Figures and figure supplements

Maturing *Mycobacterium smegmatis* peptidoglycan requires non-canonical crosslinks to maintain shape

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Figure 1. FDAAs are incorporated asymmetrically by L,D-transpeptidases. (A) Schematic of mycobacterial asymmetric polar growth. Green, old cell wall; grey, new material; dotted line, septum; large arrows, old pole growth; small arrows, new pole growth. (B) FDAA incorporation in log-phase WT Msm cell after 2 min incubation. Scale bar = 5 μm. Old pole marked with (*). (C) Schematic of Fluorescence Activated Cell Sorting (FACS)-based FDAA transposon library enrichment. An Msm transposon library was stained with FDAAs, the dimmest and brightest cells were sorted, grown, sorted again to enrich for transposon mutants that are unable or enhanced for FDAA incorporation. (D) Results from 1C screen. For each gene, the contribution to low or high staining population was calculated from transposon reads per gene. Plotted is the ratio of the population contribution from the second sort of low FDAA staining (L2) over the second sort of high FDAA staining (H2) cells compared to the Mann-Whitney U p-value. (E) Representative image of FDAA incorporation in log-phase WT, ΔLDT and ΔLDTcomp cells. Scale bar = 5 μm. (F) Profiles of FDAA incorporation in log-phase WT (N = 98), ΔLDT (N = 40), and ΔLDTcomp (N = 77) cells. Thick lines represent mean incorporation profile, thin lines are FDAA incorporation in single cells. DOI: https://doi.org/10.7554/eLife.37516.003
Figure 1—figure supplement 1. Peptidoglycan synthesis overview. (A) *Escherichia coli* and *Bacillus subtilis* lateral cell wall growth. Unlike mycobacteria, *E. coli* and *B. subtilis* insert new cell wall along the lateral cell body, mixing old and new peptidoglycan. Green portion represents old cell wall; grey portion represents new material. (B) Cartoon of penicillin-binding proteins (PBPs), L,D-transpeptidases (LDTs), and both 4–3 and 3–3 crosslinking. PBPs utilize a pentapeptide substrate found on new peptidoglycan, ending in D-Alanine-D-Alanine. Class A PBPs (aPBPs), like PonA1, have ability to polymerize glycan strands (transglycosylation) and to crosslink (transpeptidation). Class B PBPs (bPBPs) are only capable of transpeptidation. LDTs utilize a tetrapeptide substrate found on processed peptidoglycan. TP, transpeptidase; TG, transglycosylase.

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Figure 1—figure supplement 2. Time-lapse microscopy maps FDAA incorporation pattern to old and new poles. (A) Schematic of asymmetric polar growth in mycobacteria, where green portion corresponds to Alexa Fluor™ 488 NHS ester (ALEXA) stained cell wall, and grey portion represents outgrowth of new, unstained cell wall. (B) Image of WT Msm cell stained with ALEXA and FDAA at time = 0 min. (C) Graph quantifying FDAA and ALEXA incorporation along the length of the cell in (B). (D) Image of the same cell as (B) at 30 min. The blue line highlights the growth from the old pole. (E) As in C, a graph of the distribution of FDAA and ALEXA. The blue box corresponds to the blue line in (D), where the old pole has grown out. DOI: https://doi.org/10.7554/eLife.37516.005
Figure 1—figure supplement 3. Fluorescent D-amino acid screen validation. (A) FDAA incorporation in WT, ΔldtAEB, and ΔldtAEB complemented with LdtE-mRFP as measured by flow cytometry. (B) FDAA incorporation in WT, ΔLDT, and ΔLDT complemented with LdtE-mRFP (ΔLDTcomp).

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3–3 crosslinks are not detectable in ΔLDT cells. Peptidoglycan from *M. smegmatis* ΔLDT cells was isolated, hydrolyzed in NH₄OH, and analyzed by LC-MS/MS (see Materials and methods). Peaks corresponding to 3–3 crosslinks were identified by mass (Kumar et al., Baranowski et al. eLife 2018;7:e37516. DOI: https://doi.org/10.7554/eLife.37516).
Figure 1—figure supplement 4 continued

(A, B) Extracted ion chromatograms of 3–3 crosslinks from WT, ΔLDT and ΔLDT<sub>comp</sub> peptidoglycan where both donor and acceptor stems are tripeptides. (C) Table of observed 3–3 crosslink masses. ND = not detected. (D) Proposed structures of the detected 3–3 crosslinks with all four possible sites of amidation shown in blue. Tri-tri refers to the 3–3 crosslink where both donor and acceptor peptides are tripeptides (903.45; with three sites amidated- 904.43). Tri-tetra refers to the 3–3 crosslink where the donor stempeptide is a tripeptide, and the acceptor is a tetrapeptide (974.49; with three sites amidated- 975.47). The crosslink amide bond is shown in red.

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Figure 2. 3–3 crosslinks are required for rod shape maintenance at aging cell wall. (A) Msm ΔLDT time-lapse microscopy of cells switched from high- to iso-osmolar media (top row, see Figure 2—video 1), or iso- to high osmolar media (bottom row, see Figure 2—video 2). (high = 7H9 + 150 mM sorbitol; iso = 7H9). t = time in minutes post-osmolarity switch. (B) ΔLDT or WT cells were stained with Alexa 488 NHS-ester (green) to mark existing cell wall, washed, and visualized after outgrowth (unstained material). A, B scale bar = 2 µm. (C) Mean stiffness of WT (N = 73) and ΔLDT (N = 47) Msm cells as measured by atomic force microscopy. Mann-Whitney U p-Value ****<0.0001. (D) Representative profile of cell height and height-normalized stiffness (modulus/height) in a single ΔLDT cell. Pink-shaded portion highlights location of a bleb. (E) Maximum cell width of ΔLDT cell lineages over time. Width of new pole daughters = blue circle; width of old pole daughters = orange circle. Division signs denote a division event. At each division, there are two arrows from the dividing cell leading to the resulting new and old pole daughter cell widths (blue and orange respectively). (F) Model of Figure 2 continued on next page
rod shape loss in old cell wall of ΔLDT cells compared to WT. Green portions of the cell represents old cell wall; grey portion represents new cell wall. The larger arrows indicate more growth from the old pole, while smaller arrows show less relative growth from the new pole. Dotted lines represent septa. op = old pole, np = new pole.

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Figure 2—figure supplement 1. ∆LDT cell morphological characteristics. (A) Time-lapse microscopy montage of ∆LDT cells. The white stars mark new poles. The orange arrow points to the first new pole daughter cell of this series. The red arrow indicates the second resulting new pole daughter cell. In the last frame, white arrows point to all new pole daughter cells (besides the orange arrow and red arrow). (B) Time-lapse microscopy montage of ∆LDT_{comp} cells expressing LdtE-mRFP. All scale bars = 5 µm.

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Figure 2—figure supplement 2. Inheritance of old cell wall and occurrence of blebs in new pole daughter cells. (A) WT Msm stained with Alexa Fluor™ 488 NHS ester, washed and visualized over time. New material is unstained, old material is stained green. Orange arrows indicate a new pole. Orange stars mark new pole daughter cells. All scale bars = 5 μm. (B) Maximum cell width of ΔLDT cell lineages over time. Width of new pole daughters = blue circle; width of old pole daughters = orange circle. Division signs denote a division event. At each division, there are two arrows from the dividing cell leading to the resulting new and old pole daughter cell widths (blue and orange, respectively).

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Figure 3. *Mycobacterium smegmatis* is hypersensitive to PBP inactivation in the absence of LDTs. (A) Fold change in the number of reads for transposon insertion counts in ΔLDT cells compared to WT Msm. p-value is derived from a rank sum test ([DeJesus et al., 2015](https://doi.org/10.7554/eLife.37516)). (B) Transposon insertions per TA dinucleotide in *pbpA* and *ponA2* in WT (grey) and ΔLDT (blue) cells. (C) Schematic of L5 allele swapping experiment. (D) Results of WT or transpeptidase null *ponA1* allele swapping experiment in ΔLDT cells.

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L5 allele swapping to test essentiality of PonA1's ability to form 4 to 3 crosslinks (transpeptidation). (A) Schematic of L5 allele swapping experiment. Adapted from (Kieser et al., 2015b).

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Figure 4. Peptidoglycan synthesizing enzymes localize to differentially aged cell wall. (A) Representative fluorescence image of PonA1-RFP (magenta, see Figure 4—video 1), LdtE-mRFP (cyan, see Figure 4—video 2), and DacB2-mRFP (green, see Figure 4—video 3). Scale bars = 5 μm. (B) Average Figure 4 continued on next page.
Figure 4 continued

PonA1-RFP (N = 24), LdtE-mRFP (N = 23) or DacB2-mRFP (N = 23) distribution in cells before division. (C) Schematic of the in vitro experiment to test D, D-carboxy- and D,D-endopeptidase activity of DacB2 (top). Lipid II extracted from B. subtilis is first polymerized into linear (using SgtB) or crosslinked (using B. subtilis PBP1) peptidoglycan and then reacted with DacB2. The reaction products are analyzed by LC-MS. Extracted ion chromatograms of the reaction products produced by incubation of DacB2 with peptidoglycan substrates (bottom).

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Figure 4—figure supplement 1. PG synthetic enzyme localization at birth, 30, 60 and 90 min post-birth and at the frame before division (pre-division) in a representative cell. (A) Distribution of PonA1-RFP, (B) LdtE-mRFP, and (C) DacB2-mRFP. DOI: https://doi.org/10.7554/eLife.37516.023
A flowthrough
2. wash 1
3. wash 2
4. elute 1
5. elute 2

37 kD
25 kD

37 kD
25 kD

1000 100 0 PenG (U/mL)
Bocillin FL (µM)

75 kD
25 kD

All lanes 2µM DacB2

Fragment A

Theoretical Isotope Distribution: [M+H]**

Observed Mass: [M+H]**

Fragment B

Theoretical Isotope Distribution: [M+H]**

Observed Mass: [M+H]**

Fragment C

Theoretical Isotope Distribution: [M+2H]**

Observed Mass: [M+2H]**

Fragment D

Theoretical Isotope Distribution: [M+2H]**

Observed Mass: [M+2H]**
**Figure 4—figure supplement 2.** MSMEG_2433 (DacB2) functions as a D,D-carboxypeptidase and D,D-endopeptidase in vitro. (A) Coomassie-stained gel of purified His$_6$-DacB2. (B) Bocillin-FL and Penicillin G binding assay of purified DacB2. (C) Mass spectra of the reaction products of DacB2 digestion reactions.

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Figure 4—figure supplement 3. CRISPRi knock-down of dacB2 in Msm lacking LDTs reduces bleb size. An anhydro-tetracycline (aTc)-inducible guide RNA targeting dacB2 was cloned into Msm containing a dCas9 as described previously (Rock et al., 2017). Bleb size was measured in ΔLDT cells without the dacB2 targeting guide RNA (grey) as well as ΔLDT cells with (+aTc) and without (-aTc) induction of the guide RNA. Mann-Whitney U test p-value=0.0070. DOI: https://doi.org/10.7554/eLife.37516.026
Figure 5. Drugs targeting both PBPs and LDTs kill mycobacteria more rapidly when combined (A, B). Killing dynamics of Msm (A) and Mtb (B) (expressing the luxABCDE operon from *Photorhabdus luminescens* [*Andreu et al., 2010*]) measured via luciferase production (RLU = relative light units). Amoxicillin (AM) (Msm-1.25; Mtb-3.125 µg/mL); Meropenem (MR) (Msm-10; Mtb-6.25 µg/mL); Amoxicillin + Meropenem: Msm-1.25 µg/mL AM +10 µg/mL MR; Mtb-3.125 µg/mL AM +6.25 µg/mL MR. Biological triplicate are plotted for Mtb. All drugs were used in combination with 5 µg/mL clavulanate.

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Figure 5—figure supplement 1. Light production (RLU) correlates to colony-forming units (CFU) in mycobacterial cells expressing luxABCDE in drug treatment. (A) *Mycobacterium smegmatis* CFU and luminescence (RLU) during drug treatment.

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Figure 5—figure supplement 2. Meropenem and Amoxicillin killing kinetics and minimum inhibitory concentration data. (A, B) Killing dynamics of ΔLDT (A) and ΔLDT<sub>comp</sub> (B) (expressing the luxABCDE operon from Photorhabdus luminescens [Andreu et al., 2010]) measured via luciferase production (RLU = relative light units). Amoxicillin (AM) (ΔLDT<sub>comp</sub> = 0.625 mg/mL; ΔLDT<sub>comp</sub> = 0.156 mg/mL); Meropenem (MR) (ΔLDT<sub>comp</sub> = 0.625 mg/mL; ΔLDT<sub>comp</sub> = 0.156 mg/mL); Amoxicillin + Meropenem: ΔLDT<sub>comp</sub> = 0.156 μg/mL AM + 0.625 μg/mL MR; ΔLDT<sub>comp</sub> = 0.156 μg/mL AM + 0.625 μg/mL MR. (C) Minimum inhibitor concentration (MIC) data calculated via alamar blue (resazurin). All drugs were used in combination with 5 μg/mL clavulanate. Synergy by fractional inhibitory concentration (FIC) is defined as a sum of FIC for each drug in the combination less than 0.5 (‘Synergism Testing: Broth Microdilution Checkerboard and Broth Macrodilution Materials and methods,’ 2016).

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Figure 6. Model for PG enzyme and substrate distribution as governed by polar growth and PG segregation by age. (A) A model for PG age, PG enzyme and crosslink segregation via polar growth in mycobacteria. First, 4–3 crosslinks are made by PBPs at site of new growth, where the pentapeptide substrate resides. Then, these 4–3 crosslinks can be cleaved by D,D-endopeptidases (END). This action would leave a free tetrapeptide. Lastly, LDTs can utilize this tetrapeptide to generate 3–3 crosslinks. As this is occurring over time and during polar growth, the aging cell wall moves toward mid-cell (new growth at the poles moves away from the existing cell wall). (B) Schematic of PG segregation by age (top). 2 min FDAA pulse (cyan), 45 min outgrowth, followed by 2 min FDAA chase (magenta) in WT Msm cells (bottom). Newest cell wall (magenta), older cell wall (cyan). Scale bar = 5 μm.

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