lipoteichoic acids (LTA), and PG. The first would likely require both racemization and a highly promiscuous aminoacyl tRNA synthetase.[21,22] To address this possibility directly, we reacted lysates from azDala-treated *L. monocytogenes* with alkyne-biotin and analyzed the products by immunoblot. No azide-labeled proteins were detected (Supplementary Figure 4). Although D-alanine incorporation systems for LTA are generally very specific,[23] we nonetheless addressed the second theoretical possibility that D-alanine analogues might label this biopolymer. We reacted LTA enriched from azDala-treated *L. monocytogenes* with phosphine-FLAG and probed by immunoblot as above. We were unable to detect azide-labeled species in these cell wall preparations (Supplementary Figure 5). Furthermore, a mutant that does not produce LTA[24] labeled with identical efficiency to wild-type bacteria (Supplementary Figure 5). These data suggest that the D-alanine derivatives do not incorporate into

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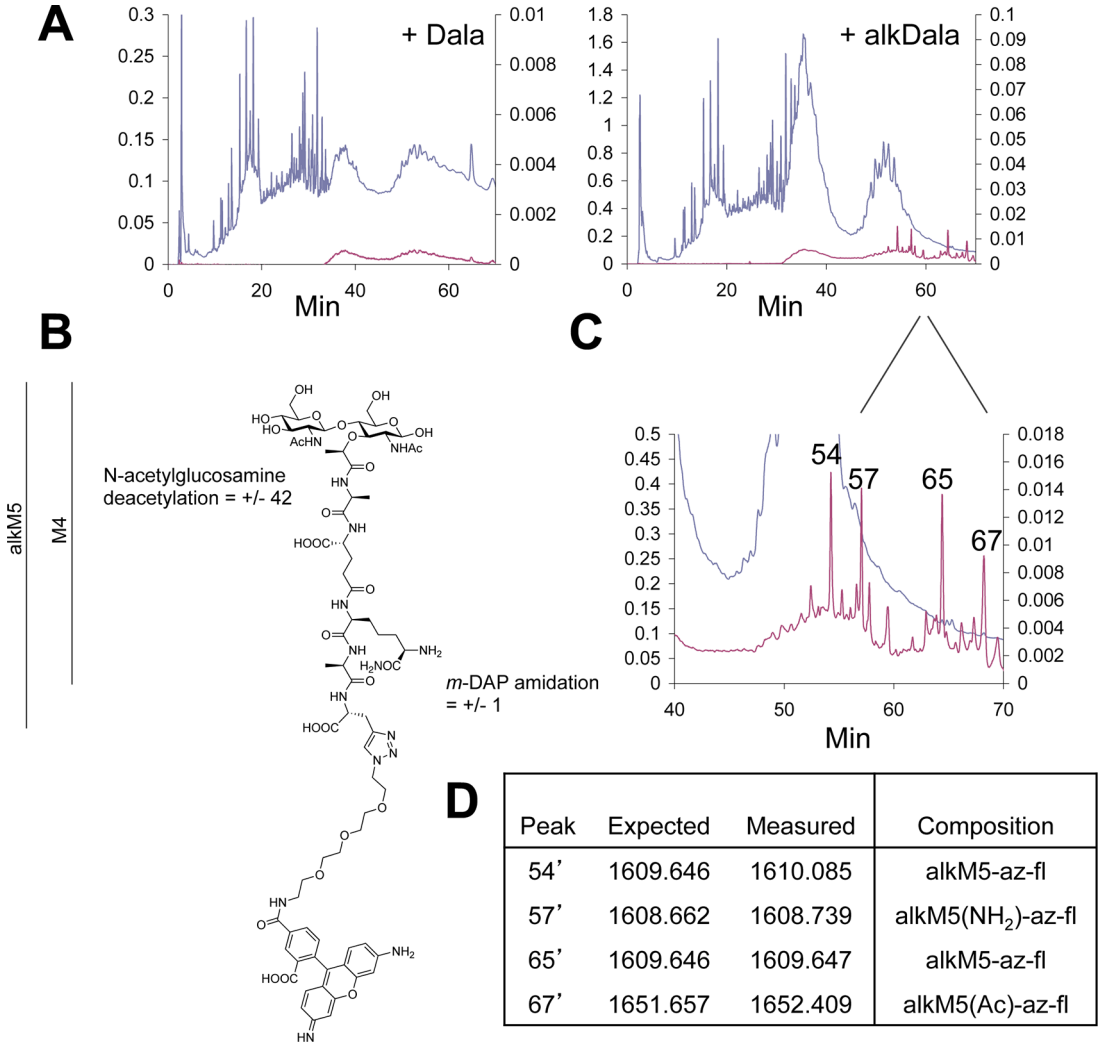

D

Peak	Expected	Measured	Composition
54'	1609.646	1610.085	alkM5-az-fl
57'	1608.662	1608.739	alkM5(NH_2)-az-fl
65'	1609.646	1609.647	alkM5-az-fl
67'	1651.657	1652.409	alkM5(Ac)-az-fl

Figure 2. AlkDala incorporates into *L. monocytogenes* PG. (A) HPLC chromatograms of nonreduced muropeptides from *L. monocytogenes* incubated in the presence of 5 mM D-alanine (left) or alkDala (right) then reacted with azido-fluor 488. Absorbance at 204 nm, blue, and at 500 nm, red, are shown. The trace for the alkDala-treated sample is enlarged in panel C. The most abundant peaks detected at 500 nm were collected and subjected to analysis by mass spectrometry (D) to identify the chemical structure of the alkDala-containing muropeptides conjugated to azido-fluor 488 (B).

proteins or LTA. We note that D-alanylation of the other *L. monocytogenes* teichoic acid polymer, wall teichoic acid (WTA), has not been observed.[25,26]

Having ruled out other potential sites of D-alanine derivative labeling, we next sought direct evidence of its incorporation into PG. PG comprises a repeating disaccharide to which is conjugated a short peptide, termed the stem peptide (Figure 1A). Although newly synthesized PG stem peptide usually terminates in D-alanine-D-alanine, diverse bacterial phyla produce and incorporate D-amino acids other than D-alanine into those positions.[17,18] The process is flexible; various natural and unnatural D-amino acids appear to incorporate, albeit at varying efficiencies.[16−18] To test whether D-alanine derivatives incorporate into PG, we first incubated *Escherichia coli* or *L. monocytogenes* with alkDala, reacted the cells with azido-fluor 488, then purified PG from the cells for further analysis. After digesting the PG with muramidase, we used HPLC to detect muropeptides by absorbance at either 204 nm (to visualize all species) or 500 nm (to identify fluorophore-containing fragments). Finally, we collected the most abundant peaks at 500 nm and used mass spectrometry to assign their chemical structures. This analysis showed that alkDala inserts into the fourth position of the stem peptide in *E. coli* PG (Supplementary Figure 6) and the fifth position in *L. monocytogenes* PG (Figure 2 and Supplementary Figure 7). We further analyzed PG samples from *E. coli* incubated in azDala alone and determined that the fraction of D-alanine that had been replaced with the synthetic analogue was roughly 50% of the tetrapeptide pool and 15% of the total muropeptide population. Importantly, even long periods of alkDala incubation did not appreciably change PG structure compared to D-alanine incubation performed in parallel (Supplementary Figure 8).

The positional selectivity of alkDala in PG implies a biosynthetic pathway of incorporation. There are two primary mechanisms for insertion of D-amino acids into PG: periplasmic editing of the mature polymer and cytosolic incorporation into PG precursors.[18,27] The first process is an L,D- or D,D-transpeptidation reaction that, respectively, results in a new D-amino acid at the fourth or fifth position of the PG stem peptide. The second process is catalyzed by intracellular ligases and results in a new D-amino acid only at the fifth position. The pentapeptide substrates that support D,D-transpeptidase incorporation of D-amino acids in other bacteria are short-lived in *L.*

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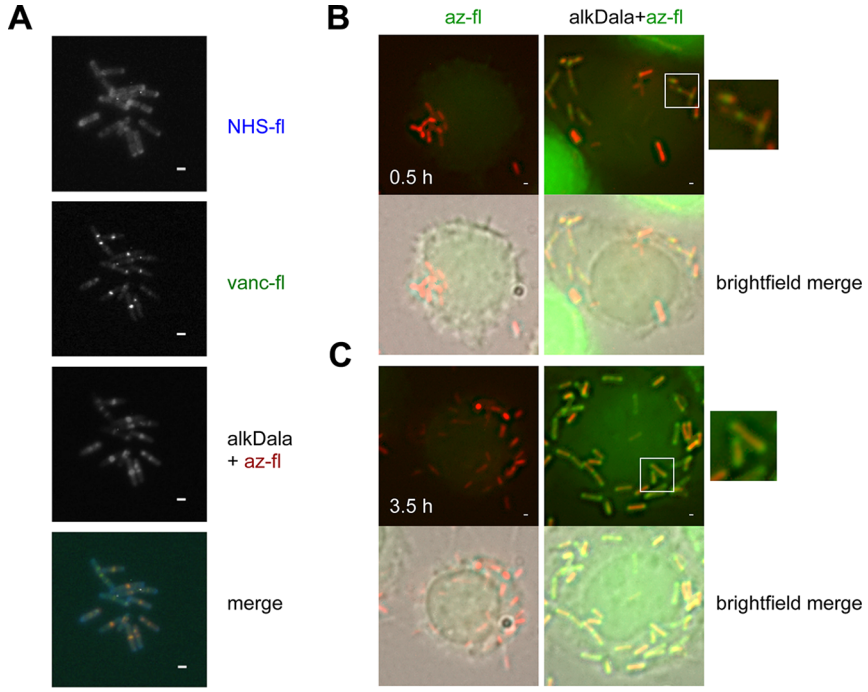

Figure 3. AlkDala labels newly synthesized *L. monocytogenes* PG *in vitro* and *in vivo*. (A) Fluorescent signals from vancomycin-BODIPY (vanc-fl) and alkDala + azido-fluor 545 (az-fl) colocalize. *L. monocytogenes* were incubated in Alexa Fluor 350 succiminidyl-ester (NHS-fl) to nonspecifically label cell wall proteins, then washed and resuspended in alkDala for 1 h and vanc-fl for the last 30 min. (B,C) AlkDala labels intracellular *L. monocytogenes*. J774 cells were infected with *L. monocytogenes* bearing red fluorescent protein (RFP) under the *actA* promoter.[38] Because this promoter is regulated by PrfA and induced upon escape from the phagosome, RFP expression correlates with entry into the cytosol.[38] Extracellular bacteria were washed away after 30 min, and the infected cells were incubated in fresh medium containing gentamicin. AlkDala was added for the remaining 3.5 h (C) or for the last 30 min only (B). Infected cells were fixed, permeabilized, and reacted with azido-fluor 488. Top rows, fluorescent images; bottom rows, fluorescent and brightfield merge. Scale bars, 1 μm.

monocytogenes because they are rapidly lost during PG maturation (Figure 1A[28−30]). Thus, the observation of alkDala in the fifth position suggests that D-alanine analogues incorporate into newly synthesized *L. monocytogenes* PG. Three additional lines of evidence support this notion. First, D-alanine analogue labeling was greatest at the peak of new cell wall production, in exponential phase growth (Supplementary Figure 9). Next, incubating *L. monocytogenes* in alkDala for one generation followed by reaction with azido-fluor 545 resulted in signal that colocalized with that of vancomycin-BODIPY, a marker of nascent PG (Figure 3A[8]). Finally, treatment of bacteria with fosfomycin, a drug that inhibits PG synthesis very early in the pathway, completely abrogated alkDala labeling, whereas treatment with penicillin and meropenem, antibiotics that target periplasmic editing enzymes, had a much weaker effect (Supplementary Figure 10).

The location of newly synthesized PG of bacteria grown *in vitro* is well-established.[1] Nearly all species deposit nascent PG at midcell during division. During elongation, however, bacteria such as *E. coli* and *L. monocytogenes* deposit new PG along the length of the cell, whereas organisms such as *C. glutamicum* and *M. tuberculosis* extend from the poles. We asked whether the spatial localization of labeling might identify it as new or mature PG. We compared short-term (approximately 10% of one doubling) and long-term (one doubling) labeling across four species (Figure 1C, compare middle and bottom rows) growing in asynchronous culture and found that the patterns displayed by the Gram-positive bacteria were consistent with nascent PG derived from both division and elongation. Since the observation of alkDala in the fourth position of *E. coli* PG points to L,D-transpeptidase-mediated incorporation,[18] occasional septal fluorescence at the earlier time point may suggest that these enzymes have activity on newly synthesized PG. Low signal on the lateral walls of *L. monocytogenes* likely reflects carboxypeptidase activity as alkDala labeling is much higher in the absence of PBP5, the major D,D-carboxypeptidase of *L. monocytogenes* (Supplementary Figure 11).[29,31]

L. monocytogenes naturally infects macrophages where it can escape from the phagosome and proliferate in the cytosol. The *dal*$^-$ *dat*$^-$ D-alanine auxotroph shows wild-type infectivity in cultured cells when D-alanine is added to the tissue culture medium.[20] This observation suggests that D-alanine and perhaps other D-amino acids are effectively taken up by macrophages at levels sufficient to support *L. monocytogenes* growth. Moreover, because eukaryotic cells do not generally produce D-amino acids, we reasoned that D-alanine analogues might selectively label bacteria inside of host cells. To test this hypothesis, we infected J774 macrophages with *L. monocytogenes*, removed extracellular bacteria, and treated the coculture with alkDala. Cells were incubated in alkDala for less than one *L. monocytogenes* generation, to label newer PG, or for several generations, to label both new and mature PG. Importantly, we did not detect toxicity to either the macrophages or intracellular bacteria under these conditions (Supplementary Figure 12). We adapted a chemical method used previously for labeling intracellular proteins in mammalian cells[32] to visualize PG by reaction with azido-fluor 488. We observed that alkyne-dependent signal varied according to alkDala incubation time: *L. monocytogenes* labeled more at the septa when incubated for a short pulse and at the septa and

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poles when labeled for a longer one (Figure 3B). The spatial distribution of fluorescence on intracellular *L. monocytogenes* approximated that observed on bacteria grown alone in broth (Figure 1C). As well, pulse chase experiments revealed that the temporal patterns of cell surface labeling were conserved *in vitro* and *in vivo*: labeled poles generally corresponded to older PG and labeled septa, to newer PG (Supplementary Figure 13).

Although we developed the method using *L. monocytogenes*, we note that the D-alanine analogues incorporate into several different bacteria and will promote investigation of diverse PG dynamics both *in vitro* and during infection. Indeed, while this paper was under review, a related study found that fluorophore-labeled D-alanine analogues are metabolically incorporated into PG of a wide variety of bacterial species.[33] This observation underscores that unnatural substrate tolerance in D-alanine metabolism can be exploited to monitor PG biosynthesis. We anticipate that D-alanine analogues containing chemical reporters will expand the scope of PG analysis even further. Judicious use of the ever-expanding, azide and alkyne-reactive probe kit should permit integration of biochemical, genetic, and cell biological data that historically have been collected and analyzed in isolation.

METHODS

***In Vitro* Labeling.** The origin and identity of bacterial strains are detailed in Supporting Information. Alexa Fluor 350 succiminidyl-ester (Invitrogen) was prepared exactly as described.[34] Vancomycin-BODIPY (Invitrogen) was used as a 1:1 mixture with unlabeled vancomycin at a final concentration of 1 μg/mL. D-Alanine and its derivatives were added directly to the culture medium at 0.5−10 mM from a 1 M stock. Analysis of PG by HPLC and mass spectrometry is included in the Supporting Information. Specific labeling conditions for Figure 1C and Supplementary Figure 1 are detailed in Supplementary Table 1. Strain-promoted cycloaddition was performed by incubating live bacteria in 10 μM DIFO-488 conjugate in PBS for 30 min.[35] CuAAC was performed on cell surfaces and lysates as described.[36] For cell surface labeling, bacteria were washed in PBS, fixed with 2% formaldehyde, and washed again in PBS prior to reaction. Bacteria were resuspended in a reaction mixture of half the volume of the original culture. This mixture contained 1 mM $CuSO_4$, 128 μM TBTA, 1.2 mM freshly prepared sodium ascorbate, and 20 μM of azide or alkyne conjugate (Click Chemistry Tools or Life Technologies) in PBS. The reaction components were added in the exact order listed, with sodium ascorbate and azide or alkyne-fluorophores immediately before incubation. CuAAC reactions were performed at RT for 30−60 min. Species-specific details for labeling are provided in the Methods and Supplementary Table 1 of the Supporting Information. The Staudinger ligation was performed by incubating cell lysates in 500 μM phosphine-FLAG[37] overnight at 37 °C.

Imaging Analysis. Details on microscopy are provided in the Supporting Information. For multicolor images (Figure 3), fluorescence intensities were adjusted in SLIDEBOOK software (Intelligent Imaging Innovations) to images of both untreated, nonfluorescent control cells as well as controls labeled with a single reagent or fluorophore. Contrast was increased for the greyscale images in ImageJ. Images stacks for Figure 3B were made into a *z*-projection image using the average setting and deconvolved in ImageJ using PSF generator and iterative deconvolver plugins developed by OptiNav, Inc. Separate channel images were merged together to form an RGB composite. For single-color images (Figure 1C), fluorescence intensities were normalized to controls lacking alkDala or azDala in the SLIDEBOOK software.

***In Vivo* D-Alanine Derivative Labeling.** *L. monocytogenes* were grown overnight at 30 °C without shaking. The next day, the bacteria were washed in PBS then added to J774 cells growing in chamber slides at a multiplicity of infection of 5. After 30 min, the coculture was washed in PBS and incubated in fresh medium. Gentamicin was added after an additional 30 min. Ten millimolar alkDala or azDala were then added at the stated time points. After 4 h, cells were washed in PBS, then fixed immediately at −20 °C in precooled, 70% ethanol for 10 min. Fixed cells were rinsed in PBS, permeabilized with 1% Triton-X, then washed 3 × 5 min in PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton-X (PBSTB). After rinsing in PBS, the CuAAC reaction was performed similarly to the *in vitro* labeling above, except that the reaction mixture contained PBS with 0.01% BSA and 0.1% Triton-X instead of PBS alone. Cells were rinsed in PBS and washed 3 × 5 min in PBSTB after azide-fluorophore reaction or 9 × 5 min following alkyne-fluorophore reaction. After a final rinse in PBS, the slides were mounted in Vectashield (Vector Laboratories) for imaging.

ASSOCIATED CONTENT

Supporting Information

Complete Methods section and additional figures and tables. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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