Microorganisms are often studied as populations but the behaviour of single, individual cells can have important consequences. For example, tuberculosis, caused by the bacterial pathogen Mycobacterium tuberculosis, requires months of antibiotic therapy even though the bulk of the bacterial population dies rapidly. Shorter courses lead to high rates of relapse because subpopulations of bacilli can survive despite being genetically identical to those that are easily killed. In fact, mycobacteria create variability each time a cell divides, producing daughter cells with different sizes and growth rates. The mechanism(s) that underlie this high-frequency variation and how variability relates to survival of the population are unknown. Here we show that mycobacteria actively create heterogeneity. Using a fluorescent reporter and a fluorescence-activated cell sorting (FACS)-based transposon screen, we find that deletion of lamA, a gene of previously unknown function, decreases heterogeneity in the population by decreasing asymmetric polar growth. LamA has no known homologues in other organisms, but is highly conserved across mycobacterial species. We find that LamA is a member of the mycobacterial division complex (the ‘divisome’). It inhibits growth at nascent new poles, creating asymmetry in polar growth. The kinetics of killing individual cells that lack lamA are more uniform and more rapid with rifampicin and drugs that target the cell wall. Our results show that mycobacteria encode a non-conserved protein that controls the pattern of cell growth, resulting in a population that is both heterogeneous and better able to survive antibiotic pressure.

To identify genetic determinants of single-cell heterogeneity, we first identified a reporter of heterogeneity that was relevant to antibiotic efficacy appropriate for a genetic screen. Calcein AM is a non-fluorescent hydrophobic molecule that enters cells through passive diffusion. Once inside, calcein AM is cleaved by esterases to produce calcein, a charged fluorescent molecule that is trapped in the cytoplasm unless exported through active mechanisms. Both molecules are substrates for ABC transporters in eukaryotic cells. Therefore, we hypothesized that: (1) cells would not stain uniformly because of differences in uptake, esterase activity, and efflux; and (2) these differences between cells would account, in part, for differences in antibiotic susceptibility. When we stained a population of Mycobacterium smegmatis cells we saw heterogeneous fluorescence by flow cytometry spanning two orders of magnitude (Fig. 1a). By time-lapse microscopy we found that much of this heterogeneous appearance appeared to begin at the time of cell division (Supplementary Video 1). Daughter cells accumulated calcein unevenly, although the calcein intensity before division was uniform throughout the mother cell. The daughter cell that inherited the new pole from the previous round of division was consistently brighter than its sister cell, which inherited the old pole (Fig. 1b). To test whether heterogeneity in calcein accumulation could explain the variation in response to certain drugs, we used time-lapse microscopy and microfluidics to determine whether calcein fluorescence of individual cells before drug treatment correlated to survival after treatment with the antibiotic rifampicin, a first-line drug for tuberculosis (Fig. 1c). We found that bright cells were less likely to divide following antibiotic treatment than dim cells (Fig. 1d). Thus, calcein accumulation predicts susceptibility to rifampicin on a single-cell level.

To determine the mechanism underlying the variability in calcein staining, we designed a genetic screen to identify transposon mutants with altered calcein accumulation. We hypothesized that there could be two different types of mutations—those that altered the average behaviour of the population and those that changed the variation of the population. To detect each of these, we designed a FACS-based screen that would identify alterations in both the median and distribution of calcein staining in M. smegmatis transposon mutants (Fig. 2a). After staining with calcein, we used FACS to sort cells into eight bins, each representing around 12.5% of the population (Fig. 2b) and used deep sequencing to quantify transposon insertions per gene in each bin (Fig. 2c). Using the total insertions in the entire population, we calculated the effective fluorescence distribution for each gene as compared to wild-type (Fig. 2d).

We found many mutations that affected the average fluorescence (Fig. 2e, Supplementary Table 1). Several of these were in genes involved in transport/permeability across the membrane. For example, among those with higher fluorescence, we found insertions in an operon that encodes two putative efflux pumps (msmeg5660-5659) and enzymes required for the modification of mycolic acids that constitute a major component of the cell wall (Fig. 3).

Figure 1 | Heterogeneity is important for survival in rifampicin. a. Flow cytometry of calcein-stained M. smegmatis cells (coefficient of variation (CV) = s.d./mean × 100). b. Wild-type cells were imaged over time in a microfluidic device while calcein AM was continuously added. At the time of cell division, the average calcein intensity of each daughter cell was measured. For 58 sister cell pairs, the ratio of the average calcein intensity of the new pole sister to the average calcein intensity of the old pole sister is calculated. c, d. Using the experiment outlined in c, the fluorescence of 96 individual cells is measured and compared to the number of progeny that same cell produced during and after rifampicin treatment, shown in d.

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portion of the cell wall (umaA). Likewise, we found that insertions in mspA, a gene that encodes a well-characterized porin known to permit entry of molecules through the M. smegmatis cell wall, resulted in lower median fluorescence. We constructed targeted deletions for several genes (Supplementary Table 2) and found that all had the predicted phenotypes (Fig. 3a, Extended Data Fig. 1a).

We expected that some, but not all, mutants would have altered killing by rifampicin. Indeed, we found mutations that might alter rifampicin accumulation substantially changed antibiotic susceptibility, either positively (ΔumaA, ΔtetR, ΔmmpS3) or negatively (ΔmspA, ΔlepA), whereas others that encode predicted esterases, enzymes that may cleave calcein AM (Δmsmeg_2500 and ΔcaEA), did not alter rifampicin killing in the predicted fashion (Extended Data Fig. 1b). Thus, our mutational data strongly suggest that calcein AM fluorescence correlates with the single-cell accumulation of rifampicin.

In addition to having an altered average fluorescence, several of the gene-deletion strains also had a different degree of cell-to-cell variation (Fig. 3b). The most notable difference was observed with ΔmsmA, which had a fluorescence distribution that was both broader and decreased in average. By contrast, two others—ΔmmpS3 and Δmsmeg5660-5659—produced both an increase in fluorescence and a narrowing of the distribution by both flow (Fig. 3b) and microscopy (for ΔmmpS3) (Extended Data Fig. 1c).

Heterogeneity in drug susceptibility has typically been studied in subpopulations of non-growing cells that are refractory to antibiotic treatment\(^2\). In time–kill curves this manifests as a biphasic curve, indicating two distinct subpopulations\(^1\). We hypothesized that the loss of heterogeneity in calcein (and, by proxy, drug) accumulation, would also lead to a difference in killing of the population. Indeed, by time-lapse microscopy, we observed that at drug concentrations close to the minimum inhibitory concentration (MIC) (Extended Data Table 1), some cells stopped growing or died whereas others continued to grow in the presence of drug. The growing subpopulation itself produced variable daughters: those that grew and those that did not, indicating a lack of heritability (Extended Data Fig. 2a, Supplementary Video 2).

We thus hypothesized that in a wild-type population of M. smegmatis cells, there were multiple subpopulations, each with a different effective MIC. Furthermore, a less-variable mutant population would result in a more ‘all or nothing’ phenotype in response to different concentrations of drug (Fig. 3c). To test this, we measured cell growth at different sub-inhibitory drug concentrations (Extended Data Fig. 2b). We reasoned that the growth of the bulk population at a fixed time is proportional to the percentage of growing cells in the population and their individual growth rates. To assess variability in the population, we fit the data to a sigmoid curve and calculated the slope; a steeper slope is associated with less variability. We then compared the two mutants ΔmmpS3 and ΔumaA. Both accumulated approximately twice the average amount of calcein as a wild-type population (Fig. 3a), but one varied less (ΔmmpS3), and the other more (ΔumaA) as compared to wild type (Fig. 3b). Consistent with our hypothesis, the slope was steeper for ΔmmpS3 and shallower for ΔumaA than wild type (Fig. 3d) and correlated extremely well with the measured calcein CV for the three strains tested (Fig. 3e). Consistently, by time-lapse microscopy, nearly all ΔmmpS3 cells stopped growing at drug concentrations that allowed for growth of a subpopulation of wild-type cells (Supplementary Video 2). Likewise, at drug concentrations normalized to the average calcein accumulation of each strain to correct for the intracellular accumulation of rifampicin, ΔmmpS3 survival decreased whereas the more variable ΔumaA strain had increased survival compared to wild type (Extended Data Fig. 2c). Together, these data suggest that ΔmmpS3 both accumulates drug more homogenously and is more uniformly susceptible to rifampicin than a wild-type population. We renamed mmpS3 as lamA (loss of asymmetry mutant A).

lamA is a previously uncharacterized gene. Although the protein product shares limited homology to the four other mspS genes in the M. tuberculosis genome (Extended Data Fig. 3a), it is distinctly different from the others (Extended Data Fig. 3b). It is over twofold larger and is the only mspS homologue that is not in an operon with an mspL gene. lamA homologues are found across mycobacterial species\(^12\), including in the minimal genome of M. leprae, but not in other organisms.

How does lamA create and maintain heterogeneity in a population of genetically identical M. smegmatis cells? Consistent with our observation in Fig. 1b, at the time of division, the asymmetry in daughter cell accumulation of calcein in ΔlamA cells was much reduced (by approximately 60%) (Fig. 3f, Supplementary Video 3). As a result, we hypothesized that LamA was involved in some aspect of mycobacterial cell growth and/or division.

Figure 2 | Screen for mutants altered in calcein distribution. a, b, An M. smegmatis transposon library was stained with calcein AM (a) and 1,200,000 cells were sorted into 8 bins by FACS (b), with each bin representing 12.5% of the population. c, d, Each of these binned libraries was deep sequenced (c) and distributions were made for each gene that represents the fraction of reads in each bin for that gene (d). Below are cartoons of potential distributions that are analogous to an effective fluorescent distribution for a single mutant. e, For each gene or intergenic region, the mean of the effective fluorescent distribution is determined and plotted against a Mann–Whitney U-test P value, which is derived from comparing the distribution of each gene to a calculated wild-type (WT) distribution. We picked nine genes (shown in red) and made eight gene-deletion strains (Supplementary Table 2).
Each of the eight gene-deletion strains is stained with calcein and three biological replicates are analysed by flow cytometry. a, The mean ratio of the median calcein fluorescence for each mutant compared to wild type. b, The mean ratio of the mutant CV compared to wild type CV. **P < 0.01, *P < 0.05, calculated by a two-sided Student's t-test in comparison to wild type; error bars represent s.d. assuming independence in the measurements of wild type and mutant (a, b). c, An illustrative summary of two potential mutants. In wild type, a subpopulation of cells can grow at drug concentrations that stop growth of the majority of the population. In a less variable mutant, this population represents less of the total population, and cells exhibit a more 'all or nothing' phenotype. In a mutant that is altered in the average behaviour, but maintains wild-type-level variability, the growing subpopulation continues to exist, although it stops growing at lower concentrations than wild type. The MIC (purple arrow) is lower in either case. d, Cell density in the presence of a range of rifampicin concentrations compared to a no-drug control for three strains (n = 2 biological replicates): wild type, ΔlamA, and ΔlamAΔ. The solid line represents the fit of the data to a sigmoid function. e, The ratio of calcein accumulation between sister cells is measured for wild-type and for ΔlamA cells (n = 58 sister pairs for wild type (grey); n = 49 sister pairs for ΔlamA (blue); dark black lines represent medians; ***P < 0.001, calculated by two-sided Student's t-test with Welch’s correction in comparison to wild type).

In contrast to rod-shaped bacteria that insert cell wall material along their sidewalls, mycobacteria insert new cell wall at or near their poles. Under our growth conditions, polar growth is asymmetric: for a single cell, the new pole grows less over the course of the division cycle, than the old pole. Surprisingly, loss of lamA changes this pattern of growth. In ΔlamA cells, new cell wall material is added at more similar rates between the old and new poles (Fig. 4a, Extended Data Fig. 4a, b), although the overall growth of individual cells (Extended Data Fig. 4c, d) and the population (Extended Data Fig. 4e, f) is the same as wild-type cells. Thus, lamA is at least partially responsible for asymmetric polar growth in mycobacteria.

The molecular details of cell growth and division in mycobacteria are poorly understood as compared to other rod-shaped bacteria. Mycobacteria do not encode obvious homologues of many cell elongation and division factors. Therefore, to further understand lamA-mediated asymmetric growth, we visualized the two best-characterized factors in the division and elongation complexes, FtsZ and Wag31 (DivIVA), in wild-type and mutant cells. FtsZ and Wag31 localize early to the site of division or elongation, respectively, and recruit other factors into active complexes. In cells that expressed FtsZ–mCherry2B and eGFP–Wag31, by time-lapse microscopy, we observed dynamic changes in the localization of each of these proteins in the absence of lamA during the transition between division and growth at the new pole. The division complex, as defined by FtsZ–mCherry2B, more rapidly decreased at the end of the cell cycle (Extended Data Fig. 5a–d, Supplementary Videos 4 and 5), whereas the elongation complex, as defined by eGFP–Wag31, was recruited more rapidly to the site of division and, thus, to the site of the new pole (Extended Data Fig. 5a, c, d, Supplementary Videos 6 and 7). These data suggest that LamA functions at the site of division, during the switch between division and elongation. Indeed, a GFPmut3–LamA (ref. 14) fusion protein localizes to the septum (Fig. 4b) at the same time that FtsZ–mCherry2B becomes stabilized in wild-type cells (Extended Data Figs 5a, b, Supplementary Video 8).

Therefore, we hypothesized that LamA is a member of the mycobacterial division complex (the ‘divisome’) that inhibits polar growth at the nascent new pole. To test whether LamA is a member of the division complex, we immunoprecipitated an epitope-tagged LamA and identified co-precipitating proteins using mass spectrometry. We found three unique peptides corresponding to PonA1, but none was present in a control sample that lacked the epitope tag (Supplementary Table 5). PonA1 is the major peptidoglycan-synthesizing enzyme in mycobacteria and is known to be a member of the division complex and an early member of the elongation complex. To verify this interaction, we constructed a strain that expressed lamA and ponA1, each tagged with a distinct epitope. We found that LamA could be precipitated with an antibody directed against the PonA1 epitope (Fig. 4c) only in the presence of epitope-tagged PonA1.

To test whether LamA inhibits cell wall synthesis, we overexpressed LamA using an inducible promoter on a multi-copy plasmid. Almost immediately after induction, we observed that, on average, cells added less cell wall material between successive generations (Extended Data Fig. 6a, Supplementary Video 9) suggesting that, indeed, LamA inhibits cell wall synthesis. Furthermore, as we followed individual cells over
several generations, we observed that not all induced cells behaved in the same way. Cells with the oldest poles—poles that were established before inducer was added—were almost unaffected by the addition of the inducer, whereas cells with the newest poles—poles that were established before inducer was added—were killed more rapidly than wild-type or complemented cells by vancomycin, rifampicin (Extended Data Fig. 7f) and by time-lapse microscopy (Supplementary Video 10). Thus, LamA may be an attractive drug target for pathogenic mycobacterial species. In fact, M. tuberculosis cells that lacked lamA were killed more rapidly than wild-type or complemented cells by vancomycin and rifampicin (Fig. 4d).

To determine how the amount of LamA-mediated heterogeneity compares to other organisms, we measured the length of cells just before division. We found that the amount of variation in wild-type M. smegmatis cells (19%) is much higher than ΔlamA M. smegmatis cells (12%) (Fig. 4e) and other rod-shaped bacteria17,18, including Corynebacterium glutamicum (Extended Data Fig. 8), a close relative of mycobacteria with similar cell wall architecture and polar elongation but that does not encode LamA. Importantly, this loss of heterogeneity could be restored in ΔlamA cells by complementation with lamA (Extended Data Fig. 9).

Together, these data show that LamA is a member of the mycobacterial division complex. It functions to inhibit cell wall synthesis at the new pole during the switch between division and elongation. Consequently, asymmetric growth is established between the new and old poles, which in turn leads to a more heterogeneous population of cells. Reducing heterogeneity in pathogen populations could decrease the rate of treatment failure. For example, some compounds subvert variability in HIV populations19. In M. tuberculosis, targeting LamA could lead to synergistic therapies that shorten treatment duration and increase the rate of cure.

**Figure 4 | LamA creates asymmetry in polar growth and is a member of the divisome.** a, Using a pulse–chase experiment with an amine-reactive dye, as described in ref. 2, the amount of growth at the new and old poles is measured over the duration of the cell cycle. Left, the ratio of growth (new pole/old pole) for single cells (n = 137 cell for wild type; n = 125 cells for ΔlamA; dark black lines represent medians; the absolute values are shown in Extended Data Fig. 6a). Right, Using the same pulse–chase experiment, growth is followed after septation at finer time increments. Time refers to the time after septation (old pole, open circles; new pole, closed circles; points represent averages of 20 cells). b, A representative image at a single time point of GFPmut3–LamA expression in wild-type M. smegmatis cells. Data were recorded for around 100 cells at t = 0. c, Anti-streptactin western blot of immunoprecipitates by indicated method (streptactin or anti-Flag). Pull-down with anti-Flag was performed twice and LamA–strept was seen both times. (i) and (ii) are different strains with pona1-Flag or lamA-strept integrated at the indicated phage sites (L5 or twee (tw)). d, Survival of wild-type M. tuberculosis (grey), ΔlamA (blue), and a complemented strain (yellow) is measured over time in the presence of 0.3 μg ml⁻¹ rifampicin or 4 μg ml⁻¹ vancomycin for three biological replicates. (Dotted line represents limit of detection; *P < 0.05, Student’s t-test in comparison to wild type.) e, The distribution for the length of cells just before division (mother cells) for wild-type (n = 120) and ΔlamA (n = 120) cells. ***P < 0.001, two-sided Student’s t-test with Welch’s correction to compare either mean (a) or variation (e). to grow and divide in the presence of vancomycin. In fact, wild-type M. smegmatis cells were able to grow even at vancomycin concentrations above the MIC (Supplementary Video 10) and, thus, as has been previously observed with other antibiotics in mycobacteria16, the bacterial number in the presence of drug, as measured by outgrowth on agar plates, reflects a balance of growing and non-growing or dead cells. In a ΔlamA strain, this heterogeneity is markedly reduced as measured by growth of the population as a function of drug concentration (Extended Data Fig. 7f) and by time-lapse microscopy (Supplementary Video 10). Thus, LamA may be an attractive drug target for pathogenic mycobacterial species. In fact, M. tuberculosis cells that lacked lamA were killed more rapidly than wild-type or complemented cells by vancomycin and rifampicin (Fig. 4d).

**Online Content** Methods, along with any additional 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.

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Author Contributions E.H.R. and E.J.R. designed experiments, analysed data, and wrote the paper. E.H.R. performed all microscopy experiments, flow cytometry experiments, the transposon screen, and the immunoprecipitation experiments. R.E.A. generated the M. tuberculosis strains and performed all M. tuberculosis experiments. E.H.R. and R.E.A. performed the M. smegmatis time-kill curves. E.H.R. developed all analysis tools including the image analysis tools and the transposon screen analysis. E.J.R. supervised the project.

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METHODS
Bacterial strains and culture conditions. Mycobacterium smegmatis MC2 155 was grown in 7H9 salts (Becto-Dickinson) supplemented with: 5 g 1−1 albumin, 2 g 1−1 dextrose, 0.85 g 1−1 NaCl, 0.003 g 1−1 catalase, 0.2% glycerol and 0.05% Tween80, or plated on LB agar. M. tuberculosis H37Rv was cultured in Middlebrook 7H9 salts supplemented with OADC (oleic acid, albumin, dextrose, catalase (BD Biosciences)), 0.25% glycerol, and 0.05% Tween-80 or plated on 7H10 agar. E. coli DH5α was used for cloning. Antibiotic concentrations used for M. smegmatis and M. tuberculosis were: 25 μg ml−1 kanamycin, 20 μg ml−1 zeocin, and 20 μg ml−1 hygromycin. Antibiotic concentrations for E. coli were: 50 μg ml−1 kanamycin and 100 μg ml−1 hygromycin. All strains were grown at 37° C. Coryne glutamicum was grown in Brain–Heart Infusion Broth.

Strain construction. All knockouts were made using recombineering. In M. smegmatis, the genes in Supplementary Table 2 were replaced with a zeocin resistance cassette, byloxP sites. Constructs were designed to be assembled into a pUC57 vector by isothermal assembly. Primers are listed in Supplementary Table 3: homology to M. smegmatis genome shown in lowercase; homology to pUC57 vector (upper primer for each gene) and zeocin resistance cassette (lower primer for each gene) shown in uppercase. For the M. tuberculosis knockout, a construct was synthesized comprised of 500 bp of homology (shown assembled into a pUC57 vector by isothermal assembly. Primers are listed in Supplementary Table 2) surrounding a HygR cassette and oxoP sites. For genes in operons, internal–gene deletions were made. The complementing strains were made by amplifying lambA (MSEG, 426S or Rv2198C) with 300 bp of the sequence upstream of the gene (primers listed in Supplementary Table 4) to capture the native promoter. These were assembled into a KanR plasmid and integrated in single copy at the L5 phage integration site. The fluorescent protein fusions: ftsZ-mcherry2B, eGFP-wag31 were expressed under a medium-strength promoter (p’pb21’); lambda GFP-pmut3 was constructed using the pUV15 promoter. Each was integrated into the M. smegmatis genome at phage integration sites (L5 for eGFP-wag31 and GFP-pmut3-lambA; twenty (tw) for ftsZ-mcherry2B) as merodiploids. To test the interaction between PonA1 and LamA constructs were made in which lamA and/or ponA1, both under the pUV15 promoter, were integrated onto the chromosome at different phase integrate sites (L5 for ponA1 (ref. 15), twenty for lamA). To make the inducible overexpression strain, LamA was sub-cloned into a multi-copy, episomal vector carrying both the tet operator and the tet repressor.

Flow cytometry. Mid-log-phase cultures were stained with 0.5 μl ml−1 calcein for 1 h and then filtered through a 10-μm filter. These cells were then analysed by flow cytometry (MACSQuant VYB excitation: 488 nm, emission filter: 525/50). To determine the appropriate gates for single cells, a pilot experiment was performed in which equal numbers of RFP- and GFP-expressing cells were grown together in triplicate (or duplicate for Fig. 3c to measure all three stains on the same 96-well plate, with shaking at 37 °C. Drugs were added once at the beginning of the experiment. For measurements using anti-Flag beads, all steps were the same except for the addition of SDS, protease inhibitors. This was then split between two tubes with glass beads and beads were beat 8 times for 1 min each, in-icing in between. For pulldowns using streptactin, SDS was added to the lysate (final concentration of 1%), and then was pre-cleaned of endogenously biotinylated proteins using avidin agarose for 1 h at room temperature. This was then spun down and the supernatant was added to beads coated with streptactin, and incubated for 30 min at room temperature. The beads were washed three times with Buffer W + 1% SDS, and eluted using 50 μl Buffer containing 10 mM biotin. For experiments using anti-Flag beads, all steps were the same except for the addition of SDS, the pre-clearing with avidin, and the elution was carried out with Flag peptide.

Survival measurements. Strains were grown to mid-log phase, diluted to OD 0.05 in triplicate, and treated with the indicated antibiotic in 7H9 and incubated with shaking at 37°C. Drugs were added once at the beginning of the experiment. Cultures were treated in culture tubes or ink wells. A small volume of cells was taken out at the specified time point, serially diluted, and plated for survivors by colony-forming units. Each of the M. smegmatis time–kill curves was performed at least twice, each time in triplicate, with similar results. The rifampicin kill curve in M. tuberculosis was performed twice—once in duplicate and then again in triplicate (this one is shown in Fig. 4d)—with similar results, whereas the M. tuberculosis vancomycin time–kill curve was performed twice at different vancomycin concentrations with similar results.

OP60nm measurements. Strains were grown to mid-log phase, diluted to OD 0.2 in triplicate (or duplicate for Fig. 3c to measure all three stains on the same 96-well plate). Water was added to the outermost wells of a 96-well plate. Two-fold serial dilutions of the appropriate drug were added to inner wells of the plate. Both a no-drug control and a no-bacteria control were included. Cells were added for a final OD of 0.1. This was incubated at 37°C with shaking in a TecoB 200 plate reader, and optical density measurements at 600 nm were measured automatically every 30 min for approximately 18 h. The optical density of the wells exposed to drug were background subtracted and divided by the no-drug control.

MIC determination. The MIC of M. smegmatis and M. tuberculosis strains were performed using the Alamar Blue assay as described in ref. 15.

Statistical analysis. All experiments were performed at least twice, unless otherwise noted. Means were compared using a two-sided Student's t-test. In the cases where sample variances differed we used Welch's correction, which does not assume equal variances. To compare the average and/or variation of two samples. For the data shown in Fig. 2e, we used a non-parametric test (Mann–Whitney), as some genes displayed non-normal distributions. P < 0.05 was considered significant. For most microscopy experiments, roughly 100 cells were analysed (or 50 sister cell pairs). For experiments that followed cells over shorter time increments,
approximately 20 cells were analysed. For ratios, as in Fig. 3 and Extended Data Fig. 1, error in the ratio (in terms of standard deviation) was calculated assuming independence in the two measurements. No statistical methods were used to predetermine sample size, and the researchers were not blinded to sample identity. **Data availability.** Source Data and Supplementary Information are provided in the online version of the paper. All other data that support the findings of this study are available from the corresponding author(s) upon reasonable request.

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Extended Data Figure 1 | Validation of screen and identification of mutant with decreased heterogeneity. 
a. The average of three biological replicates of the eight gene-deletion strains is stained with calcein and analysed by flow cytometry. The median of the distribution of each strain (in units of wild-type s.d.) is compared to the value expected from the screen (slope = 1; \( R^2 = 0.95 \); error bars represent s.d. assuming independence in the measurements of wild type and mutant).
b. Survival of three biological replicates of each strain is measured by plating and counting survivors after 42 h in rifampicin treatment and compared to wild type (**\( P < 0.01 \), *\( P < 0.05 \), calculated by a two-sided Student's \( t \)-test in comparison to wild type).
c. The average fluorescence value of calcein-stained \( M. \) smegmatis cells measured by microscopy (\( n = 147 \) for wild type; \( n = 174 \) for \( \Delta \)lamA).
Extended Data Figure 2 | Loss of lamA results in a more homogenous response to drug. a, Two examples of growing lineages of wild-type M. smegmatis cells exposed to 5 μg ml⁻¹ rifampicin. Blue, growing cells; pink, non-growing cells. The thick dotted line indicates the time at which rifampicin was added. Thin dotted lines represent division events. Data for around 100 of cells were recorded. The behaviour of single cells was highly variable: many cells stopped growing immediately whereas others were able to grow and divide in a non-heritable manner. Bulk measurements were recorded in Fig. 3d as a way of quantifying the variability over many more cells (approximately 1,000,000 cells). b, The geometric mean of two biological replicates for wild-type and mutant M. smegmatis cells growing in sub-MIC concentrations of rifampicin. c, The mean survival of four biological replicates at 40 h in rifampicin at normalized drug concentrations: wild type = 10μg ml⁻¹; ΔlamA = 5μg ml⁻¹; ΔumaA = 5μg ml⁻¹.
Extended Data Figure 3 | *mmpS3* is different than the other *mmpS* genes. a, A clustal omega alignment of all the mycobacterial membrane protein, small (*mmpS*) genes in the *M. tuberculosis* genomes. b, The percentage identity of the *mmpS* genes are compared to the others.
Extended Data Figure 4 | Growth properties of ΔlamA cells compared to wild type.  

**a**, Using a pulse–chase experiment as in Fig. 4a, the amount of growth at the new and old poles (total growth = new pole + old pole) over the duration of a cell cycle is measured for both wild-type (grey, \( n = 137 \)) and ΔlamA (blue, \( n = 125 \)) cells. Dark black lines represent medians.  

**b**, Slopes were fitted to the data displayed in Fig. 4a. A \( P \) value was calculated using a \( t \)-test to compare the slopes of wild type and ΔlamA.  

**c, d**, Growth of wild type (grey), ΔlamA (blue), and complement (yellow) as measured by elongation rate (c, \( n = 49 \) for wild type; \( n = 71 \) for ΔlamA) and cell cycle time (d, \( n = 49 \) for WT; \( n = 71 \) for ΔlamA). Dark black lines represent medians.  

**e, f**, Optical density (e) and colony-forming units (f) are measured over time for three biological replicates.
Extended Data Figure 5 | LamA functions during the switch between division and elongation. a, The maximum values of FtsZ–mCherry2B, eGFP–Wag31, and GFPmut3–LamA in the middle of the cell are measured as a function of cell cycle time in both wild-type and ΔlamA cells. Shaded areas represent standard deviations across 20 cells for each strain. b, An example of the time lapse images that were quantified for a. Each panel is 15 min apart. Arrows, point to the appearance of either FtsZ or LamA at the septum. Data was recorded around 100 cells, and 20 cells were used for fine quantification shown as shown in a. Scale bar, 5 μm. c, d, For each phase indicated in a, the data were fit to a line and the slope was calculated; the averaged data are shown in c, while slopes for individual cells are plotted in d.
Extended Data Figure 6 | Overexpression of LamA inhibits growth at the new pole. **a**, The birth length of a strain carrying a replicating plasmid with LamA under an inducible promoter, pFetOR (circles), is compared to wild type (squares, \( n = 142 \)) in the presence (red) or absence (grey) of inducer (aTc) over many division cycles (\( n = 10 \) division 0; \( n = 20 \) division 1; \( n = 36 \) division 2; \( n = 60 \) division 3). **b**, Average birth length of 10 cells as a function of birth length before inducer is measured for individual cells overexpressing LamA. Oldest pole cells have poles that were established before inducer was added. Newest pole cells have poles that were the newest in the presence of inducer. (***\( P < 0.001 \) by a two-sided Student's \( t \)-test with Welch's correction.)
Extended Data Figure 7 | Loss of lamA leads to more uniform drug response for a variety of antibiotics. a, Single-cell intensity of fluorescent-vancomycin-stained cells ($n = 151$ wild type; $n = 140$ ΔlamA; $n = 126$ ΔlamA L5::lamA). Black lines represent fit of the data to a Gaussian curve. Survival over time for wild-type M. smegmatis (grey), ΔlamA (blue), and the complemented strain (yellow) are measured using colony-forming units ($n = 3$ biological replicates). b, Rifampicin 20 μg ml$^{-1}$; c, teicoplanin 100 μg ml$^{-1}$; d, ceftrioxone 50 μg ml$^{-1}$ with 5 μg ml$^{-1}$ clavulanate; e, vancomycin 3 μg ml$^{-1}$. (*P < 0.05 by a two-sided Student's $t$-test in comparison to wild type, dotted line represents limit of detection.) f, Cell density of three biological replicates in the presence of a range of vancomycin concentrations compared to a no-drug control for two strains: wild-type M. smegmatis and ΔlamA. The solid line represents the fit of the data to a sigmoid function. $s$ is the best-fit value for the slope.
Extended Data Figure 8 | The variation in a lamA deletion population is similar to the variation seen in other rod-shaped bacteria. a. The variation in length of cells at the time of division (mother cells) for a variety of rod-shaped bacteria. b. Length of 130 C. glutamicum cells before division. Black line represents the fit of the data to a Gaussian function.

| Organism        | CV of mother cell length | Reference |
|-----------------|--------------------------|-----------|
| E. coli         | 11%                      | 17        |
| C. crescentus   | 12%                      | 17        |
| B. subtilis     | 11-12%                   | 18        |
| C. glutamicum   | 10%                      | panel b   |
Extended Data Figure 9 | The addition of a copy of lamA onto the chromosome under the native promoter complements all phenotypes and restores heterogeneity: Wild-type (grey), ΔlamA (blue), and complement (yellow). a, Ratio of daughter cells at the time of division (n = 71 sister cell pairs for wild type; n = 63 sister cell pairs for ΔlamA; n = 60 sister cell pairs for complement). b, c, The length of cells at the time of division (b; n = 71 cells for wild type; n = 63 cells for ΔlamA; n = 60 cells for complement) and birth (c; n = 142 cells for wild type; n = 126 cells for ΔlamA; n = 120 cells for complement). Using a pulse–chase experiment as in Fig. 4a and Extended Data Fig. 6a, growth at the poles is measured. d, e, The absolute values (d) and the ratio (e) of new pole growth to old pole growth. n = 137 cells for wild type; n = 125 cells for ΔlamA; n = 45 cells for complement. Dark black lines represent medians.
Extended Data Table 1 | MICs of various strains

| Strain   | Drug      | MIC       |
|----------|-----------|-----------|
| Msm (WT) | Rifampicin| 2.5 μg/ml |
| Msm ΔλmA | Rifampicin| 0.625 μg/ml |
| Msm ΔurnaA | Rifampicin| 2.5 μg/ml |
| Msm (WT) | Vancomycin| 2.5 μg/ml |
| Msm ΔλmA | Vancomycin| 0.3125 μg/ml |
| Mtb (WT) | Vancomycin| 2.25 μg/ml |
| Mtb ΔλmA | Vancomycin| 0.28 μg/ml |

The MIC (minimum inhibitory concentration) of *M. smegmatis* and *M. tuberculosis* strains used in this study exposed to either vancomycin or rifampicin.