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

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METHODS

Bacterial strains, media and reagents. *Escherchia coli* K12 MG1655, *Bacillus subtilis* 168, *Streptomyces coelicolor* A3(2) M145 and *Corynebacterium glutamicum* 534 were obtained from American Type Culture Collection. *Mycobacterium tuberculosis* H37Rv and *Listeria monocytogenes* 10403S are lab stocks from the Bertozzi and Portnoy laboratories, respectively. Bacteria were labeled in the exponential phase of growth.

Construction and validation of the *L. monocytogenes* mutants *dal*⁻ *dat*⁻, Δ*lmo2555* and *prfA** (*prfA* G145S, NF-L1177), wildtype 10403S expressing *actA*-RFP (PL512) and wildtype and *lmo2754::tn* EGD-e were previously described.(*1-5*) All *L. monocytogenes* strains were grown shaking in Brain Heart Infusion (BHI) medium at 37 °C unless otherwise noted. The *dal*⁻ *dat*⁻ D-alanine auxotroph was supplemented with 1 mM D-alanine, washed and resuspended in medium without D-alanine for the experiment in Figure 1D. The strain was supplemented with 1 mM D-alanine to obtain data in Figure 1E and Supplementary Figure 5. MICs were calculated for wildtype and *prfA* L. monocytogenes* as the minimum amount of antibiotic that prevented visible growth in a 96-well plate.

M. tuberculosis were grown in 7H9 medium to mid-log phase and centrifuged at RT at 150 x g for 5 min to remove clumps. Bacteria were aliquoted to inkwells for labeling with D-alanine derivative. After washing once in PBS containing 0.5% BSA and 0.1% tween-20 (PBSTB) *M. tuberculosis* were fixed for 10 min in 2% formaldehyde at RT then washed in PBSTB. The CuAAC reaction was performed in PBSTB after 10 passages through a 26 G needle. After washing in PBSTB, bacteria were fixed again for 1 h in 4% formaldehyde at RT prior to removal from the BSL-3 facility.

For in vitro pulse chase experiments, *L. monocytogenes* growing in BHI medium were incubated in D-alanine or alkDala for 40 min, washed once in BHI medium, then incubated in BHI containing D-alanine or azDala for a final 5 min. Bacteria were fixed in 2% formaldehyde and reacted with Alkyne-Fluor 488 (Click Chemistry Tools) for 45 min to detect azDala then washed in PBS and reacted with Azide-Fluor 545 (Click Chemistry Tools) for 45 min to detect alkDala. For in vivo pulse chase experiments, *L. monocytogenes*-infected J774 cells were incubated in D-alanine or alkDala for 60 min, washed in DMEM medium for 5 min, then incubated in DMEM containing D-alanine or azDala for a final 20 min. The coculture was fixed at -20 °C in 70% ethanol then reacted in two rounds of CuAAC as above.

Toxicity assays. J774 cells were incubated in 10 mM D-alanine, alkDala or azDala for 4 h and viability was assessed by ATP quantitation (CellTiter-Glo, Promega).

To assess bacterial viability in vivo, J774 cells were infected with *L. monocytogenes* at a multiplicity of infection of 5. After 30 min, the coculture was rinsed in PBS then incubated in fresh DMEM medium containing 10 mM D-alanine, alkDala or azDala for 3.5 h. Gentamicin was added 30 min after addition of D-alanine derivatives. Cells were rinsed in PBS then lysed in PBS + 0.2% Triton-X for 5 min at 4 °C. Serial dilutions of the lysate were spotted on LB plates and incubated at 37 °C overnight prior to enumeration.

To test for strain-promoted cycloaddition toxicity, *C. glutamicum* was treated with 5 mM D-alanine or azDala for 90 min then washed and incubated in PBS containing 10 μM DIFO-488 at RT. After 30 min, serial dilutions of bacteria were spotted on LB plates and incubated at 30 °C overnight prior to enumeration.

Microscopy. Labeled bacteria were imaged live or after fixation. After washing in PBS, the cells were either resuspended in PBS or air dried and mounted in Vectashield (Vector Labs) and imaged on a glass coverslip using a Zeiss Axiovert 200M inverted microscope equipped with a 100x/1.30 Plan-Neofluar oil immersion lens. Image stacks were acquired using a CoolSNAP HQ charged-coupled device camera (Roper Scientific).

Flow cytometry. Triplicate samples of labeled bacteria were washed in PBS and resuspended to a final volume of 400 μL. Flow cytometry was performed on a BD Biosciences FACSCalibur instrument. Between 50,000-100,000 cells were analyzed for each sample and the mean fluorescence intensities for ungated samples were plotted in arbitrary units.

High performance liquid chromatography and mass spectrometry. *E. coli* and *L. monocytogenes* were grown overnight in 150 or 50 mL of medium, respectively, containing 5 mM D-alanine or D-alanine analog. Bacteria were washed in PBS then reacted with reacted with azido-fluor 488 for 1 h. PG from labeled cells was purified by the boiling SDS extraction method and muramidase digestion treatment (Cellosyl) as previously described.(*6*) Solubilized muropeptides were then either directly injected into the HPLC system (non-reduced samples) or subjected to sodium borohydride reduction as described previously.(*6*) Muropeptides were analyzed using a binary-pump Waters HPLC system (Waters Corporation, Milford, USA) fitted with a reverse phase RP18 Aeris peptide column (250 x 4.6 mm; 3.6 μm particle size) (Phenomenex, USA) and a dual wavelength absorbance detector. Elution conditions were: flow rate 1 mL/min; temperature 35 ºC; 3 min isocratic elution in 50 mM sodium phosphate, pH 4.35 followed by a 57 min linear gradient to 75 mM, sodium phosphate, pH 4.95 in 15% (v/v) methanol

[or 90 mM sodium phosphate, pH 5.2 in 30% (v/v) methanol when azido-fluor 488 was used], and 10 min isocratic elution under the gradient final conditions. Elution was monitored setting one channel to 204 nm and the second to a wavelength appropriate for detection of corresponding muropeptide derivatives. Muropeptides of interest were collected following HPLC separation, vacuum dried, and subjected to MALDI-mass spectrometry as described.(*6*)

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Supplementary Table 1. In vitro labeling conditions for Figure 1C and Supplementary Figure 1.

Bacterial species	D-alanine analog	Fluorescent probe
S. coelicolor (Sc)	azDala (10 mM, 4.5 h)	DIFO-488[a] (10 μM, 30 min)

C. glutamicum (Cg)	azDala (5 mM, 10 or 90 min)	DIFO-488[a] (10 μM, 30 min)
L. monocytogenes (Lm)	alkDala (5 mM, 5 or 40 min)	Azido-Fluor 488[b] (20 μM, 30 min)
M. tuberculosis (Mt)	alkDala (5 mM, 1 or 26 h)	Azido-Fluor 488[b] (20 μM, 60 min)
E. coli (Ec)	alkDala (5 mM, 2 or 20 min)	Alexa Fluor 488 azide[b] (20 μM, 30 min)
B. subtilis (Bs)	alkDala (5 mM, 90 min)	Azido-Fluor 488[b] (20 μM, 60 min)

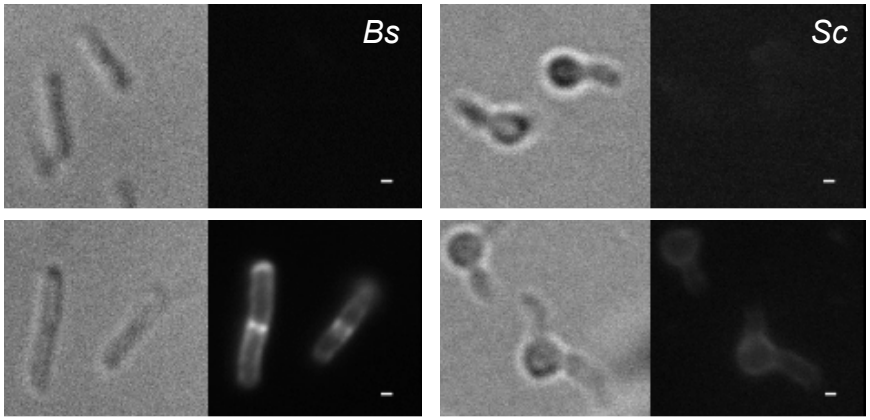
[a](*8*)We obtained the best results for the strain-promoted cycloaddition reaction with this cyclooctyne-fluorophore combination. Of note, we were unable to obtain signal over background in *E. coli* using this reagent. The reaction was performed on live cells and did not result in any measurable toxicity (Supplementary Figure 2).

[b]CuAAC reaction with Azido-Fluor 488 (Click Chemistry Tools), which contains a PEG spacer, resulted in the best labeling for Gram-positive bacteria. In contrast, Alexa Fluor 488 azide (Life Technologies) was the superior choice for *E. coli*. The reaction was performed on fixed cells to avoid potential complications from copper toxicity.

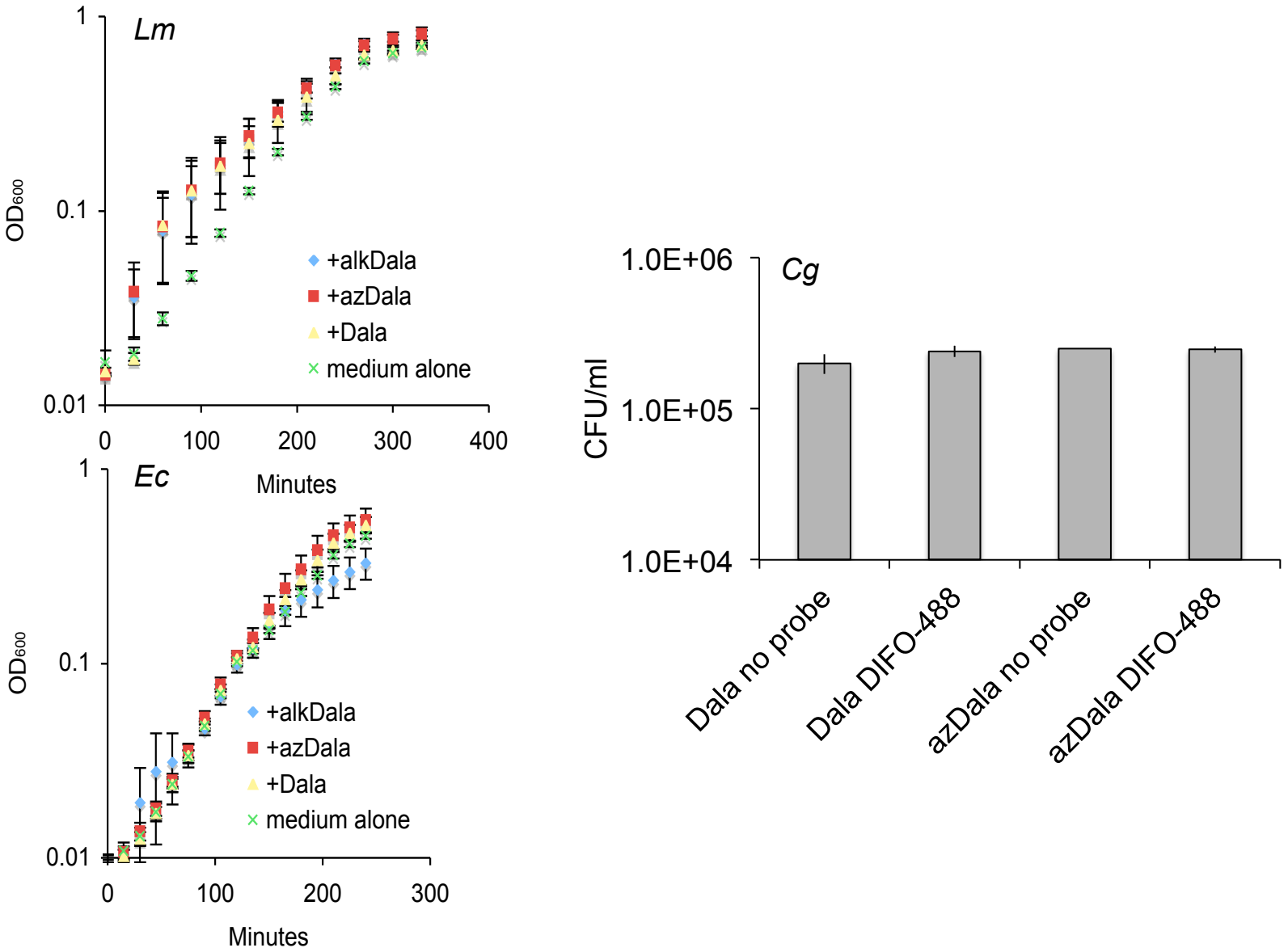
Supplementary Table 2. Minimal inhibitory concentrations (μg/ml) for wildtype and *prfA* L. monocytogenes.*

	wildtype	***prfA****
penicillin G	0.25	0.25
meropenem	0.5	0.5
D-cycloserine	12	96
fosfomycin	resistant[a]	0.75

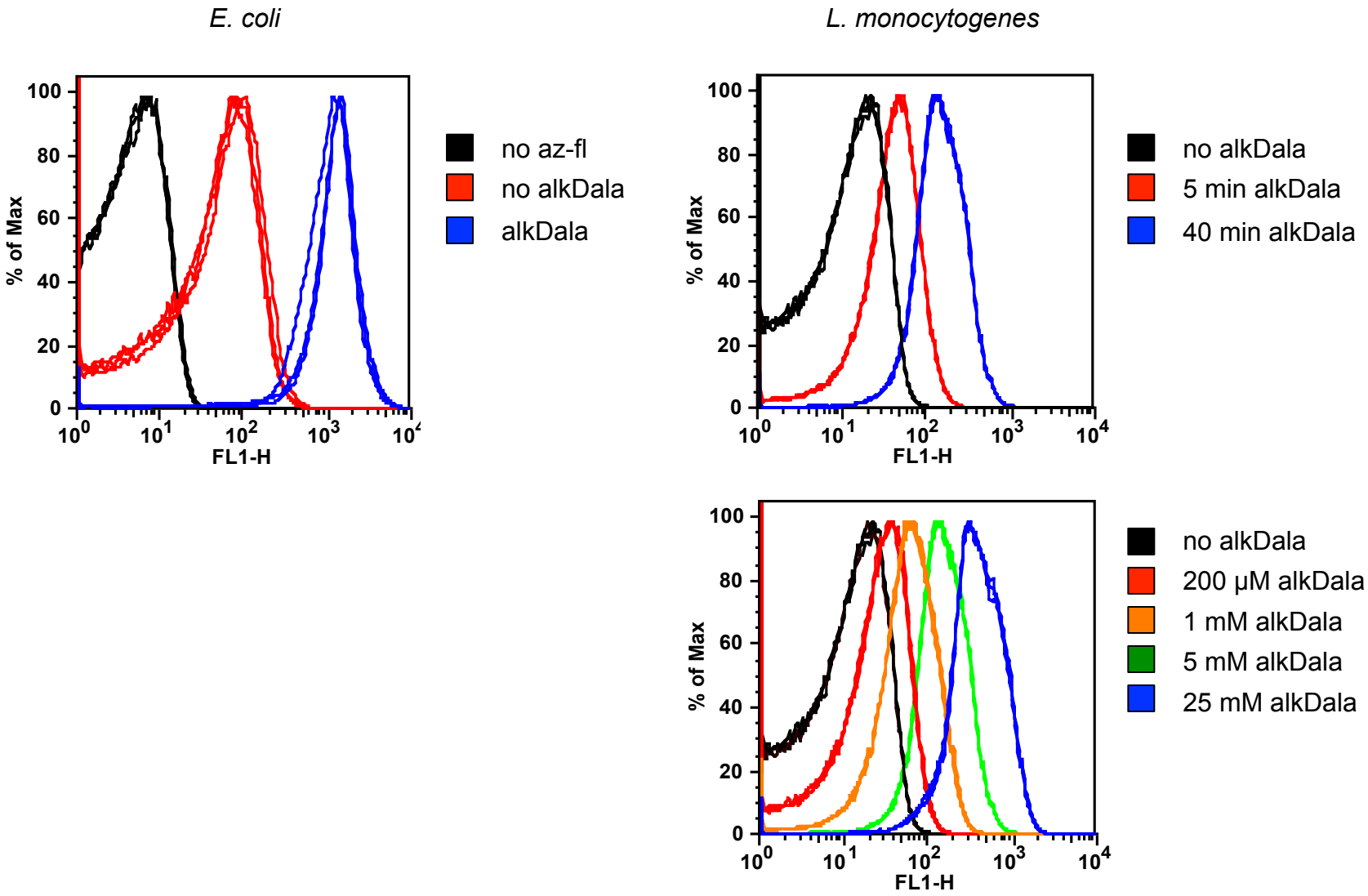
[a]As described in Supplementary Figure 10



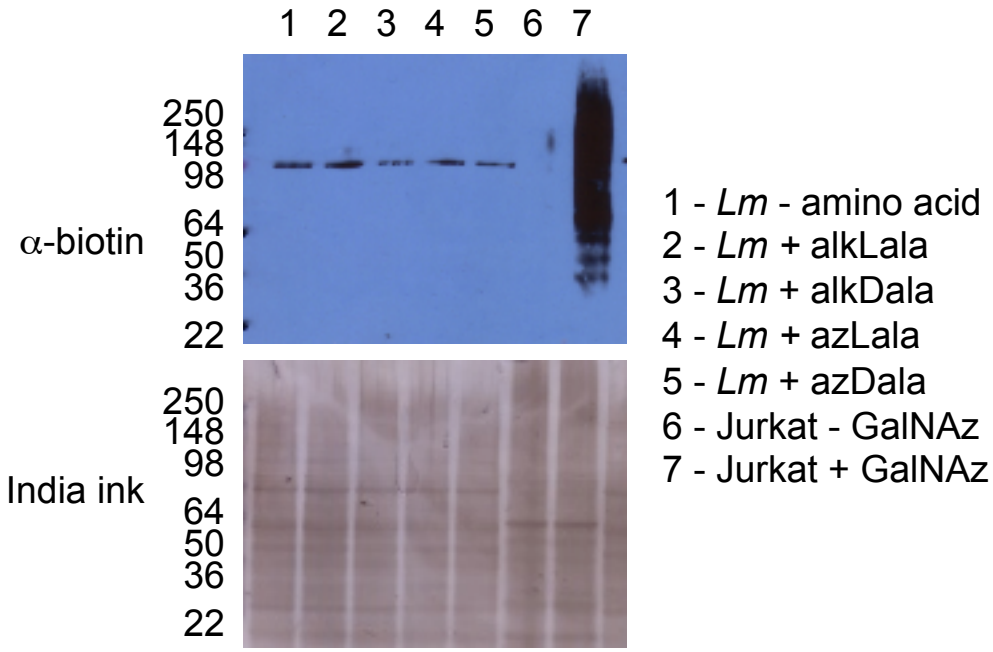
Supplementary Figure 1. Incubation of *Bacillus subtilis* and *Streptomyces coelicolor* in D-alanine analogs followed by reaction with click chemistry probes results in cell surface fluorescence. Left, *B. subtilis (Bs)* and right, *S. coelicolor (Sc),* in the presence (bottom) or absence (top) of D-alanine derivative. Labeling conditions in Supplementary Table 1. Scale bars, 1 μm.



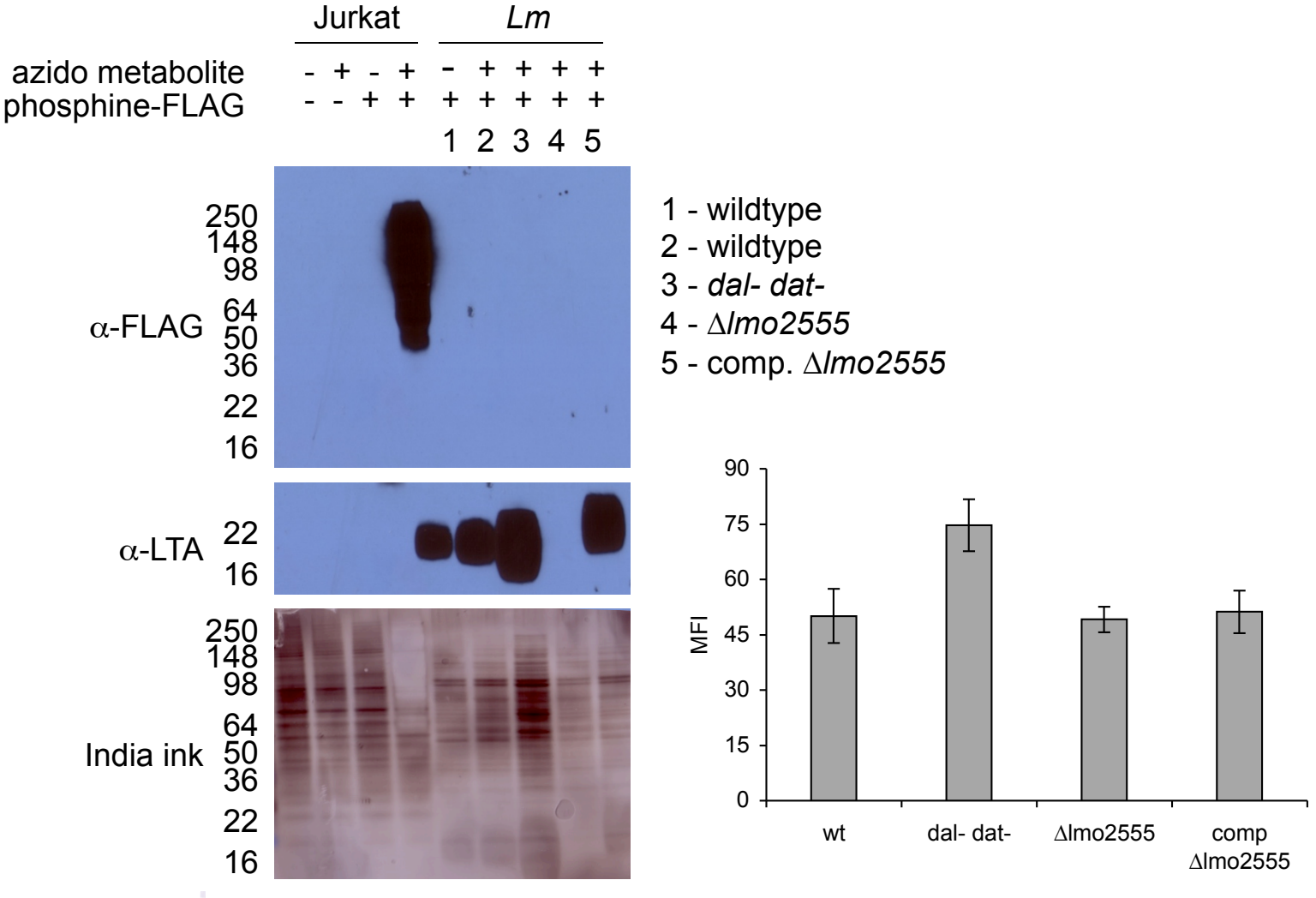
Supplementary Figure 2. Neither D-alanine derivatives nor the strain-promoted cycloaddition reaction impair bacterial growth. Overnight cultures of *L. monocytogenes* (*Lm,* top left) and *E. coli* (*Ec*, bottom left) were diluted 1:25 and 1:100 in BHI and LB growth medium, respectively, in the presence or absence of 5 mM D-alanine analog. Bacterial were grown in 96-well plates and absorbance at 600 nm was recorded. Right, colony forming units (CFU) from *C. glutamicum* (*Cg*) grown in the presence or absence of azDala for 90 min then washed and incubated in DIFO-488. Error bars, +/- s.d. Data are in triplicate and representative of two to three independent experiments.



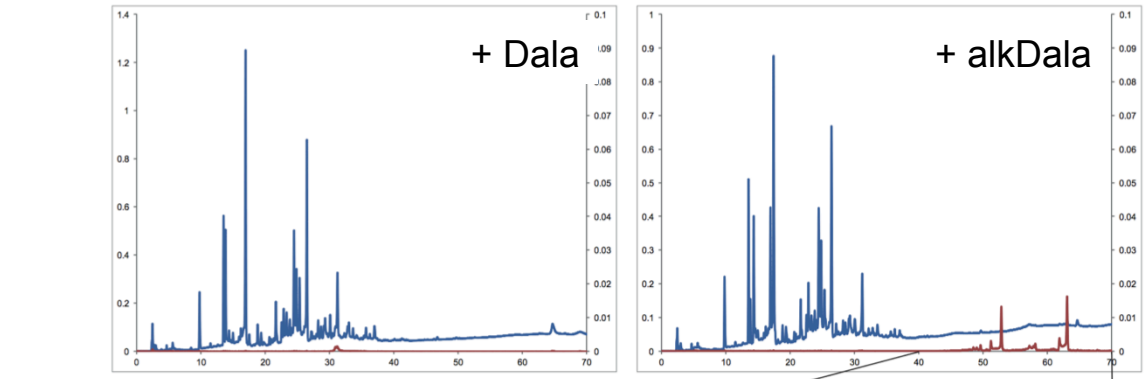
Supplementary Figure 3. alkDala labels the entire population of *E. coli* or *L. monocytogenes*. Bacteria were incubated in alkDala, reacted with azido-fluor 488 and subjected to flow cytometry. Left, *E. coli*. Top right, *L. monocytogenes* incubated in 5 mM alkDala for 5 or 40 minutes. Bottom right, *L. monocytogenes* were incubated in varying concentrations of alkDala for 40 min. Histograms of fluorescence intensity (FL1) for triplicate samples.

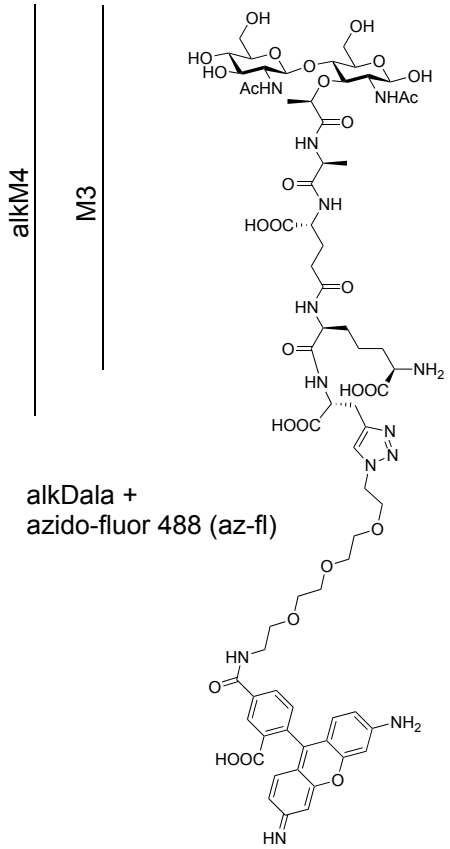


Supplementary Figure 4. azDala does not label *L. monocytogenes* proteins. Lysates from *L. monocytogenes* incubated in 5 mM alanine derivatives were reacted with alkyne-biotin and immunoblotted with anti-biotin. Jurkat cell lysate containing N-acetylgalactosamine (GalNAz)-labeled glycoproteins is a control for the CuAAC reaction.



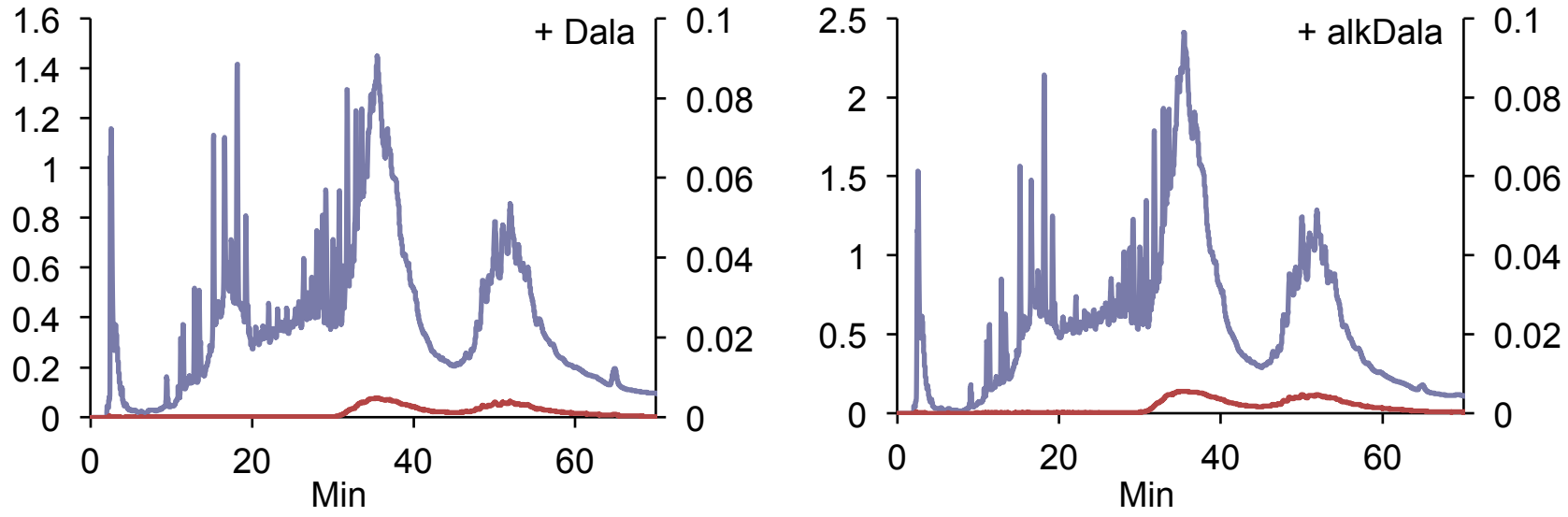
Supplementary Figure 5. azDala does not label *L. monocytogenes* lipoteichoic acids (LTA). LTA were enriched from *L. monocytogenes* grown in 5 mM azDala as described.(*3*) Preparations containing LTA were reacted overnight with phosphine-FLAG(*7*) and immunoblotted with anti-FLAG or anti-LTA (Clone 55, Hycult). Jurkat cell lysate containing N-acetylgalactosamine (GalNAz)-labeled glycoproteins is a control for the Staudinger ligation. Δ*lmo2555* is a mutant deficient in LTA synthesis.(*3*) Comp, complemented strain. MFI, mean fluorescence intensity. Error bars, +/- s.d. Data are in triplicate and representative of two independent experiments.



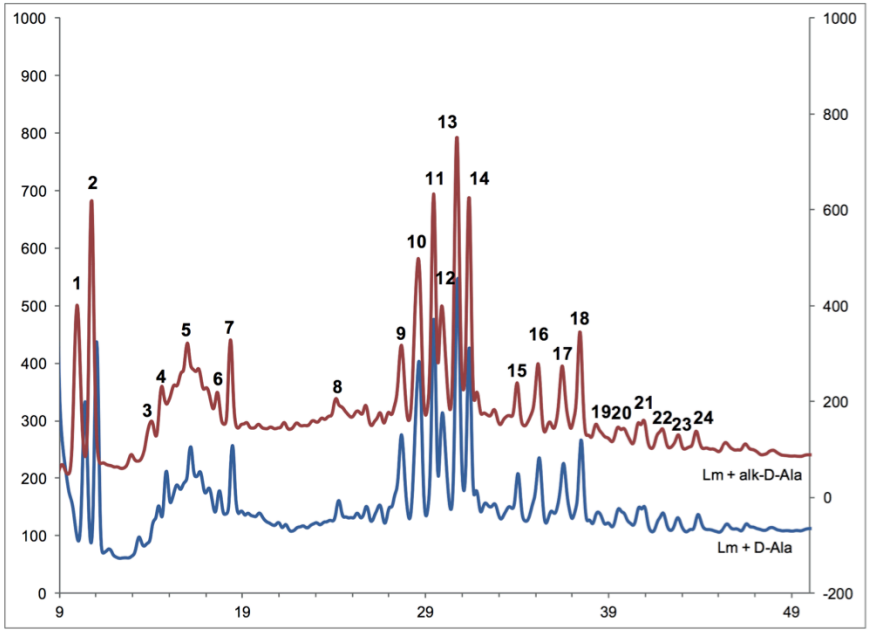


Peak	Expected	Measured	Composition
53'	1538.609	1538.601	alkM4-az-fl
63'	1538.609	1538.416	alkM4-az-fl

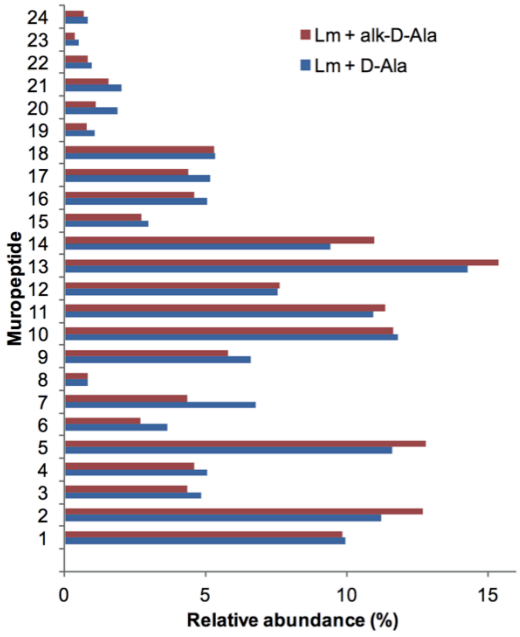
Supplementary Figure 6. AlkDala incorporates into *E. coli* PG. HPLC chromatograms of non-reduced muropeptides from *E. coli* incubated in the presence of 5 mM D-alanine or alkDala then reacted with azido-fluor 488. Absorbance at 204 nm, blue, and at 500 nm, red. The most abundant peaks detected at 500 nm were collected and subjected to analysis by mass spectrometry.



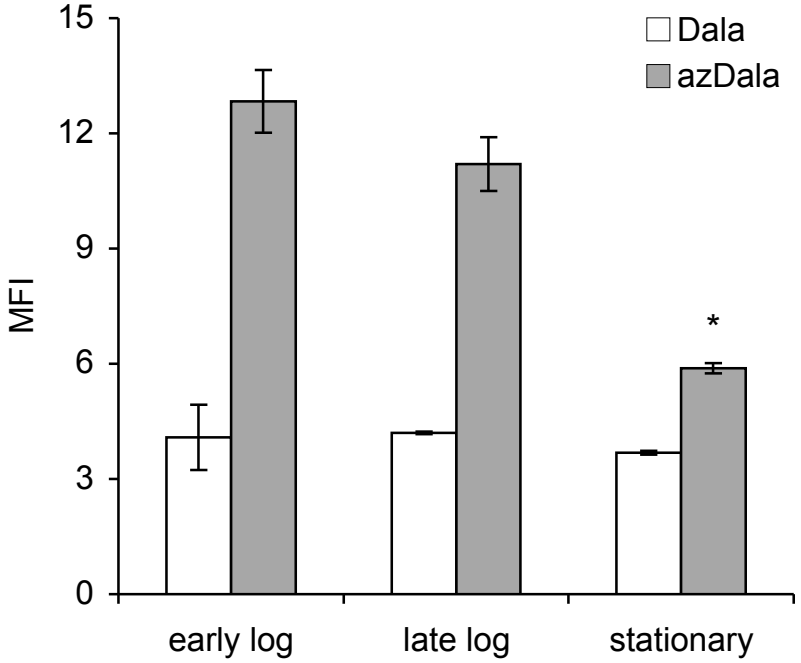
Supplementary Figure 7. HPLC chromatograms of non-reduced muropeptides from *L. monocytogenes* in the absence of azido-fluor 488. Bacteria were incubated in either 5 mM D-alanine or alkDala. Absorbance at 204 nm, blue, and at 500 nm, red.



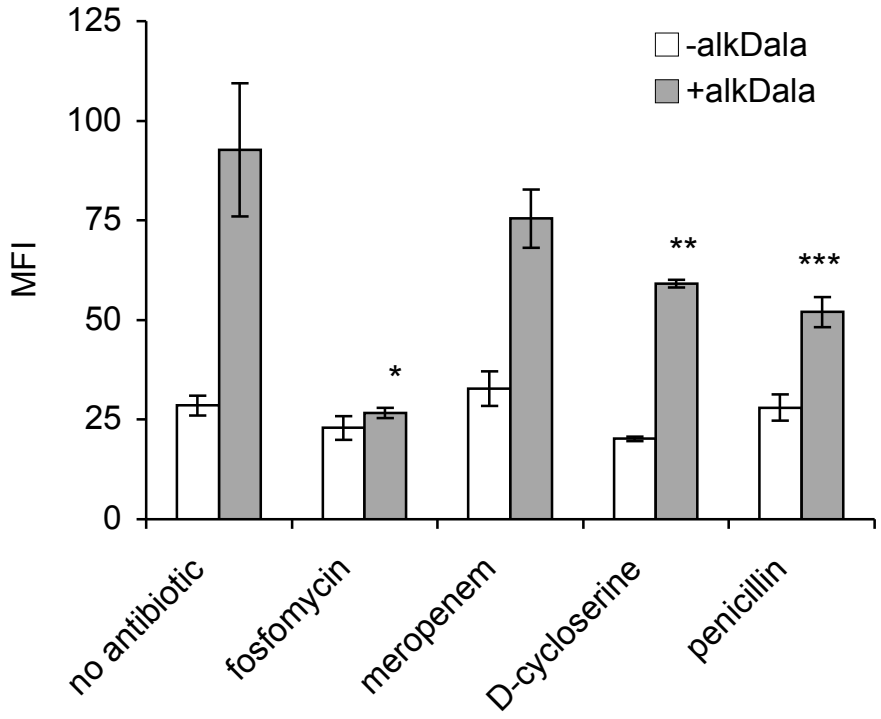
MUROPEPTIDE	Lm + D-Ala M.F. (%)	Lm + alk-D-Ala M.F. (%)
1	9.94	9.86
2	11.22	12.70
3	4.84	4.36
4	5.04	4.60
5	11.62	12.83
6	3.66	2.68
7	6.77	4.36
8	0.83	0.83
9	6.62	5.81
10	11.82	11.66
11	10.93	11.36
12	7.56	7.62
13	14.30	15.38
14	9.42	10.97
15	2.99	2.74
16	5.05	4.61
17	5.14	4.37
18	5.35	5.32
19	1.06	0.79
20	1.88	1.09
21	2.01	1.55
22	0.97	0.83
23	0.49	0.37
24	0.83	0.70



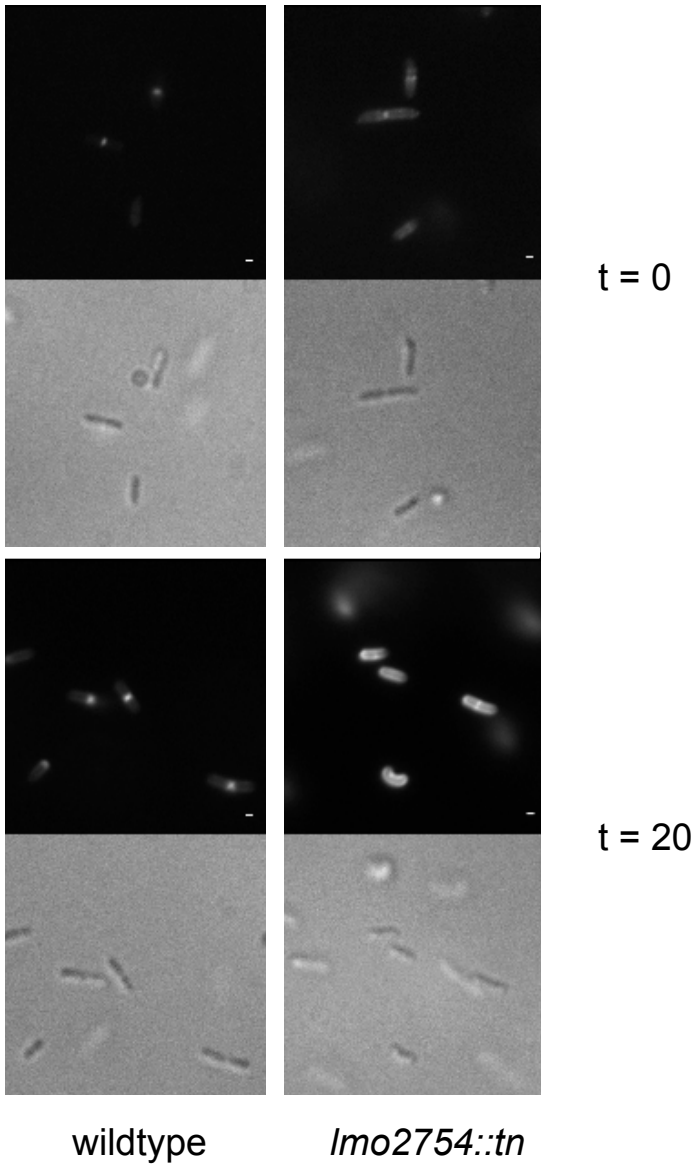
Supplementary Figure 8. AlkDala incubation does not cause major alterations to the composition of *L. monocytogenes* PG. HPLC chromatograms of reduced muropeptides from *L. monocytogenes* grown overnight in the presence of 5 mM D-alanine or alkDala. Blue, PG from bacteria treated with D-alanine and red, PG from bacteria treated with alkDala.



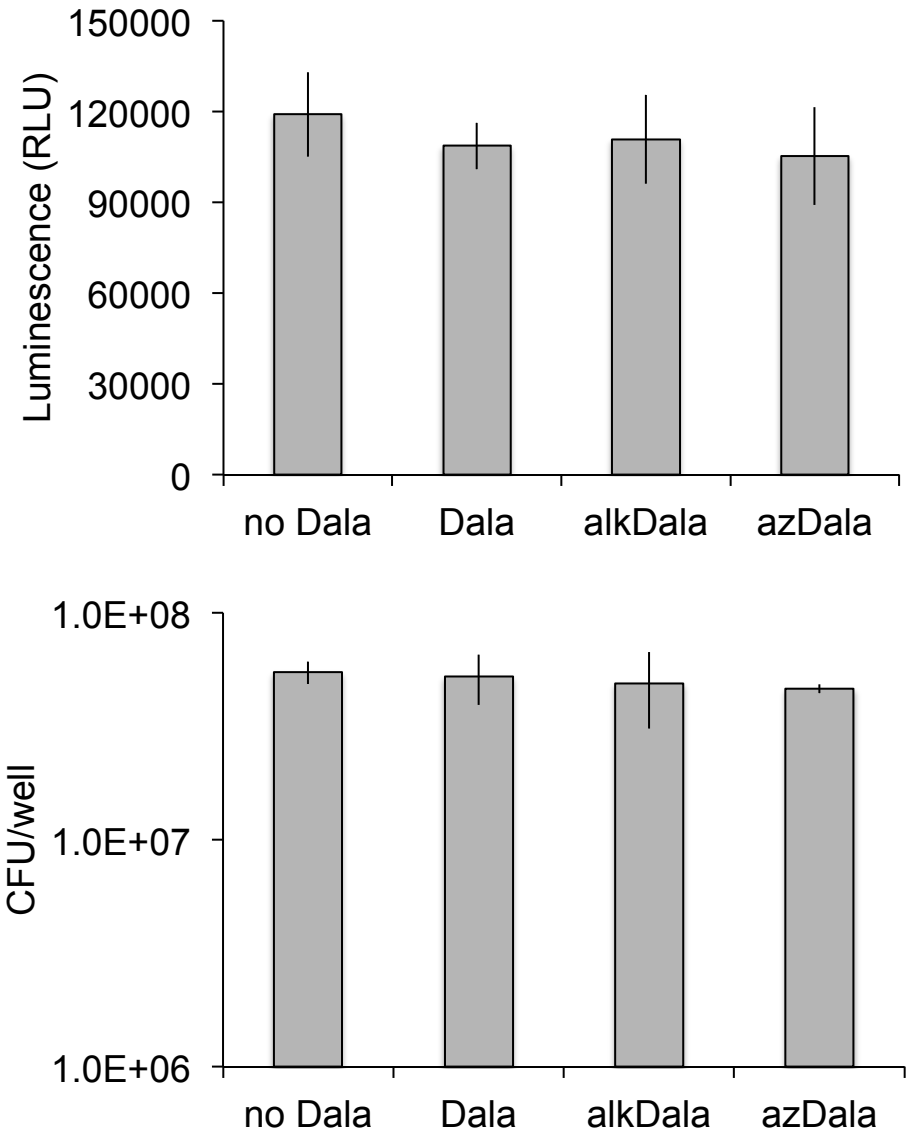
Supplementary Figure 9. AzDala labels bacteria growing in log phase better than in stationary phase. *L. monocytogenes* were labeled with 5 mM azDala for one generation starting in either early log phase (OD_{600} = 0.3), late log phase (OD_{600} = 1.0) or early stationary phase (OD_{600} = 1.8), reacted with DIFO-488,(*8*) fixed and analyzed by flow cytometry. MFI = mean fluorescence intensity. Error bars, +/- s.d. *P=0.0001, two-tailed Student's t test. Data are in triplicate and representative of two independent experiments.



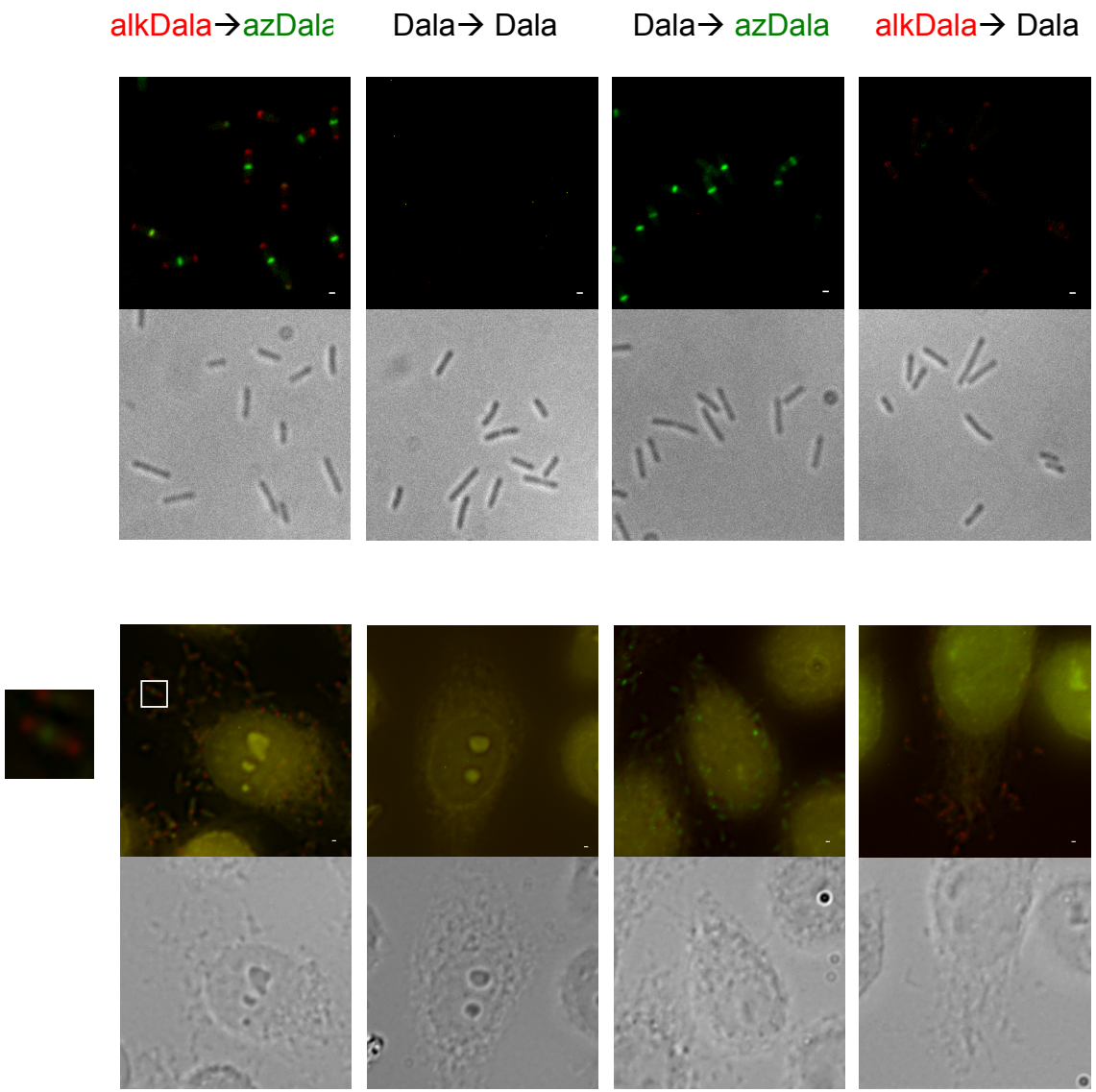
Supplementary Figure 10. Fosfomycin blocks alkDala labeling in *L. monocytogenes*. *prfA* L. monocytogenes* were labeled with or without 5 mM alkDala for 1 h in the presence or absence of antibiotics at 2X MIC (Supplementary Table 2). Importantly, growth was inhibited equally (~30% decrease) across all of the antibiotic-treated strains. The bacteria were fixed, reacted with azido-fluor 488 and analyzed by flow cytometry. We used the mutant strain because wildtype *L. monocytogenes* are naturally resistant to fosfomycin in vitro. PrfA-dependent expression of the Hpt virulence factor, either in vivo or in a *prfA** strain, mediates uptake of the antibiotic and sensitizes the bacteria to its activity.(*9*) Data for wildtype *L. monocytogenes* are the same as *prfA** for the other antibiotics (adjusting for MIC differences). MFI = mean fluorescence intensity. Error bars, +/- s.d. *P=0.002, **P=0.03, ***P=0.01, two-tailed Student's t test, compared to no antibiotic treatment. Data are in triplicate and representative of two independent experiments.



Supplementary Figure 11. alkDala labeling increases along *L. monocytogenes* sidewall in the absence of PBP5. Wildtype (left) or *lmo2754::tn* (right) were labeled with alkDala then fixed and reacted with azido-fluor 488. Bacteria were centrifuged either immediately after addition of alkDala (0 min, top) or 20 min later (bottom) then washed once prior to fixation. The *lmo2754::tn* mutation disrupts the gene encoding the D,D-carboxypeptidase PBP5.(*1*) Scale bars, 1 μm.



Supplementary Figure 12. D-alanine derivatives do not impair host cell growth or bacterial survival in vivo. Top, luminescent output of J774 cells treated with D-alanine derivatives for 4 h and lysed in the presence of luciferin substrate and luciferase enzyme. Luminescence is directly proportional to ATP content. Bottom, colony forming units (CFU) of *L. monocytogenes* treated with D-alanine analogs for 3.5 h during J774 infection. Error bars, +/- s.d. Data are in triplicate.



Supplementary Figure 13. Pulse chase labeling of *L. monocytogenes* in vitro and in vivo. Top, *L. monocytogenes* were incubated in D-alanine or alkDala for 40 min followed by D-alanine or azDala for an additional 5 min. Bottom, *L. monocytogenes*-infected J774 cells were incubated in D-alanine or alkDala for 60 min followed by D-alanine or azDala for an additional 20 min. Red, Azide-Fluor 545 (to detect alkDala), green, Alkyne-Fluor 488 (to detect azDala). Scale bars, 1 μm.