Supporting Information for

Mechanisms of incorporation for D-amino acid probes that target peptidoglycan biosynthesis

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The data that support the findings of this study are available from the corresponding author.

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SI Methods

Chemicals and proteins for in vitro experiments.

Antibiotics were purchased from Sigma. N_{α}, N_{ε}-Diacetyl-D-Lys-D-Ala-D-Ala was purchased from Bachem. Click-iT Cell Reaction Buffer Kit and Alexa Fluor™ 488 Azide were purchased from ThermoFisher Scientific. The following proteins were prepared as previously

described: PBP1A$_{Ec}$[1], PBP1B$_{Ec}$[2], PBP1$_{Bs}$[3], PBP4$_{Sa}$[4], LdtA$_{Vc}$[5], PBP4$_{Ec}$[6], DdlB$_{Ec}$[7], MurF$_{Pa}$[8]. MurF$_{Bs}$ was cloned by James Chalmers and supplied by Wayne Patrick (Dept of Biochemistry, University of Otago, New Zealand). PBP1B$_{Hi}$ was purified by the same procedure as for PBP1A$_{Ec}$.

(F)DAAs and DAADs

HADA (7-Hydroxycoumarin-3-carboxylic acid-D-alanine), HALA (7-Hydroxycoumarin-3-carboxylic acid-L-alanine), NADA (7-Nitrobenzo-2-oxa-1,3-diazole -D-alanine), BADA (BODIPY FL-D-alanine), TDL (TAMRA-D-lysine) and TADA (TAMRA-D-alanine) were synthesized as reported previously[9, 10]. EDA—DA (Ethynyl-D-alaninyl-D-alanine) and DA—EDA (D-Alaninyl-ethynyl-D-alanine) were synthesized as reported previously[11]. Natural amino acids and EDA (Ethynyl-D-alanine or D-Propargylglycine) were purchased from Sigma. Stock solutions were prepared in DMSO (Sigma-Aldrich ReagentPlus >99.5%) at concentrations of 50 - 200 mM and stored at −20°C before use.

Image acquisition and quantification.

For imaging, bacterial cells were resuspended in minimal volume of 1 x PBS. 1 μL of this cell suspension were spotted to the coverslips (typically, 24X50 mm coverslips; #1.5) and an 8x8-mm wide, 2-mm thick PBS-agarose pad (SeaKem LE Agarose) was laid on top of the cells. Phase and fluorescence images were acquired using a Nikon Ti-E inverted microscope equipped with a CFI Plan Apo Lambda DM 60X (1.4 NA, Ph3) oil objective and Andor iXon EMCCD camera. NIS-Element AR software was used for image acquisition. When quantitative comparisons were made, the samples were imaged in the same session with the same image conditions across. Image processing was performed in FIJI. Images were scaled without interpolation, cropped and rotated.

Linear adjustment was performed to optimize contrast and brightness of the images. Figure construction was performed in Adobe Illustrator. The relative fluorescence units of FDAA labeling intensity were quantified using a FIJI plugin, MicrobeJ[12], where cells were identified in the phase contrast channel with width limit from 0.3 to 2 μm and length above 1 μm. FDAA labeling intensity was then quantified and averaged within individual cells (N> 100). These individual intensity values were normalized to the highest average signal within an experiment and were further evaluated and plotted in GraphPad Prism software. To test the significance between two conditions, first D'Agostino & Pearson normality tests were performed. In cases the values did not pass the normality case nonparametric Mann-Whitney tests are used. Otherwise, the significance was determined by unpaired t tests.

Preparation of bacterial cells fixed with ethanol for in vitro experiments

Cells were grown in the following growth conditions: *E. coli* in LB at 37ºC, *Staphylococcus aureus* in LB at 37 ºC, *V. cholerae* in LB at 37ºC, *Agrobacterium tumefaciens* in LB at 25 ºC, *Caulobacter crescentus* in Peptone Yeast Extract (PYE) at 30ºC, *Asticcacaulis biprosthecum* in PYE at 25 ºC and *Myxococcus xanthus* in casitone yeast extract (CYE) at 30 °C.

The same general procedure was followed to prepare ethanol-fixed cells independent of the species. Exponentially growing bacterial cells (5 mL) were washed twice with 1x Phosphate Buffered Saline (PBS: NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4-$2H_2O$ 1.78 g/L, KH_2PO_4 0.27 g/L, pH 7.4). The cells were resuspended in 500 μL 1x PBS and ice-cold Ethanol 100% (v/v) was added to a final 70 % (v/v). The cells were fixed and permeabilized by incubation on ice for 15 min. The cells were collected by centrifugation (9,000 g, 2 min) washed twice with 1 mL 1 x PBS and resuspended in the appropriate buffer as described below. For the preparation of FDAA-pre-

labeled ethanol fixed cells; exponentially growing cells were first labeled with HADA (0.5 mM, for 30 min) and the same preparation protocol was followed.

FDAA Labeling with recombinant proteins and ethanol-fixed cell

The same general procedure was followed for experiments involving ethanol-fixed cells independent of the species. Ethanol-fixed cells were resuspended in 5 x $LdtA_{Vc}$ buffer for labeling with $LdtA_{Vc}$ and 5 x $PBP4_{Sa}$ buffer for labeling with $PBP4_{Sa}$. HADA-prelabeled ethanol fixed cells were resuspended in the 1 x D,D-CPase buffer containing 10 mM $MgCl_2$ and 50 mM HEPES pH 7.5 and $PBP4_{Ec}$. The reactions (typically done in 150 μL) contained the final concentrations of 0.5 mM - 1 mM FDAA, excess enzyme (0.1 - 0.25 mg/mL final) and 1 x of the corresponding buffers and incubated for 1h at 37°C. For optimal dual-color-labeling, cells were first incubated with 0.5 mM HADA and $PBP4_{Sa}$ (0.2 mg/mL, 1 h at 37°C) washed twice in 1 x PBS and incubated with 0.5 mM BADA and $LdtA_{Vc}$ (0.15 mg/mL, 1 h at 37°C). The labeled cells were washed twice in 1 mL 1 x PBS before imaging.

FDAA labeling of exponentially growing cells

In principle, cells are labeled as discussed before[13]. To exponentially growing cells (typically 1 mL in rich media unless noted otherwise) (F)DAAs were added to 0.5 – 1 mM and incubated at optimal growth conditions for ~ 1 generation (unless noted otherwise). The cells were then fixed by directly adding ice-cold ethanol 100% (v/v) to a final 70 % (v/v) and incubating on ice for 15 min. The cells were washed three times with 1 mL 1x PBS by centrifugation (9,000 g, 2 min) and imaged. An exception to this general protocol was *B. subtilis* cells. When appropriate, fixation of *B. subtilis* cells was avoided and, in instances where fixing was required, they were

only washed 1x with PBS and not 3x as described above. For cells labeled with EDA, click reaction were performed using Click-iT Cell Reaction Buffer Kit and Alexa Fluor™ 488 Azide. After the ethanol fixation and washes, cells were resuspended in 200 μL reaction buffer containing 20 μM Alexa Fluor™ 488 Azide and incubated for 45 min at 25°C. The cells were washed twice with 1 mL 1 x PBS again and imaged.

FDAA labeling of exponentially growing cells with drugs or other competitors

In labeling experiments involving inhibitors, the cells are were pretreated for 1-5 min with the drug prior to addition of the (F)DAAs. After addition of the (F)DAAs, the cells were incubated no longer than 20 min in order to minimize cell death. All the labeling experiments were repeated at least twice. The specific drug and FDAA concentrations in experiments leading to figures were the following: **Figure 2a**; 5 min pretreatment with 4 mM fosfomycin, 4 μM vancomycin, 31 μM penicillin G or 31 μM DCS followed by 15 min with 100 μM TDL. **Figure 2d**; see below. **Figure 3d**; see below. **Figure SI1c**; exponentially growing *B. subtilis* cells were first pre-labeled by 250 μM TDL for 45 min, washed and resuspended in 1xPBS containing 1% LB and 1 mg/mL Ampicillin. After 2 min pretreatment, HADA was added to 1 mM and cells were further incubated for 13 min at 37°C. Cells were washed and resuspended in LB containing 125 μM BADA and were further incubated for 20 min 37°C. **Figure SI2b**; Ethanol fixed *E. coli* cells were pretreated 1 min with DCS (5 mM) and $LdtA_{Vc}$ (0.1 mg/mL) in $LdtA_{Vc}$ buffer followed by 20 min incubation in TADA (0.1 mM), HADA (0.1 mM), or NADA (0.1 mM) at 37°C. **Figure SI2c**; *B. subtilis* cells exponentially growing in $S7_{50}$ were pretreated with DA—DA (2.5 mM), D-Alanine (2.5 mM), or D-Tyrosine (2.5 mM) for 5 min and HADA was added to 0.5 mM. The cells were further incubated for 40 min at 37°C. **Figure SI6c**; see below. **Figure SI6d**; see below.

Production and purification of M4.

Soluble murotetrapeptides (M4) were collected from muramidase digested and HPLC-separated *Vibrio cholerae* PG samples processed as previously described[14]. Specifically, 1 L of *V. cholerae* (grown in LB medium) was harvested and muropeptides were isolated by boiling the cell suspension were boiled in SDS 10% (w/v) for 2 h. Then, sacculi were repeatedly washed with MilliQ water by ultracentrifugation (110000 ×g, 10 min, 20 °C TLA100.3 Beckman rotor; OptimaTM Max ultracentrifuge Beckman, Beckman Coulter, California, USA). Samples were treated with Pronase E (100 µg/ml, 1 h, 60 °C) for Braun's lipoprotein removal. The reaction was heat-inactivated, and sacculi were further washed by ultracentrifugation as described above. Samples were digested with muramidase (Cellosyl 100 µg/ml) for 16 h at 37 °C and heat-inactivated. Coagulated proteins were removed by 10 min of centrifugation at 14000 ×g. For sample reduction, samples were first adjusted to pH 8.5–9.0 with borate buffer, and then a freshly prepared NaBH4 2M solution was added to a final concentration of 10 mg/ml. After 30 min at room temperature, samples were adjusted to pH 3.5 with phosphoric acid.

The muramidase treated sample was run on a Waters HPLC equipped with an Aeris peptide reverse-phase column (250 x 4.6 mm; 3.6 µm particle size) (Phenomenex, California, USA) and detected at 204 nm. Muropeptides were separated at 45°C using a linear gradient from buffer A [formic acid 0.1% (v/v)] to buffer B [formic acid 0.1% (v/v), acetonitrile 40% (v/v)] in a 25 min run with a 1 ml/min flow. The M4 peak was collected, samples were pooled and concentrated using a Speedvac, and finally resuspended in water. The purity and quantity of M4 was checked by running a small aliquot in the UPLC, and its nature was confirmed by MS/MS analysis using a Xevo G2-XS QTof system (Waters Corporation, USA) as described below.

UPLC separation of muropeptides

The UPLC separation of muropeptides was performed on a Waters UPLC system (Waters, Massachusetts, USA) equipped with an ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 150 mm (Waters) and a dual wavelength absorbance detector. Elution of muropeptides was detected at 204 nm. Muropeptides were separated at 45 °C using a linear gradient from buffer A [formic acid 0.1% (v/v)] to buffer B [formic acid 0.1% (v/v), acetonitrile 40% (v/v)] in a 12 min run with a 0.250 ml min-1 flow, and detected at 204 nm.

QTOF analysis of muropeptides

The identity of individual muropeptides was established by MSE analysis using a UPLC coupled to a Xevo G2-XS QTof quadrupole time-of-flight mass spectrometer (Waters).
Samples were separated using the UPLC method described before. The scan range was from 10 to 2000 m/z with a scan rate of 0.25s. Instrument was operated in positive electro-spray ionization mode. The capillary and sample cone voltages were 3kV and 40 V, respectively. Gas flows were set at 100 and 500 l/hr for cone gas and desolvation gas, respectively. The source temperature was 120°C and desolvation temperature was 350°C. For MSE, low collision energy was set at 6 eV while ramping the high collision energy from 15-40 eV. Leucine-enkephalin reference was used as the lockmass at a concentration of 200 pg/mL with a continuous flow of 5µL/min and 0.25s scan time, to maintain the accuracy of the analysis. All the acquisition and analysis of data were controlled by Waters UNIFI software. Structural characterization of muropeptides was performed based on their MS data and MS/MS fragmentation pattern.

***In vitro* L,D-TPase assay**

To test the L,D-TPase activity, M4 and different DAAs (D-Met, HADA or DCS) were used as substrates. LD-transpeptidase assays were performed with 10 µg of $LdtA_{Vc}$, in 50 µl of $LdtA_{Vc}$ buffer (50 mM Tris HCl, pH 7.5, 100 mM NaCl) containing 7 µg of M4, and (F)DAAs (1 mM D-Met or HADA or 20 mM D-Met or DCS) at 37ºC for 12h. All enzymatic reactions were stopped by boiling the samples for 5 min, followed by centrifugation at 14,000 rpm for 15 min to discard the precipitated protein. The pH of the supernatant containing the reaction products was adjusted to 3-4 units with orthophosphoric units and then the samples were injected in the UPLC for analysis.

UPLC separation of muropeptides was performed on a Waters UPLC system equipped with an ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 150 mm (Waters) and a dual wavelength absorbance detector. Elution of muropeptides was detected at 204 nm. Muropeptides were separated at 45 °C using a linear gradient from buffer A [formic acid 0.1% (v/v)] to buffer B [formic acid 0.1% (v/v), acetonitrile 40% (v/v)] in a 12 min run with a 0.175 ml/min flow. Detection of D-Met-containing muropeptides was performed as described previously[15]: samples were incubated ± 1.5% (w/v) H_2O_2, which induces a large shift in the elution time of D-Met containing muropeptides without affecting normal muropeptides.

The structure of the $M4^{DCS}$ muropeptide was confirmed by MS/MS analysis, using a Xevo G2-XS QTof system (Waters Corporation, USA) as described above.

In vitro D,D-TPase assay with a soluble tripeptide substrate

D-methionine (D-Met) or D-cycloserine (DCS) stock solutions and acetyl-tripeptide substrate, N_α, N_ε-Diacetyl-L-Lys-D-Ala-D-Ala, (3P, 100 mM in water) were diluted with 5x $PBP4_{Sa}$ buffer (62.5 mM HEPES, pH 7.5, 10 mM $MnCl_2$, 1.25 mM Tween-20)[16] to a final concentration

of 1mM and 10 mM, respectively. The reaction was initiated by the addition of $PBP4_{Sa}$ (final concentration: 0.4 mg/ml). Reactions were incubated at 37°C for 1 h with continuous shaking. After the reaction, the mixtures were analyzed by reverse-phase HPLC using Hewlett-Packard Serries 1100 HPLC instrument controlled by Agilent ChemStation for HPLC (Version B.04.03-SP1) equipped with a Luna® 5 μM C18(2) 100 Å LC Column (250 x 4.6 mm). HPLC data (10-90% MeCN/H2O with 0.1% TFA, over 20 min) was collected monitoring absorbance at 470 nm or with evaporative light scattering detection (ELSD).

In vitro DCS competition of FDAA labeling

Ethanol-fixed cells were pretreated 1 min with 5 mM DCS and LdtAVc in 1x LdtAVc buffer before the addition of NADA, TADA, or HADA to 0.1 mM. The reactions were incubated for 1h at 37oC and the samples were processed as above.

Construction and FDAA labeling of *B. subtilis Δddl* strain

Mutation of *ddl* was performed by amplifying the regions upstream with forward primer (5'- GGCTGGCAGCGTCCATTAA -3') and reverse primer (5'- CAATTCGCCCTATAGTGAGTCGTTCCGTACACTAATCCTAGACT -3') and downstream with (F: 5'- CCAGCTTTTGTTCCCTTTAGTGAGCATACATTCTAATGAAGAAGGA -3'; R: 5'- ATCCTCAGGCGCGATGCC -3') of *ddl* using *B. subtilis* 3610 chromosomal DNA as a template, and amplifying the spectinomycin resistance cassette from pAH54 with (F: 5'- ACGACTCACTATAGGGCGAATTG -3'; R: 5'- CTCACTAAAGGGAACAAAAGCTGG -3'). The three fragments were ligated using Gibson assembly and transformed into DK1042 in the presence of 6 mM DA—DA. Mutants were selected for on $S7_{50}$ supplemented with 6 mM DA—

DA and 100 μg/mL spectinomycin. The presence of the mutation was confirmed by PCR amplification and sequencing of the *ddl* region.

The *B. subtilis Δddl* strain had to be grown in $S7_{50}$ minimal media supplemented with 6 mM DA—DA. In these conditions, *B. subtilis Δddl* had a similar growth rate to the parent. For comparative labeling with this strain; 5 mM DCS, 1 mM Ampicillin or equal volume of water (for no drug) was added to *B. subtilis Δddl* and *B. subtilis wt* strains exponentially growing at 37°C in $S7_{50}$ + 6 mM DA—DA. After 3 min, HADA or TADA was added to 0.5 mM or to 0.1 mM and incubated for another 17 min. Without fixation the cells were washed and imaged.

In vitro MurF and Ddl assays

In vitro MurF and Ddl activities were determined through use of the coupled ADP release assay that utilizes pyruvate kinase and lactate dehydrogenase, as previously described.[17] $MurF_{Pa}$ activities were determined in 50mM HEPES and 10mM $MgCl_2$ (pH 7.7) containing 200μM UDPMurNAc-L-Ala-D-Glu-*meso*-DAP and dilutions of DA—DA, EDA—DA or DA—EDA in the presence of 10% DMSO at each dilution (final). Our inability to detect turnover by $DdlB_{Ec}$ in the presence of EDA alone led us add minimal amounts of D-alanine at all times in order to occupy the high affinity N-terminal pocket to D-alanine (K_M = 3.3 μM), but prevent a high background rate of DA—DA formation[18]. Therefore, $DdlB_{Ec}$ activities were determined in 50mM HEPES and 10mM $MgCl_2$ (pH 7.7) containing 15 μM D-alanine at all times and dilutions of D-Ala or EDA in the presence of 10% DMSO at each dilution (final). This means that the Km quoted in **Figure 3e** refers to the second subsite of DdlB in its formation of either D-Ala-D-Ala or D-Ala-EDA.

In order to closely reproduce the *Bacillus subtilis* cytosol the reaction buffer for $MurF_{Bs}$ was 50mM HEPES-KOH, 10mM $MgCl_2$, 150mM KCl, pH 7.25. All reactions used $MurF_{Bs}$ with the His_6 tag cleaved and 600 µM UDPMurNAc-L-Ala-D-Glu-*meso*-DAP.

Construction of *E. coli* deletion strains and (F)DAA labeling with *E. coli Δ6ldtΔddlAB*

E. coli (BW25113) deletion strains, e.g. lacking combinations of L,D-TPase genes, were generated and combined by transferring *kan* marked alleles from the Keio *E. coli* single-gene knockout library[19] into relevant background strains using P1 phage transduction[20]. The Keio pKD13-derived *kan* cassette is flanked by FRT sites, allowing the removal of the *kan* marker via expression of FLP recombinase from plasmid pCP20 to generate unmarked deletions with a FRT-site scar sequence[21, 22].

Because the *E. coli ΔddlAB* strain[23] was labeled by FDAAs in a growth-independent way (presumably via L,D-TPases), the same two deletions were introduced into the *E. coli* BW25113Δ6LDT[24] background. The *E. coli* Δ6LDT*ΔddlAB* strain had to be grown in M9 minimal media supplemented with 5 mM DA—DA. In these conditions, *E. coli* Δ6LDT*ΔddlAB* had a similar growth rate to the parent, *E. coli* Δ6LDT. For comparative labeling with this strain; 10 mM DCS, 1 mM Ampicillin or equal volume of water (for no drug) was added to *E. coli* Δ6LDT and *E. coli* Δ6LDT*ΔddlAB* strains exponentially growing at 37ºC in M9 + 5 mM DA—DA. After 4 min, HADA or EDA was added to 0.5 mM and incubated for another 11 min, before the cells are were fixed and processed as mentioned above.

FDAA labeling of nutrient starved cells

Exponentially growing cells (1.5 mL in rich media) were washed three times with 1 mL 1 x PBS and resuspended in 1 mL 1x PBS and incubated at room temperature 1 h – 2 h. Once the cells were washed and resuspended again in 1 x PBS, FDAAs (0.5 – 1 mM) were added and the cells were further incubated up to 1 h at room temperature. The cells were washed twice with 1 mL 1x PBS again and imaged.

Construction of *E. coli* L,D-TPase complementary over expression strains

In order to construct the *ldtD* complementary overexpression plasmid, first a DNA fragment encoding full-length LtdD was amplified by PCR with a forward primer (5'-ACGAACCATATGTTGCTTAATATGATGTGT -3') and reverse primer (5'-CGAACTTCTAGATTTACCTGATTAATTGTTCCGC-3'). The amplified DNA was cloned into NdeI/EcoRI-cut pSRK_GM vector. The obtained plasmid (pSRK_GMΩldtD) was transformed into *E. coli* BW25113Δ6LDT generating the strain *E. coli* BW25113Δ6LDT *pSRK_GMΩldtD*. LdtD was overexpressed by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside to exponentially growing cells at 37 °C 20 min before the addition of HADA (1 mM) and further incubation for 40 min.

The same procedure was followed for other L,D-TPases, but using the following forward and reverse primers toward building respective overexpression plasmids:

yafK/YafK (F:5'- ATAAGCCATATGCGTAAAATCGCATTAAT-3';
R:5'-GCCAGGTCTAGAATGGCGCTTATTTTGCCTCGGGGAG-3'),

ldtC/YcsF (F:5'-ACGAACCATATGATGATCAAAACGCGTTTT-3';
R:5'-TTTTATCTAGACTTTACAGCGTTTGTGGGCTCAC-3'),

ldtB/YbiS (F:5'-ACGAACCATATGAATATGAAATTGAAAACA-3';

R:5'-AGTTTTCTAGATTAATTCAGACGAACCGGCAT-3'),

ldtE/YnhG (F:5'-ACGAACCATATGAAACGCGCGTCTTTGCTT-3';

R:5'-TTTTGTCTAGAGTTTACCGCTACTGCGTCACGCGTAACA-3')

and *ldtA*/ErfK (F:5'-ACGAACCATATGCGTCGTGTAAATATTCTT-3';

R:5'-TGCTTTCTAGATACCAACGCTCTTAAAACATCTGTCTTGAACC -3').

Production and purification of lipid II.

Lipid II substrate versions (mDAP and amidated mDAP versions) for *in vitro* PG synthesis assays were prepared as previously described[19-21]. Lipid II-meso-diaminopimelic acid was used in experiments with *E. coli* and *Haemophilus influenzae* PBPs. Radioactively labeled amidated Lipid II was used in experiments with *B. subtilis* PBP1.

Lipid II-meso-diaminopimelic acid (Lipid II-m-DAP) was synthesized using UDP-MurNAc-pentapeptide isolated from *Bacillus cereus*, essentially as described previously[22] with the following modifications. Purification was performed over a DEAE-cellulose column using a linear gradient of chloroform/methanol/water (2:3:1 v/v/v) to chloroform/methanol/1M ammonium bicarbonate (2:3:1 v/v/v). The fractions containing Lipid II-m-DAP were collected and dried under vacuum. The resulting lipid II-m-DAP was then dissolved in 1:1 chloroform: methanol and stored at -20^{o}C until use.

Radioactively labeled ([14C]-amidated) Lipid II was labeled exploiting the the ability of MurG to exchange the GlcNAc group between UDP-GlcNAc and Lipid II. Since the *E. coli* MurG used here displayed much lower affinity for amidated Lipid II-m-DAP than the nonamidated form, we first synthesized [14C]-Lipid II-m-DAP and then amidated this using AsnB from *B. subtilis*. First, 1.22 mmol of purified lipid II-m-DAP was incubated with 12.5 mCi of UDP-N-acetyl-D-[1-

14C] glucosamine (specific activity 55mCi/mmol) (Hartmann Analytic GmbH). The labeling reaction was performed in 1ml of 100 mMTris-HCl, pH 8.0, 1 mM MgCl2, and 2% (w/v) Triton X-100. The reaction was started by the addition of 1 mL purified recombinant *E. coli* MurG, which is able to exchange the N-acetyl-D-glucosamine of lipid II with that of UDP-N-acetyl-D-[1-14C] glucosamine. After incubation at room temperature for 2.5 hours, the reaction was complete as determined by liquid scintillation counting. To amidate the carboxylic acid group of the m-DAP, lipid II-DAP was incubated with *B. subtilis* AsnB in the presence of ATP and glutamine (to be published elsewhere), followed by extraction using butanol/pyridine acetate, pH 4.2, and another purification step over a DEAE cellulose column using a gradient of chloroform/methanol/water (2:3:1 v/v/v) to chloroform/methanol/0.5M ammonium bicarbonate (2:3:1 v/v/v).

In vitro Peptidoglycan synthesis and D,D-TPase assays

Peptidoglycan synthesis reactions with radiolabelled lipid II substrate were performed in an *in vitro* detergent micelle system at the following conditions. For PBP1A$_{Ec}$ PBP1B$_{Ec}$ and PBP1A$_{Hi}$: 0.5 μM enzyme in a buffer of 20 mM HEPES/NaOH pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, 1% DMSO, 0.05% Triton X-100 with 15 μM [^{14}C]mDAP lipid II (1.5 nmol, ~10,000 dpm) was incubated for 1 h at 37°C with 500 μM of D-Ala (control) or FDAAs as indicated. For PBP1$_{Bs}$: 0.5 μM enzyme in a buffer of 20 mM HEPES/NaOH, pH 7.5, 10 mM $CaCl_2$, 20 mM NaCl, 1% DMSO, 0.02% Triton X-100 with 15 μM [14C] amidated mDAP lipid II (1.5 nmol, ~10,000 dpm) was incubated for 1 h at 37°C with 500 μM of D-Ala (control) or FDAA. After reactions, samples were processed by digestion with cellosyl and reduction with sodium borohydride prior to for reversed phase HPLC analysis of the peptidoglycan products using a Prontosil 120-3-C18 AQ column on an Agilent Technologies 1260 Infinity system UV detection at 205 nm and, when

radiolabelled samples were analysed, additionally a LabLogic β-RAM online radioactivity detector. New peaks observed during the HPLC analysis of the peptidoglycan product were identified by mass spectrometry as described previously[23].

Sacculi purification

Sacculi from FDAA labeled ethanol fixed cells were isolated following the previously published protocol with the following modifications[11]: Ethanol fixed cells were labeled by recombinant TPases and FDAAs as described above. The cells were washed twice with 1x PBS and once with 1x PBS-SDS solution (0.5% SDS, w/v). The cells were then resuspended in 100 µL 1x PBS-SDS solution containing 1.5 mg/ml pronase E (Sigma-Aldrich, *Streptomyces griseus*) and incubated for 2 hours at 60°C. After the cells were collected, they were washed with H_2O once, resuspended in 4% SDS-H_2O solution (w/v) and boiled for 1 hour. The boiled pellets were washed with H_2O twice and then imaged.

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Supporting Information Figures

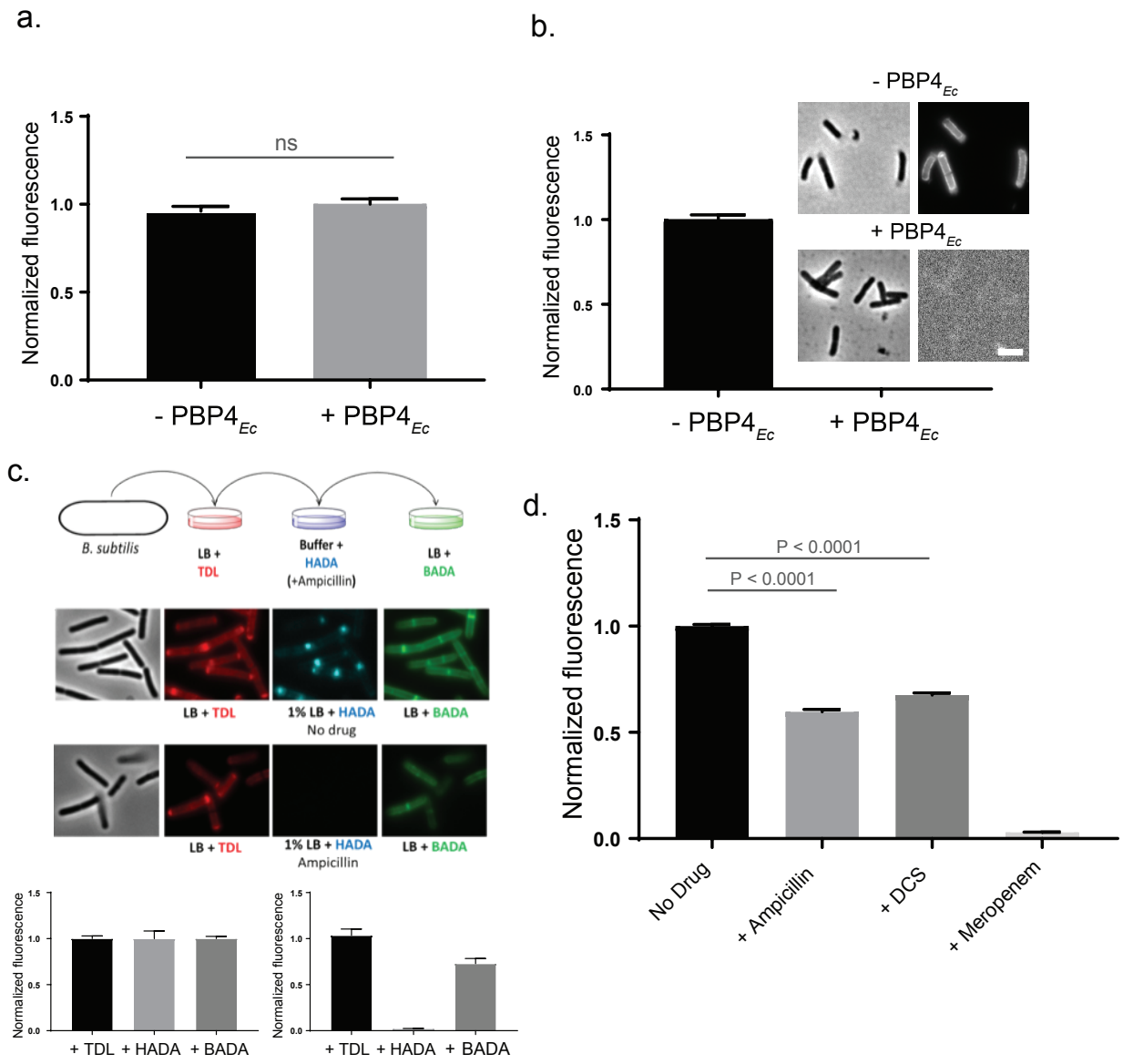

Figure SI1: a, Treatment of ethanol-fixed *E. coli* cells (known to incorporate FDAAs into their PG tetrapeptides[24]) prelabeled with FDAAs using PBP4$_{Ec}$ did not significantly change retained FDAA signal intensity. This confirms that PBP4$_{Ec}$, a D,D-CPase, has negligible side activities in this context (e.g., D,D-endopeptidation[6]). Compare also with *E. coli* BW25113Δ6LDT*ΔdacA* cells (**Figure SI4a**). **b,** Treatment of ethanol-fixed *B. subtilis ΔdacA* cells (prelabeled with HADA by PBP4$_{Sa}$) with PBP4$_{Ec}$ completely removes the HADA signal indicating that vegetative *B. subtilis* cells incorporate FDAAs solely into their muropentapeptides. The partial lysis of *B. subtilis* cells

visible in phase contrast is caused by ethanol fixation[11]. **c,** Brief ampicillin pretreatment inhibited FDAA incorporation (HADA signal) in live vegetative *B. subtilis* cells but was not toxic as shown by the cells ability to incorporate FDAAs (BADA signal) after the drug was removed by washing. See the experimental methods for details. **d,** Brief pretreatment of live *E. coli* wild-type cells with ampicillin, D-cycloserine (DCS), and meropenem, significantly inhibited FDAA incorporation. Micrographs are adjusted for qualitative comparison only. Column bar graphs represent mean relative signal quantified from at least N > 100 cells. Error bars are SEM. Scale bars, 2 μm.

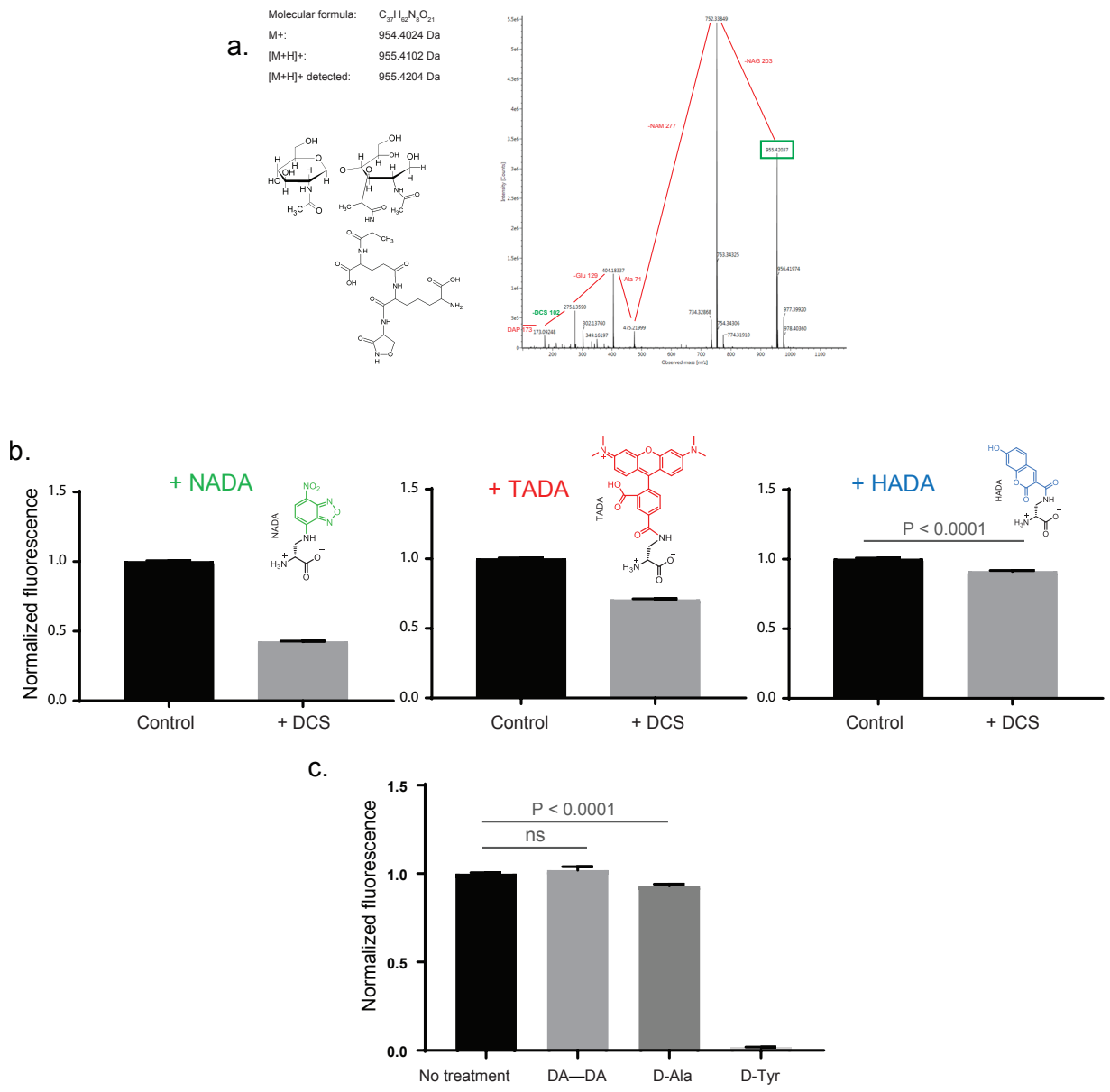

Figure SI2: a, D-cycloserine incorporation into the M4 substrate by $LdtA_{Vc}$ is confirmed by mass spectrometric analysis. **b,** Pretreatment of ethanol-fixed *E. coli* Δ6LDT cells (1 min) with excess (50 x) DCS inhibits $LdtA_{Vc}$–facilitated FDAA incorporation (after 1h, 37ºC) *in vitro*. **c,** Excess D-tyrosine and D-alanine, but not D-Ala-D-Ala (DA—DA), significantly competed with HADA incorporation in *B. subtilis ΔdacA* cells actively growing in $S7_{50}$ minimal media. Column bar

graphs represent mean relative signal quantified from at least N > 100 cells. Error bars are SEM. See SI Methods for experimental details

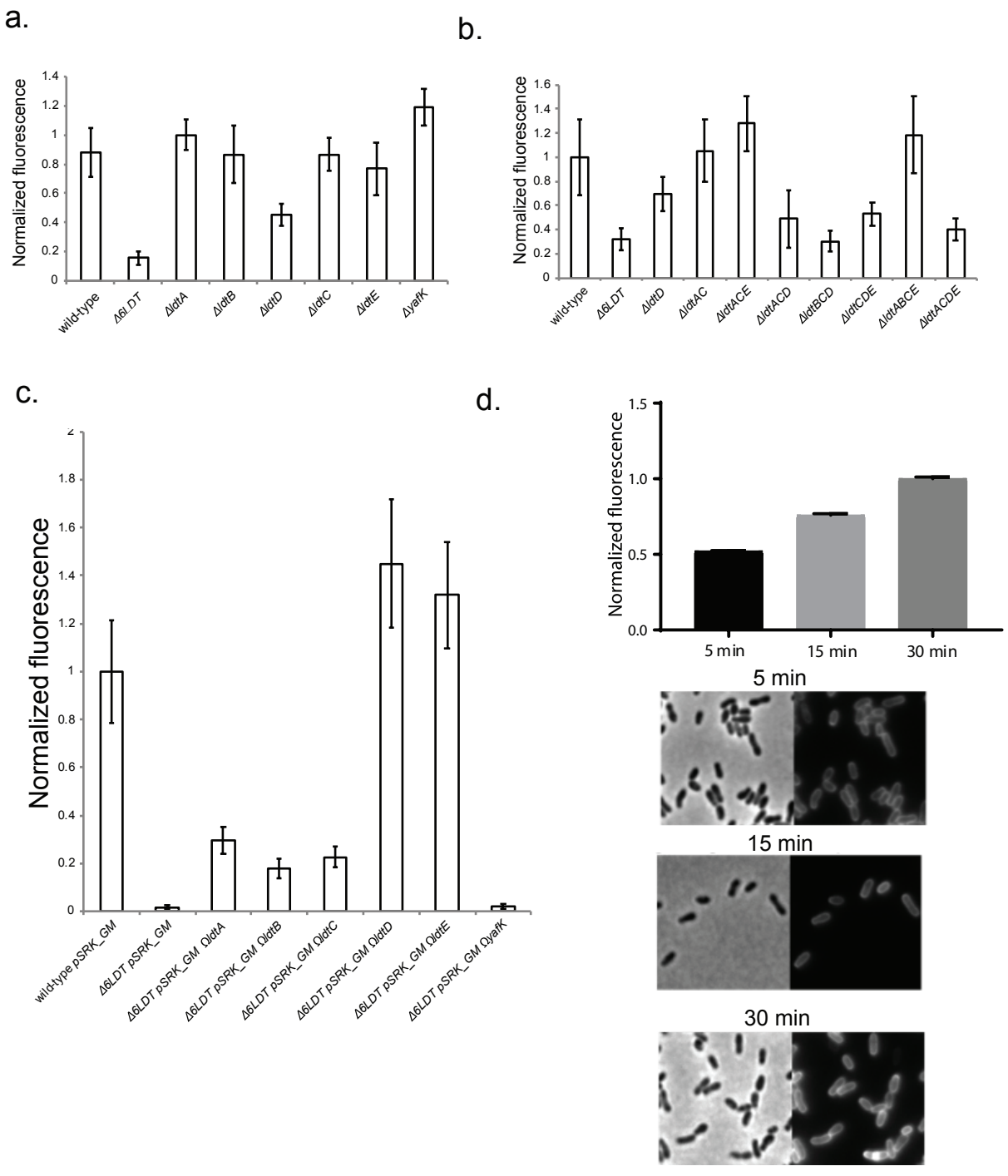

Figure SI3: a, Effects of the single L,D-TPase deletions on HADA accumulation in live *E. coli*

cells. **b,** Effects of the different combinations of L,D-TPase deletions for HADA accumulation in live *E. coli* cells. **c,** Effects of the over-expression of single L,D-TPase genes for HADA accumulation in nutrient starved live *E. coli* cells. **d,** HADA signal accumulation in nutrient starved live *Agrobacterium tumefaciens* cells over time. Micrographs are adjusted for qualitative comparison only. Column bar graphs represent mean relative signal quantified from at least N > 100 cells. Error bars are SEM. Scale bars, 2 μm.

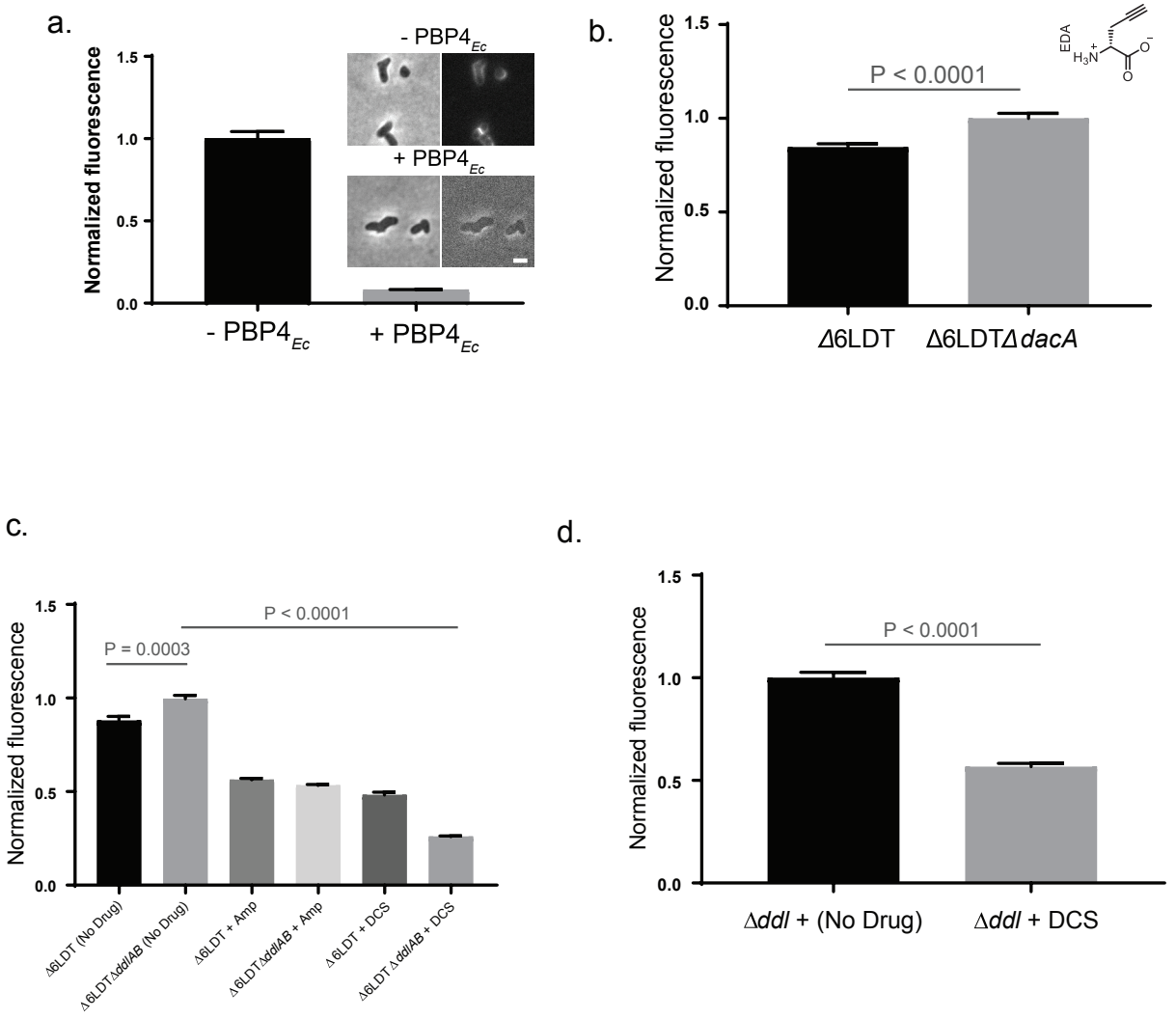

Figure SI4: a, Treatment of ethanol fixed *E. coli* Δ6LDT*ΔdacA* cells prelabeled with FDAAs by

PBP4$_{Ec}$ completely removed HADA signal indicating that these cells incorporate FDAAs solely to their pentapeptides. **b,** Live *E. coli* Δ6LDT and *E. coli* Δ6LDT*ΔdacA* cells incorporated EDA ~20-fold less compared to *E. coli* wild-type cells. Live *E. coli ΔdacA* cells accumulated significantly more EDA signal compared to wild-type, *E. coli* Δ6LDT, or *E. coli* Δ6LDT*ΔdacA* cells. **c,** Live *E. coli* Δ6LDT and *E. coli* Δ6LDT*ΔddlAB* cells, grown in M9 minimal media supplemented with DA—DA, incorporated EDA comparably. EDA incorporation could be significantly inhibited by ampicillin and DCS pretreatment even in *E. coli* Δ6LDT*ΔddlAB* cells; suggesting that EDA is incorporated by D,D-TPases and that DCS and ampicillin can both inhibit *E. coli* D,D-TPases. **d,** TADA incorporation could be significantly inhibited by DCS pretreatment even in *B. subtilis Δddl* cells; suggesting that DCS can inhibit *B. subtilis* D,D-TPases. Micrographs are adjusted for qualitative comparison only. Column bar graphs represent mean relative signal quantified from at least N > 100 cells. Error bars are SEM. Scale bars, 2 μm.

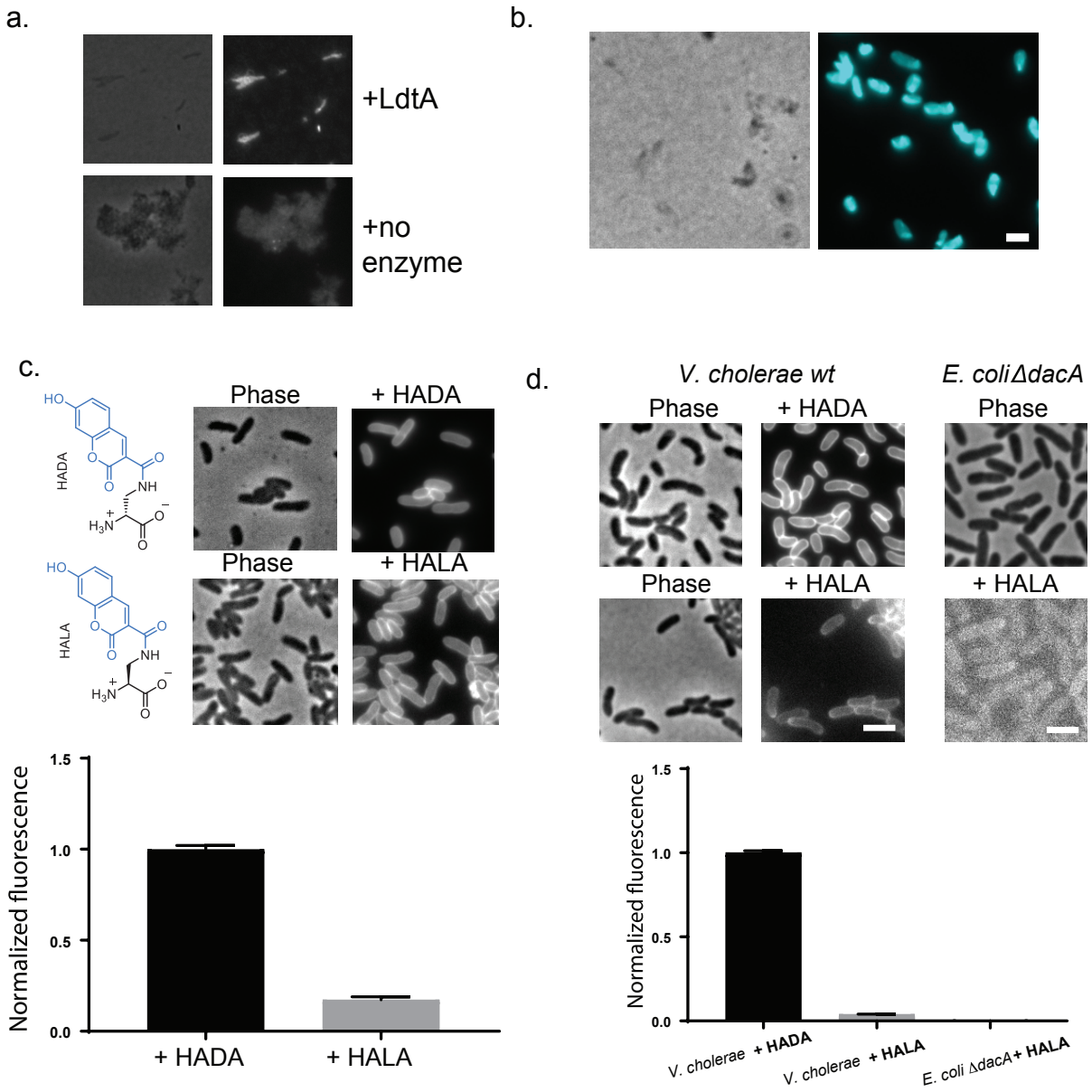

Figure SI5: a, Attempts to use isolated sacculi from *Vibrio cholerae* as a substrate for FDAA incorporation by recombinant transpeptidases failed, presumably due to the non-specific binding of FDAAs to free sacculi. **b**, FDAA labeling of sacculi isolated from ethanol-fixed *E. coli* cells incubated with $LdtA_{Vc}$ and HADA *in vitro* (1 h, 37°C). **c,** $LdtA_{Vc}$ incorporated HALA to ethanol fixed *V. chloreae* cells ~5 times less efficiently than HADA *in vitro*. **d,** Live *V. cholerae* cells incorporated HALA ~20 times less efficiently than HADA. With the same labeling and imaging conditions, *E. coli ΔdacA* cells, capable of incorporating FDAAs into both PG tetrapeptides and

pentapeptides, did not give significant labeling above the background after incubation with HALA. Micrographs are adjusted for qualitative comparison only. Column bar graphs represent mean relative signal quantified from at least N > 100 cells. Error bars are SEM. Scale bars, 2 μm.

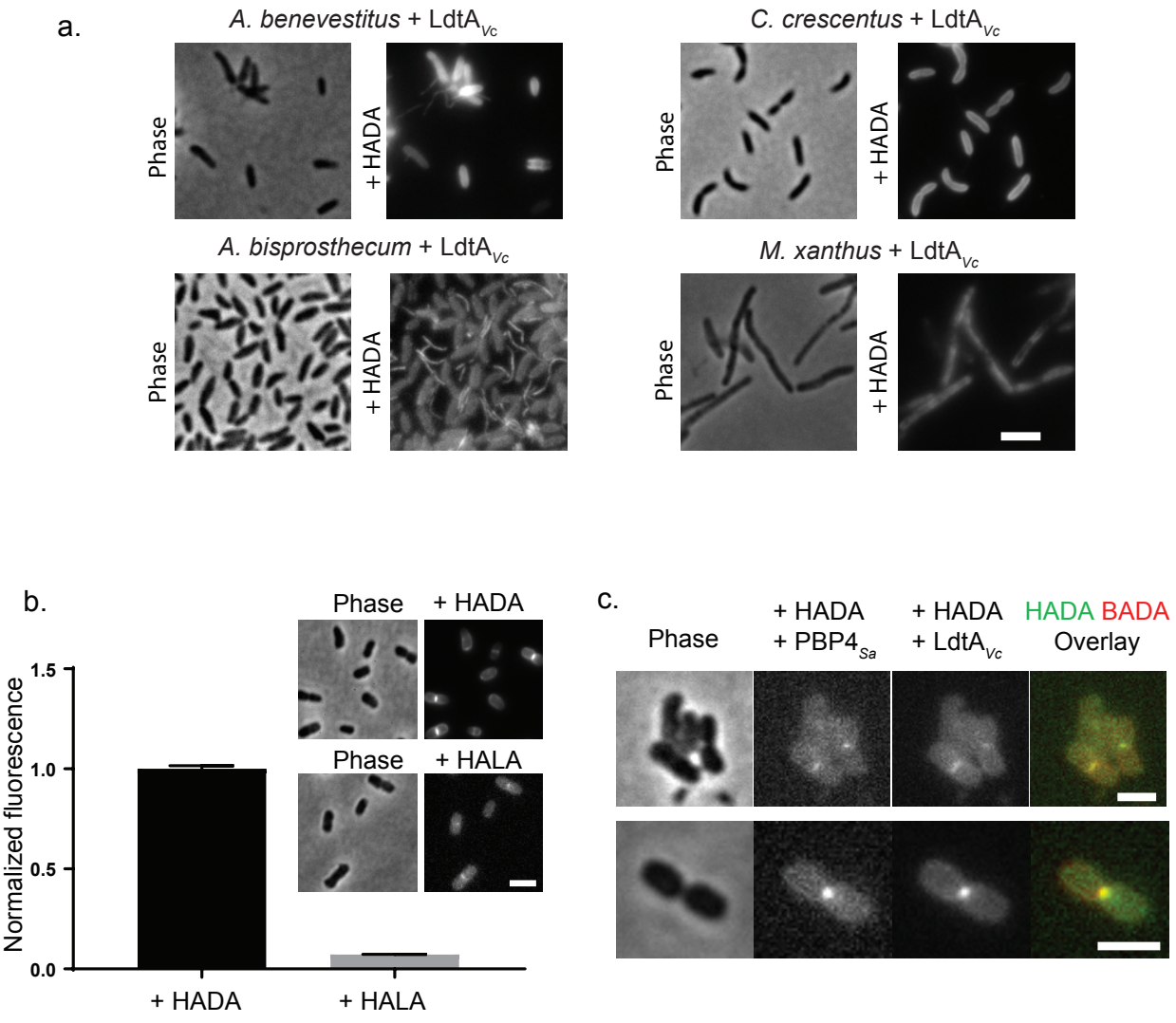

Figure SI6: a, Ethanol-fixed *Asticcacaulis benevestitus, Asticcacaulis biprosthecum, Caulobacter crescentus*, and *Myxococcus xanthus* cells were substrates for LdtA$_{Vc}$ and FDAAs (e.g. HADA), *in vitro*. **b,** Ethanol-fixed *E. coli* Δ6LDT*ΔdacA* cells were substrates for PBP4$_{Sa}$ and HADA; these cells were also substrates for PBP4$_{Sa}$ and HALA, although HALA was incorporated ~20-fold less

efficiently, *in vitro*. **c,** Sequential PBP4$_{Sa}$ (with HADA) and LdtA$_{Vc}$ (with BADA) can report on subcellular PG pentapeptide vs. PG tetrapeptide distribution in ethanol fixed *E. coli* Δ6LDT*ΔdacA* cells, that are rich in both. Micrographs are adjusted for qualitative comparison only. Column bar graphs represent mean relative signal quantified from at least N > 100 cells. Error bars are SEM. Scale bars, 2 μm.