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Evolutionary rescue of spherical *mreB* deletion mutants of the rod-shape bacterium *Pseudomonas fluorescens* SBW25

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eLife Assessment

This **valuable** study combines evolution experiments with molecular and genetic techniques to study how a genetic lesion in MreB that causes rod-shape cells to become spherical, with concomitant deleterious fitness effects, can be rescued by natural selection. The results are **convincing**, although further improvement of the statistical analyses and figure presentation, and further clarification of the concrete contribution of the paper and how it relates to previous literature, would be welcome.

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Abstract

Maintenance of rod-shape in bacterial cells depends on the actin-like protein MreB. Deletion of *mreB* from *Pseudomonas fluorescens* SBW25 results in viable spherical cells of variable volume and reduced fitness. Using a combination of time-resolved microscopy and biochemical assay of peptidoglycan synthesis, we show that reduced fitness is a consequence of perturbed cell size homeostasis that arises primarily from differential growth of daughter cells. A 1,000-generation selection experiment resulted in rapid restoration of fitness with derived cells retaining spherical shape. Mutations in the peptidoglycan synthesis protein Pbp1A were identified as the main route for evolutionary rescue with genetic reconstructions demonstrating causality. Compensatory *pbp1A* mutations that targeted transpeptidase

activity enhanced homogeneity of cell wall synthesis on lateral surfaces and restored cell size homeostasis. Mechanistic explanations require enhanced understanding of why deletion of *mreB* causes heterogeneity in cell wall synthesis. We conclude by presenting two testable hypotheses, one of which posits that heterogeneity stems from non-functional cell wall synthesis machinery, while the second posits that the machinery is functional, albeit stalled. Overall, our data provide support for the second hypothesis and draw attention to the importance of balance between transpeptidase and glycosyltransferase functions of peptidoglycan building enzymes for cell shape determination.

Introduction

The rescue of fitness-compromised mutants by selection is a useful strategy to obtain new understanding into the molecular determinants of complex phenotypes (Remigi et al. 2019; LaBar et al. 2020). The approach has shed light on factors such as the flexibility of regulatory networks (Lind et al. 2015), the diversity of structural components affecting ecological success (Lind et al. 2017), the predictability of evolution (Lind et al. 2019), the origins of new genes (Näsvall et al. 2012), and the causes of changes in interactions among bacteria and their hosts (Remigi et al. 2014). Here we turn attention to the evolutionary rescue of a *Pseudomonas fluorescens* mutant lacking MreB, a key component of cell shape (Errington 2003) that is conserved in a broad range of rod-shaped bacteria (Shi et al. 2018).

For model organisms, notably, *Escherichia coli* and *Bacillus subtilis*, the molecular mechanisms underpinning cell shape have been the subject of intensive investigation (Shi et al. 2018; van Teeffelen and Renner 2018; Egan et al. 2020). The primary determinant of bacterial cell shape in both Gram positive and Gram negative cells is peptidoglycan. Its synthesis and assembly is directed by two main multiprotein complexes that each comprise a combination of enzymes and regulatory elements involved in cell wall dynamics, DNA segregation and cell division (Daniel and Errington 2003; Young 2003; Osborn and Rothfield 2007; Young 2007; Huang et al. 2008; Young 2010). The first of these, the elongasome (or rod complex), inserts newly synthesised peptidoglycan strands into the lateral wall of the growing cell, while the divisome participates in septum positioning and cell wall constriction (van Teeffelen and Renner 2018). Both elongasome and divisome rely on penicillin-binding proteins (PBPs) to polymerize (transglycosylate) and / or to cross-link (transpeptidate) newly synthesised peptidoglycan strands.

The elongasome consists of several catalytically interacting components including the transglycosylase (TGase) RodA (Cho et al. 2016), the transpeptidase (TPase) Pbp2 (Ishino et al. 1986), that function in concert with class the A PBP (aPBP), Pbp1A, which has both TGase and TPase activities (Randich and Brun 2015). Activity of Pbp1A, while not strictly a member of the elongasome, depends on the outer membrane-anchored protein LpoA (Sardis et al. 2021), with Pbp1A interacting with Pbp2 (Banzhaf et al. 2012; Egan et al. 2020; Kang and Boll 2022). Central to elongasome function – and essential for construction of rod-shaped cells – is the actin-like protein MreB. MreB forms helical-like structures tethered to the inner membrane and coordinates the pattern of new wall growth along the lateral part of the cell cylinder through interactions with other components of the elongasome (Typas et al. 2012; Shi et al. 2018). Indicative of a balance between diffusive synthesis affected by class A PBPs and processive activities of the elongasome is the fact that deletion of classA PBPs leads to up-regulation of rod complex activity (Patel et al. 2020), with evidence suggesting that the two systems work semi-autonomously (Cho et al. 2016).

Most bacteria contain a second aPBP, Pbp1B, which like Pbp1A, has both TGase and TPase activity. The two aPBPs are partially redundant (Yousif et al. 1985), but based on localisation of Pbp1B to the mid-cell during division (Bertsche et al. 2006), the standard view is that Pbp1B contributes to divisome function (particularly through interaction with PBP3), whereas Pbp1A contributes to cell elongation (Egan et al. 2020). However, it is apparent that Pbp1B also contributes to elongation (Typas et al. 2012; Cho et al. 2016), with evidence from *E. coli* that its role may be more important than Pbp1A (Vigouroux et al. 2020). In particular it was recently shown that Pbp1B responds to cell wall damage by repairing defects (Vigouroux et al. 2020) and that the bound (enzymatically active) form of Pbp1B is elevated in the absence of Pbp1A (Vigouroux et al. 2020).

In both *E. coli* and *B. subtilis* (and most other rod-shape bacteria), MreB is required for maintenance of rod-shape (Shi et al. 2018). While deletion of the gene is lethal under normal growth conditions, inhibition of MreB function by depolymerisation via the drug A22 causes abnormal cell wall growth leading to spherical cells that swell and eventually lyse (Iwai et al. 2002). An earlier study in *B. subtilis* – where cells devoid of MreB were maintained in a viable state by culture in the presence of magnesium – found that transposon-induced mutations in *pbp1A* could rescue cell viability in the absence of magnesium, but could not return cells to rod-shape (Kawai et al. 2009).

Here, with interest in evolution of the machinery underpinning cell shape, we took advantage of prior knowledge that inactivation of *mreB* in the rod-shaped bacterium *P. fluorescens* SBW25, while reducing fitness, is not lethal (Spiers et al. 2002), and asked whether selection could compensate for this cost. Of particular interest was: 1, the capacity for compensatory evolution to restore fitness; 2, the number, timing and impact of fitness-restoring mutations and; 3, the connection between fitness-restoring mutations, growth dynamics and cell morphology.

Materials and methods

Bacterial strains and culture conditions

Escherichia coli and *Pseudomonas aeruginosa* were grown at 37°C; and *P. fluorescens* SBW25 at 28°C. Antibiotics were used at the following concentrations for *E. coli* and / or *P. fluorescens* SBW25: 12 μg ml^{-1} tetracycline; 30 μg ml^{-1} kanamycin; 100 μg ml^{-1} ampicillin. Bacteria were propagated in Lysogeny Broth (LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl per litre).

Strain construction

The Δ*mreB* and *Δpbp1A* mutants were constructed using SOE-PCR (splicing by overlapping extension using the polymerase chain reaction), followed by a two-step allelic exchange protocol[1]. All media for construction of *mreB* mutants were supplemented with 10 mM $MgCl_2$. Genome sequencing confirmed the absence of suppressor mutations. The same procedure was used to reconstruct the mutations from the evolved lines (*pbp1A* G1450A, *pbp1A* (A1084C), Δ*pflu4921-4925* (5399934-5403214del)) and ectopic Mini-Tn7T-*mreB* into ancestral SBW25 and Δ*mreB* backgrounds. DNA fragments flanking the mutation of interest were amplified using two primer pairs. The internal primers were designed to have overlapping complementary sequences that allowed the resulting fragments to be joined together in a subsequent PCR reaction. The resulting DNA product was TA-cloned into pCR8/GW/TOPO (Invitrogen). This was then subcloned into the pUIC3 vector, which was mobilised via conjugation into SBW25 with the help of pRK2013. Transconjugants were selected on LB plates supplemented with nitrofurantoin, tetracycline and X-gal. Allelic exchange mutants identified as white colonies were obtained, where possible, by cycloserine enrichment to select against tetracycline resistant cells, and tetracycline sensitive clones were examined for the deletion or mutations using PCR and DNA sequencing. Complementation of *mreB* in the Δ*mreB* background was achieved by cloning the PCR-amplified DNA fragment into pUC18-mini-Tn7T-LAC, which was subsequently integrated into the *P. fluorescens* SBW25 genome at a unique site located downstream of the *glmS* gene (Liu et al. 2014).

Growth dynamics

Strains were initially grown in 5 mL LB broth at 28°C overnight, except for Δ*mreB* which was grown for 2 days. Growth curves were started by adding 2 µl aliquots of overnight cultures to LB to a final volume of 200 µl (1:100 dilution) in 96-well microplates (3 replicates). Plates were incubated at 28°C (with shaking) for 24 to 48 hours in a BioTek Synergy 2 plate reader. Growth was measured at an absorbance of 600 nm and recorded at 5-minute intervals using the Gen5 software (Bio-Tek). Growth curves were processed as semi-log plots to identify the lag and exponential phases. Doubling times were calculated as: t = ln (2)/k.

Evolution experiment

Ten replicate populations of the Δ*mreB* strain were grown in 5 mL aliquots of LB broth at 28°C with shaking at 180 rpm. Every 24 h, 5 µL was transferred to fresh media. Every 5 days, samples of each population were collected and stored at −80°C in 15% (v/v) glycerol. The number of generations per transfer changed over the course of the experiment but was approximately ten generations per 24 h period (100 transfers (~1,000 generations) were performed).

Competitive fitness assay

Competitive fitness was determined relative to ancestral SBW25 marked with GFP. This strain was constructed using the mini-Tn*7* transposon system, expressing GFP and a gentamicin resistance marker (mini-Tn*7* (Gm)P_{rrnB} P1 gfp-a). Strains were grown to exponential phase in shaken LB at 28°C before beginning competitive fitness assays. Competing strains were mixed with SBW25-GFP at a 1:1 ratio by adding 150 uL of each strain to 5 mL LB and grown for 3 hours. Initial ratios were determined by counting 100,000 cells using flow cytometry (BD FACS Diva II). Suitable dilutions of the initial population were plated on LB agar plates to determine viable counts. The mixed culture was diluted 1,000-fold in LB, then incubated at 28°C for 24 hours. Final viable counts and ratios were determined as described above. The number of generations over 24 hours of growth were determined using the formula ln(final population/initial population)/ln (2). Selection coefficients were calculated using the regression model s = [ln (R (*t*)/R (0))]/[*t*], where R is the ratio of the competing strain to SBW25-GFP, and *t* is time. Control experiments were conducted to determine the fitness cost of the GFP marker in SBW25. For each strain, the competition assay was performed with a minimum of three biological replicates. Ancestral SBW25 had a relative fitness of 1.0 when compared to the marked strain, indicating that the GFP insert is neutral, and that the SBW25-GFP strain was a suitable reference strain for this assay.

Mutation detection

In order to detect mutations in the experimental lines, total DNA was extracted from the Δ*mreB* strain and from each of the 10 populations at 500 and 1,000 generations. Samples were submitted for 100 bp single-end Illumina DNA sequencing at the Australian Genome Research Facility in 2012. An average of 6,802,957 single reads were obtained per sample and these were assembled against the reference *P. fluorescens* SBW25 genome (GenBank accession: *OV986001.1*) using the clonal option of Breseq with default parameters (Deatherage and Barrick 2014). Mutations were identified with breseq's HTML output and in the case of predicted novel junctions manually inspected with Geneious Prime (*https://www.geneious.com*). The raw sequence files and breseq-generated data outputs are available at *https://doi.org/10.17617/3.CU5SX1*.

To detect the frequency of the *pbp1A* mutations in experimental lines at generation 50 a colony sequencing approach was used. Colony sequencing was used to prevent the confounding effects of altered cell size, and genomic content per cell, on estimates of the frequency of mutants. To ensure sequenced colonies were isogenic, the frozen glycerol stock from generation 50 was streaked on LB agar plates and resulting individual colonies were each re-streaked on LB agar. Resulting colonies were then used as a template for PCR, using primers that allowed amplification of all the mutations identified by Illumina Sequencing at 500 and 1000 generations. Sanger sequencing was used to identify mutations, with mutations only called if present in sanger sequencing from both forward and reverse primers, with sequences mapped to the *pbp1A* sequence from the SBW25 reference genome using Geneious Prime.

Microscopy

Phase contrast and fluorescence images were captured using the BX61 upright microscope (Olympus) at 100x using a Hamamatsu Orca Flash 4.0 camera. The microscope is equipped with an HXP lamp with appropriate multi-band pass filters for different channels. Cell^P/ Cellsens software (Olympus) was used to control the microscope. Cells were grown in LB, and harvested at log phase (OD_{600} 0.4). Viability assays were conducted using the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher). Viability was measured as the proportion of live cells in the total population (live/ (live +dead)). Nucleoid staining was performed using the DAPI nucleic acid stain (Thermo Fisher) following the manufacturer's protocols.

Time-lapse on agarose pads

Strains were inoculated in LB from glycerol stocks and grown with shaken overnight at 28°C. Cultures were then diluted 100-fold in fresh LB and seeded on a gel pad (1% agarose in LB). The preparation was sealed on a glass coverslip with double-sided tape (Gene Frame, Fischer Scientific). A duct was cut through the center of the pad to allow for oxygen diffusion into the gel. Temperature was maintained at 30°C using a custom-made temperature controller. Bacteria were imaged on a custom-built microscope using a 100X/NA 1.4 objective lens (Apo-ph3, Olympus) and an Orca-Flash4.0 CMOS camera (Hamamatsu). Image acquisition and microscope control were actuated with a LabView interface (National Instruments) (Julou et al. 2013). Typically, we monitored 10 different locations; images were taken every 5 min in correlation mode. Segmentation and cell lineage were computed using a MatLab code implemented from Schnitzcell (Locke and Elowitz 2009). Bacteria were tracked for 3 generations. In order to limit the number of generations, we monitored bacteria harvested from a liquid stationary phase. Since, bacteria shorten after entering stationary phase, bacteria lengthened when resuming growth under the microscope, so that the elongation rate is larger than the septation rate for the first generations (**Fig. 3B**). To obtain the control line in our experimental configuration, we measured these rates in three species (ancestral *P. fluorescens* SBW25, *E. coli* MG1655 and *P. aeruginosa* PAO1). Points below the line indicate a reduction of cell volume beyond the fact that cells simply resumed growth.

Scanning Electron Microscopy (SEM)

Cells were grown in LB, and harvested in log phase. Cells were fixed in modified Karnovsky's fixative then placed between two membrane filters (0.4μm, Isopore, Merck Millipore LTD) in an aluminum clamp. Following three washes of phosphate buffer, the cells were dehydrated in a graded-ethanol series, placed in liquid CO_2, then dried in a critical-point drying chamber. The samples were mounted onto aluminum stubs and sputter coated with gold (BAL-TEC SCD 005 sputter coater) and viewed in a FEI Quanta 200 scanning electron microscope at an accelerating voltage of 20kV.

Image analysis

Compactness and estimated volume measurements of cells from liquid culture. The main measure of cell shape, compactness or C, was computed by the CMEIAS (Liu et al. 2001) software as: √(4Area/π)/major axis length (Liu, J. 2001). Estimated volume or V_e was estimated with different formula, according to cell compactness, for spherical cells that have a compactness ≥ 0.7, V_e was computed using the general formula for ellipsoids: V=4/3π (L/2) $(W/2)^2$, where L=length and W=width. V_e of rod-shaped cells, defined as having a compactness value ≤ 0.7, were computed using the combined formulas for cylinders and spheres: $V_e = (\pi\ (W/2)^2\ (L\text{-}W)) + (4/3\pi\ (W/2)^3)$.

Cell volume and division axis of cells on agarose pads. Cell volume was computed from the mask retrieved after image segmentation. Masks were fitted to an ellipse. The elongation axis is given by the direction of the major axis. The division axis relative to the mother was computed by comparing the elongation axis between mother and sister cells using the following formula: $|sin\theta|$, where θ is the angle between mother and sister cells. $|sin\theta|=0$ means that mother and daughter cells are aligned, $|sin\theta|=1$ means that they are perpendicular.

Probability p to pass to the next generation

For the second generation on the agar pad, we computed the probability to pass to the next generation as the capability of cells to progress through the cell cycle and divide. Bacteria that did not grow or stopped elongating before dividing were classified as non-proliferating. For all non-proliferating bacteria, we confirmed that no division occurred during the next 5 hours. The *p* ratio was then simply computed as the number of viable daughter cells divided by the expected number. Error on the probability is estimated at the 95% confidence interval by the formula: 1.96 $\sqrt{p(1 - p)/N}$, where N is the total number of cells.

Elongation rate, septation rate, growth asymmetry and the precision of septum positioning

The elongation rate, *r*, measured in min^{-1} is the rate at which cell volume increases: $V(t)=V_0e^{rt}$, where *t* is the relative time in the cell cycle. The septation rate is given by $\frac{ln(2)}{\tau}$, where τ is the average division time, *i.e.*, the time between birth and septation. For all sister cell pairs, we computed relative difference in growth between sister cells by measuring the relative elongation rate (*i.e.*, volume extension rate) of sister cells at generation $F1: |\frac{r_2-r_1}{r_1+r_2}|$, where $r_{1,2}$ is the elongation rate for each cell of sister pairs. The population average $C_g = <abs(r_1-r_2)/(r_1+r_2)>_{cells}$. was computed on the proliferating sub-population in order to avoid trivial bias due to cell proliferation arrest of one of the two sister cells. We did the same for the relative difference in division time and measured $C_T=<abs(T_1-T_2)/(T_1+T_2)>_{cells}$., where $T_{1,2}$ are the division time of sister cells. For all cells at generation F1, we computed the precision of septum positioning by measuring the relative cell volume of the two daughter cells: $|\frac{V-V_1}{V+V_2}|$, where $V_{1,2}$ are the volumes of each daughter cell. The population average $C_s=<abs(V_1-V_2)/(V_1+V_2)>_{cells}$ was computed for every F1 cell having offspring.

Laplace argument

For a given geometry, cell volume is determined, at steady state, by an analog of the Young-Laplace law for thin elastic membranes, which relates the pressure discontinuity ΔP at the cell wall to membrane tension and radius of curvature R_s. The pressure jump is powered by turgor pressure in the cytoplasm. For spheres, having two identical curvature radii R_s, the law is $\Delta P_s = \frac{2T_s}{R_s}$. For cylinders, having only one finite radius of curvature R_c, the law is $\Delta P_c = \frac{T_c}{R_c}$. Assuming that turgor pressure and membrane tension are the same, $\Delta P_S = \Delta P_c$ and $T_S = T_C$, we obtain $R_s = 2R_c$. Hence, the ratio of volumes between the two geometries can be expressed as a function of the original ancestral SBW25 length L and width $2R_C$: $\frac{V_s}{V_c} = \frac{2^5 R_c}{3L}$, where V_S and V_c are the volume of the sphere and the cylinder, respectively. Hence a change from cylindrical to spherical shape should result in a volume increase by a factor 1.78 for *P. fluorescens*, with a diameter of 1µm and a length of 3µm (**Fig. 3A**).

Protein sequence alignment and modelling

Protein sequences were obtained from NCBI BLAST (*http://blast.ncbi.nlm.nih.gov*) and The *Pseudomonas* Genome Database (Winsor et al. 2016), and aligned using MEGA7 (Kumar et al. 2016). The sequence alignment was visualised using ESPript 'Easy Sequencing in PostScript' (Robert and Gouet 2014). Protein visualisation was performed using Visual Molecular Dynamics (VMD) (Humphrey et al. 1996) based on the crystal structure of *Acinetobacter baumannii* Pbp1a in complex with Aztreonam as the base model (Pbp1A from *A. baumannii* shares a 73% amino acid sequence identity (E value = 0.0) to the Pbp1A of *P. fluorescens* SBW25). Sequences were aligned, and locations of the mutations in the evolved lines were mapped in the corresponding regions. The PDB file was downloaded from the RCSB Protein Data Bank (*www.rcsb.org*) using PDB ID 3UE0.

Membrane preparation and detection of penicillin-binding proteins

For the membrane preparation of the different genotypes of *P. fluorescens* SBW25, 150 mL cell cultures were grown to an OD_{600} of 0.6 and harvested by centrifugation at 15,000 x g for 15 minutes at 4 °C (Beckman JLA-16.250 rotor, Beckman Avanti J-25, Beckman Coulter). Pellets were washed with 20 mM potassium phosphate (pH 7.5) and 140 mM sodium chloride buffer. Following another 30 minutes of centrifugation at 360 x g at 4 °C, cells were disrupted by passing twice through a French press ('Pressure Cell' Homogenizer FPG12800, Stansted Fluid Power Ltd). Cell lysates were subjected to centrifugation at 360 x g for 10 minutes at 4 °C. Supernatant fractions were collected and centrifuged at 75,600 x g for 90 minutes at 4 °C (Beckman JA25.50 rotor, Beckman Avanti J-25, Beckman Coulter). The resulting pellets were washed once again and resuspended in 100 µL potassium phosphate buffer. The concentration of the membrane preparations was measured using Bradford reagent (BioRad) with bovine serum albumin as a standard (Sigma).

For detection of PBPs, membranes were incubated for 30 minutes at 37 °C with a fluorescent labelling agent, Bocillin FL Penicillin (Invitrogen, Thermo Fisher Scientific) (final concentration: 0.05 mM). Finally, the samples were denatured with 5x SDS loading buffer with ß-mercaptoethanol as a reducing agent at 100 °C for 5 minutes, followed by centrifugation to remove membrane debris. 20 µL of reaction mixture was subjected to SDS-PAGE analysis on an 8% polyacrylamide gel, protected from the light. Bocillin FL-labelled PBPs were visualised with a laser scanner, Typhoon FLA 9500 (GE Healthcare Life Sciences) at 473 nm with a 530DF20 emission filter.

Peptidoglycan isolation and analysis

For peptidoglycan profiling and analysis, *P. fluorescens* genotypes were grown in LB medium to an OD_{600} of 0.2 and harvested. Purification of peptidoglycan was carried out as previously described (Desmarais et al. 2013) with some minor changes. Briefly, the cell pellets were boiled in an equal volume of 5% (w/v) SDS for approximately an hour and left stirring overnight at room temperature. The sacculi were washed repeatedly with MilliQ water by ultracentrifugation (150 000 × g, 15 min, 20 °C TLA100.3 Beckman rotor; OptimaTM Max ultracentrifuge Beckman, Beckman Coulter) until SDS was eliminated from the samples. The clean sacculi were digested with muramidase (Cellosyl 100 $\mu g.mL^{-1}$) overnight at 37 °C. The enzyme reaction was stopped by heat-inactivation at 100 °C. Coagulated proteins were removed by centrifugation (21,000 x g for 15 min, Heraeus^{TM} Pico^{TM} 21 Microcentrifuge, Thermo Fisher Scientific). For sample reduction, supernatants were adjusted to pH 8.5 - 9.0 with borate buffer, followed by incubation for 30 minutes at room temperature with freshly prepared $NaBH_4$ solution (final concentration of 10 $mg.mL^{-1}$). Finally, the pH was adjusted to 3.5 by the addition of phosphoric acid. Muropeptides were separated by UPLC on a Waters UPLC system (Waters) equipped with a Kinetex C18 UPLC Column, 130 Å, 1.7 µm, 2.1 mm × 150 mm (Phenomenex) and a dual wavelength absorbance detector. Elution of muropeptides was detected at 204 nm. Separation of the muropeptides was carried out 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 an 18 min run with 0.250 $mL.min^{-1}$ flow.

Fluorescent D amino acid labeling

Short-pulse staining of FDAA incorporation into growing *P. fluorescens* cell walls was accomplished using FDAA BODIPY-FL 3-amino-D-alanine (BADA) (Hsu et al. 2017) (green; emission 502 nm, excitation 518 nm). Briefly, BADA was diluted in dimethyl sulfoxide to a concentration of 100 mM. For each test strain, one mL of exponential culture (OD 600 of 0.4) was pelleted by centrifugation (10,000g : 2,000g for Δ*mreB*) and resuspended in 100 uL. BADA was added to achieve a final concentration of 1mM BADA. Incubation at 28°C for 20% of strain generation time. Following this, 230 μL of ice-cold 100% ethanol was added to culture. An additional 1 mL of ice cold 70% ethanol was added to remove excess dye. Cells were fixed on ice for 15 minutes before being washed with 1 mL of PBS three times to remove excess BADA. Finally, cells were resuspended in 20-100 μL of PBS and subjected to standard fluorescent microscopy on agarose pads. Fluorescent microscopy was performed as described above.

Results

Deletion of *mreB* from *P. fluorescens* SBW25 generates viable spherical cells

Previous observations of viable *mreB* mutants of SBW25 came from transposon screens of adaptive (wrinkly spreader) mutants (Spiers et al. 2002). To establish that cells can indeed proliferate despite the absence of MreB, *mreB* was deleted from ancestral *P. fluorescens* SBW25 (hereafter SBW25). In LB broth, SBW25 Δ*mreB* cells were viable, spherical and variable in cell volume (**Fig. 1A**). DNA sequencing showed no evidence of cryptic compensatory mutations (see breseq output *https://doi.org/10.17617/3.CU5SX1*).

Cell shape, width and length were used to estimate volume (V_e). The volume of Δ*mreB* mutant cells was larger (20.65 ± 16.17 μm^3) than those of ancestral SBW25 (3.27 ± 0.94 μm^3) cells. A negative correlation between V_e and compactness (the ratio of spherical to circle shape with a compactness of 1.0 being a circle) as a result of cell elongation was evident for SBW25 (R^2 = 0.63) (**Fig. 1B**). In contrast, only a weak correlation was observed for the Δ*mreB* mutant (R^2 = 0.13) (**Fig. 1B**). As cell volume increases, DNA content is expected to increase provided that DNA replication continues independently of septation. Increased DNA content and spherical cell shape are both predicted to further perturb cell division (Jun and Mulder 2006; Jun and Wright 2010). Ancestral SBW25 and Δ*mreB* cells were stained with a nucleic acid stain (DAPI) and analysed using flow cytometry. In both genotypes DNA content scaled with cell volume. The largest Δ*mreB* cells had many times the DNA content of ancestral cells, scaling approximately with volume (**Supp. Fig. 1**, **Supp. Fig. 2**); small cells yield a signature consistent with maintenance of DNA (**Supp. Fig. 1**).

In comparison with SBW25, the Δ*mreB* mutant had reduced fitness (**Fig. 1C**) with a longer effective generation time, extended lag phase, and lower maximum yield (**Supp. Fig. 3**). Complementation of Δ*mreB* with the ancestral gene restored rod-shaped morphology and fitness (**Supp. Fig. 4**).

To understand the causes of reduced fitness, the division of single cells was monitored by time-lapse video microscopy on agar pads. Although SBW25 Δ*mreB* cells appear spherical, measurement of cell aspect ratio revealed existence of an elongation axis that remained constant throughout the cell cycle (**Supp. Fig. 5**). As in rod- and ovococci-shaped bacteria, the septum formed perpendicularly to the elongation axis. After cell division, the elongation axis rotated 90° relative to the mother cell (**Supp. Fig. 6**, Supp. Movie 1), which is reminiscent of ovococci (Pinho

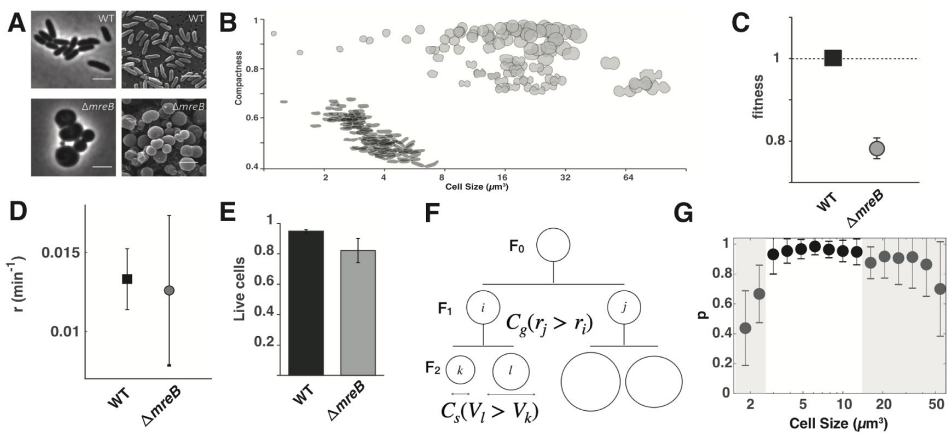

Figure 1.

Characterisation of ancestral SBW25 and Δ*mreB* strains. Figure shows (A) photomicrographs of ancestral SBW25 and Δ*mreB*. Scale bars, 3 μm. B) The relationship between cell shape and estimated volume (V_e) is represented using compactness, a measure of roundness. One hundred representative cells from ancestral SBW25 and Δ*mreB* populations are shown as cell outlines. C) Fitness of ancestral SBW25 GFP and the Δ*mreB* mutant relative to ancestral SBW25 when both are in exponential phase during pairwise competition assays. Data are means and standard deviation of 50,000 cells each of ancestral SBW25 and Δ*mreB*, respectively, and 100,000 cells total, after competition. D) Elongation rate measured for SBW25 and Δ*mreB* SBW25. Error bars represent mean and standard deviation (N_{WT} = 94; $N_{\Delta mreB}$ = 99) E) Proportion of live cells in ancestral SBW25 (black bar) and ΔmreB (grey bar) based on LIVE/DEAD BacLight Bacterial Viability Kit protocol. Cells were pelleted at 2,000 x g for 2 minutes to preserve ΔmreB cell integrity. Error bars are means and standard deviation of three biological replicates (n>100). F) Schematic of asymmetric size production in cell lineages at generation F0 and F1. Asymmetries from differential growth rate between sister cells (*i,j*) are captured by C_g (see methods), while asymmetries in daughter (*l,k*) cell sizes at septation are measured by C_s. G) probability *p* to pass to the next generation as a function of cell size in Δ*mreB* cells. The shaded gray areas are indicative of regions where survival significantly drops.

et al. 2013). But more surprisingly, measurement of the rate of volume extension (hereafter termed elongation rate measured in min^{-1}) showed that during exponential phase it was indistinguishable from the cell elongation rate of ancestral SBW25 (**Fig. 1D**).

Since elongation rate was unchanged upon deletion of *mreB*, reduced fitness of the mutant was likely due to production of non-viable cells. We thus measured cell viability in LB broth with a "live / dead" assay and showed that 95% of ancestral SBW25 cells were viable, while the fraction of viable Δ*mreB* mutant cells was reduced to 81%. Thus, the decreased fitness of the mutant is primarily a consequence of the production of non-viable cells (**Fig. 1E**). To confirm this result, we performed time lapse microscopy of cells grown on agar pads to measure the probability p to pass to the next generation. For ancestral SBW25, the probability p of producing offspring equals 1, whereas for Δ*mreB* cells, p = 0.85. In instances where the probability to pass to the next generation p is not equal to one, the equation for growth dynamics is represented by dN=p*r*N*dt, where N is the number of cells at time t and r is the cell elongation rate. Hence, the effective growth rate of the population is given by p*r. Because Δ*mreB* and ancestral SBW25 cells elongate at the same rate, the fitness of Δ*mreB* cells is directly given by p. Consistently, the probability to pass to the next generation p = 0.85±0.05 (N=141), as measured in single cell experiments, is not significantly different from the fitness of 0.77±0.025 for Δ*mreB* in bulk experiments (**Fig. 1C**).

We next examined the causes for decreased proliferation capacity by measuring, at the single cell level, the correlation between the probability p to pass to the next generation and the fidelity of cell division. To this end, the precision of septum positioning, C_s, and correlation between the relative difference in elongation rate between sister cells at generation F1, C_g, and probability p of producing offspring at the end of generation F1 (**Fig. 1F**) were calculated. As a proxy to determine the precision of septum positioning, we measured the relative difference C_s in cell volume V_e, between two daughter cells immediately after division at generation F2. In ancestral SBW25, septum position was accurately placed, with only 5 % difference in the volume of sister cells. In Δ*mreB* mutant cells, the accuracy of septum position was significantly altered, with 19.4 % difference in the volume of sister cells. To determine if sister cells had different growth dynamics, we measured the relative difference C_g in elongation rate r between two sister cells at generation F1. In ancestral SBW25, the elongation rate in pairs of sister cells differed by 5 %, whereas in Δ*mreB* cells the difference in rate was on average 55 %. Combined together, error in septum positioning and growth asymmetry between sister cells explains why the Δ*mreB* mutant produces cells of variable volume, which then have different proliferative capacity. To examine how cell volume affects proliferation ability, the probability p to pass to the next generation was plotted as a function of cell volume. As evident in **Fig. 1G** the volume distribution has two extreme tails, with both large and small cells being unlikely to produce viable offspring.

Selection rescues fitness of a compromised *mreB* deletion mutant

To determine whether selection could discover mutation(s) that might compensate for reduced fitness of Δ*mreB*, SBW25 Δ*mreB* was propagated for 1,000 generations in shaken broth culture with daily transfer. The competitive fitness of derived types was measured relative to ancestral SBW25 using neutral markers to distinguish competitors. Fitness of all replicate lines (relative to ancestral SBW25) significantly improved (**Fig. 2A**) to the point where fitness was comparable to ancestral SBW25 (**Supp. Fig. 7**). Subsequent measurement of fitness at generations 50, 250 and 500 showed that compensation of fitness was rapid, with a highly similar trajectory for each of the 10 replicate populations (**Supp. Fig. 8**). Such rapid restoration of fitness is indicative of a small number of readily achievable compensatory mutations.

Examination of derived genotypes at generation 1,000 by microscopy showed that all lines retained the coccoid shape (**Fig. 2B**, **Supp. Fig. 9**). Cell volume decreased compared to that of the ancestral cell type (**Fig. 2B**) and did not overlap with the Δ*mreB* population (**Fig. 1B** and

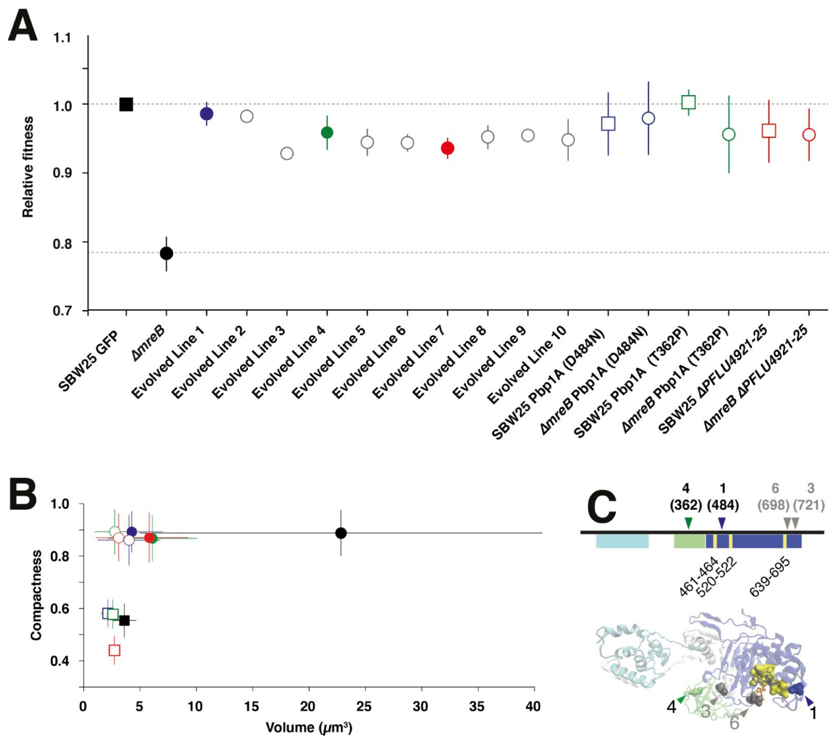

Figure 2.

Characterisation of evolved lines at 1,000 generations. A) Relative fitness of the Δ*mreB* mutant, derived lines after ~1,000 generations, and mutant reconstructions in Δ*mreB* and SBW25, relative to SBW25 (dashed line) in pairwise competition experiments. Error bars represent standard deviation. B) Compactness versus estimated volume (Ve) of derived lines: Line 1 in blue, Line 4 in green and line 7 in red; derived mutations reconstructed in Δ*mreB*, and SBW25 backgrounds. Data points are means and SD of X 100 cells. Square data points represent rod-like cells, whereas circles (both open and filled) represent spherical cells. Ancestral genotypes are labeled in black. Open squares and open circles depict the reconstruction of mutations in the SBW25 and Δ*mreB* backgrounds, respectively. Filled circles represent the 1000 generation evolved lines from where the mutations have been identified. Colors as follows; blue is Line 1 or Pbp1A D484N, green is Line 4 or Pbp1A T362P, red is Line 7. C) Domain map and model of Pbp1A (PFLU0406) showing the 2 major domains; the glycosyltransferase (GT) domain (cyan) and the transpeptidase (TP) domain (blue). The oligonucleotide/oligosaccharide binding (OB) domain is shown in green. The active site of the TP domain is also shown (yellow). The locations of mutations from Lines 1 and 4 are indicated with coloured arrows in the *pbp1A* gene map and in the model of Pbp1A. The mutations in Lines 3 and 6 are also shown in grey but were not characterised further in this work.

2B) showing that the derived cells define a new phenotype and not a subset of the spherical Δ*mreB* ancestor. Analysis of the pattern of cell division in the derived lines showed evidence of an elongation axis, which rotated at cell division, as observed for Δ*mreB* mutant cells (**Supp. Fig. 6**).

Identifying compensatory mutations

In order to identify the genetic basis of adaptation, populations were sequenced at generations 500 and 1,000 with the ensuing reads mapped to the genome of SBW25 (Table 1) and mutations identified using Breseq (Deatherage and Barrick 2014). A single gene, *pbp1A* (PFLU0406) had mutations in seven independent lines. In four of these, Line 1 (D484N), Line 3 (D721A), Line 4 (T362P) and Line 6 (N698K), the mutation had risen to high frequency, but only in Line 6 was the mutation fixed. Some lines (4, 9 and 10) had more than a single *pbp1A* mutation and in some instances the mutation detected at generation 500 differed from that evident at generation 1,000. Clonal interface between competing mutations thus seems likely. In the three lines, with no evidence of *pbp1A* mutations at generations 500, or 1,000, two exhibited mutations in genes involved in septum formation (*ftsA* for Line 2 and *ftsZ* for Line 5). Finally, Line 7, contained a five-gene deletion spanning PFLU4921 to PLFU4925 which included *oprD*, but also has a non-synonymous mutation in *ftsE* that was not detected at generation 500, but composed 65% of sequencing reads at generation 1,000. The prominence of *pbp1A* mutations led us to further study two lines showing *pbp1A* mutations (Line 1 and Line 4) and to compare them with the five gene deletion (PFLU4921 to PLFU4925 Δ5399934-5403214) evident in Line 7.

The *pbp1A* gene is a class A penicillin-binding protein with both transpeptidase (TP) and transglycosylase (TG) activities. It is semi-autonomous and interacts with the rod complex and specifically with PBP2 (Banzhaf et al. 2012; Geisinger et al. 2020). Pbp1A contains three known domains (**Fig. 2C**). Structure mapping of the mutations shows that the mutation in Line 1 occurred in the transpeptidase (TP) domain, which is proximal to the active site (**Supp. Fig. 10**). Similar mutations in *Streptococcus pneumoniae* cause a loss-of-function (Job et al. 2008) and rescue viability of both *mreC* and *mreD* mutants (Land and Winkler 2011). The mutation in Line 4 occurred in the oligonucleotide/oligosaccharide binding or Outer membrane Pbp1A Docking Domain (OB/ODD) domain[6]. Each mutation was reconstructed by allelic exchange in SBW25 and SBW25 Δ*mreB* backgrounds.

Given the very rapid fitness increase (**Supp. Fig. 8**) we looked for evidence of *pbp1A* mutations at generation 50. The gene was amplified by PCR from 16 colonies from each replicate population and the product Sanger sequenced. All lines had at least one mutation (out of the 16 colonies) demonstrating the relative ease at which *pbp1A* mutations arise. Results are shown in **Supp. Table 2**.

Analysis of *pbp1A* mutations

Both Δ*mreB pbp1A* reconstructed genotypes (hereafter Δ*mreB pbp1A**) produced spherical-to-ovoid cells with near ancestral SBW25 volume (Δ*mreB pbp1A* (D484N) M = 3.86±0.89 μm^3; Δ*mreB pbp1A* (T362P) M = 5.51±0.87 μm^3) and DNA content (**Fig. 2B**, **Supp. Fig. 2**). Analysis of the pattern of cell division in the reconstructed lines showed evidence of an elongation axis that rotated at cell division as observed for SBW25 Δ*mreB* (**Supp. Fig. 6**). Δ*mreB pbp1A** genotypes showed growth dynamics and fitness effects similar to ancestral SBW25 (**Supp. Fig. 11**) (**Fig. 2A**), indicating that these *pbp1A* mutations are each sufficient to restore ancestral fitness and to recapitulate the major phenotypes of derived Lines 1 and 4. Elongation rate was identical to SBW25 for SBW25 Δ*mreB* carrying the reconstructed mutations, (**Supp. Fig. 12**). Fitness of a genetically engineered Δ*mreB* Δ*pbp1A* mutant (relative to Δ*mreB*) was 1.35 (SD±0.04, n = 6) was indistinguishable from fitness of the Line 4 mutation reconstructed in the same Δ*mreB* background (1.38; SD±0.11, n = 6) demonstrating that loss-of-function of *pbpB1A* is sufficient to restore fitness (**Supp. Fig. 13**). Loss-of-function is also consistent with the finding that at generation 50 all lines harboured mutations in *pbp1A* (**Supp. Table 2**).

When reconstructed in ancestral SBW25, *pbp1A* mutations had little effect on population dynamics (**Supp. Fig. 11A**), or shape (**Fig. 2B**), however, elongation rates were reduced (**Supp. Fig. 12**), suggesting that the *pbp1A* mutations result in a reduction – or more likely even complete loss-of-function. In addition, cells were narrower than WT cells: 0.89 µm (SD±0.07) and 0.94 µm (SD±0.05) for Pbp1A TP and OB/ODD mutations, respectively, compared to 1.00 µm (SD±0.06) for ancestral SBW25. This resulted in smaller cell volumes (**Fig. 2B**, **Supp. Figs 14** & **15**). The reduced widths / volumes -- which also correlate with reduced DNA content (**Supp. Fig. 2**) – might be indicative of reduced enzymatic function leading to reduced lateral cell wall synthesis as the case for loss-of-function mutations in *pbp1A* reported in both *B. subtilis* and *E. coli* (Murray et al. 1998; Claessen et al. 2008; Kawai et al. 2009; Banzhaf et al. 2012).

Analysis of Δ*pflu4921-4925*

The five-gene deletion (Δ5399934-5403214 (Δ*pflu4921-4925*)) in derived Line 7 (**Supp. Fig. 16**, **Supp. Table 1**) includes three hypothetical proteins, a cold shock protein (PFLU4922, encoding CspC), and an outer membrane porin, PFLU4925, which encodes OprD. The latter is responsible for the influx of basic amino acids and some antibiotics into the bacterial cell (Skurnik et al. 2013). The deletion was also reconstructed and characterised in the Δ*mreB* and ancestral SBW25 backgrounds. The relative fitness of the Δ*mreB* Δ*pflu4921-4925* genotype was similar to the ancestral SBW25 type (**Fig. 2A**), but with an extended lag time (**Supp. Fig. 11**). Cells were spherical with an average Ve of 5.26 μm^3 (SD±3.13) (**Fig. 2B**, **Supp. Figs 14** & **15**) and harboured less DNA compared to Δ*mreB* SBW25, but more than the Pbp1A* reconstructions in the same background (**Supp. Fig. 2**). In addition, a fraction of the cell division events were defective, resulting in clumps of spherical cells with imperfectly formed septa (**Fig. 2B**, **Supp. Fig. 14**-**17**). Spherical cells with notably incomplete septa were not observed in the derived Line 7 population (**Supp. Table 1**).

When genes *pflu4921-4925* (Δ5399934-5403214) were deleted from ancestral SBW25 the cells remained rod-shaped and displayed aberrant growth characteristics relative to the ancestral strain (**Supp. Fig. 14**). The elongation rate was slower (**Supp. Fig. 12**), cells were significantly thinner (width = 0.74µm SD±0.06; $p < 0.001$, two sample *t*-test), and had a smaller average Ve (2.47 μm^3, SD±1.18) (**Fig. 2B** and **Supp. Fig. 15**). As in the Δ*mreB* background, a fraction of cells failed to completely separate at cell division and DNA was dispersed between incomplete septa (**Supp. Figs 14**-**17**). The average DNA content of SBW25 Δ*pflu4921-4925* cells was greater than the *pbp1A** reconstructions in the same background (**Supp. Fig. 2**).

Cell volume homeostasis

The large average cell volume observed in stationary phase SBW25 Δ*mreB* cells (**Fig. 2B**) suggests that some of the physical parameters governing cell volume may have been altered. Cell volume at steady state is determined by the equilibrium between turgor pressure (Rojas and Huang 2018) and membrane tension through the Laplace law for thin elastic shells (Verge-Serandour and Turlier 2021) (see Materials and Methods for elaboration). Since it is difficult to imagine a scenario where the cell-wall would strengthen in the absence of *mreB*, *mreB* loss is expected to result in a significant decrease of internal pressure compared to ancestral SBW25 cells, synonymous with the influx of water through pores formed in the cell-wall.

During exponential growth, the cell volume of daughter cells is limited by the amount of new material that can be incorporated by the mother cell. According to the “adder” model (Campos et al. 2014; Taheri-Araghi et al. 2017), the average cell volume in the population converges within a few generations towards the average added volume. We thus quantified the added volume for all strains (**Fig. 3A**) and confirmed that cell volume rapidly reduced for exponentially growing SBW25 Δ*mreB* cells (Supp. Movie 2). Since, the volume of Δ*mreB* cells in exponential phase is much lower than in stationary phase, it suggests that cells swell during stationary phase, probably due to defects in cell wall integrity with deleterious consequences for cell viability. Hence, without restoring cell wall integrity, cell volume homeostasis cannot be maintained. Remarkably, the added-volumes of spherical cells in each of the derived mutant lines (**Fig. 3A**) were close to the volumes corresponding to the passage from a cylindrical to spherical geometry with constant turgor pressure and membrane tension (see Materials and Methods). This suggests that adaptive mutations restored the mechanical integrity of the cell wall.

To maintain cell volume homeostasis, elongation and septation rates must be balanced so that cells maintain a steady volume throughout successive generations. Indeed, if septation is faster than elongation, cell volume of progeny decreases, while if elongation is faster than the rate of septation rate, cells increase in volume. In both instances the fate of populations is extinction. The rate of septation was significantly higher than the elongation rate in Δ*mreB* cells showing that the distribution of cell volume is unstable and confirms that the average volume is expected to decrease in subsequent generations (**Fig. 3B**). In contrast to SBW25 Δ*mreB* cells, the rates of elongation and septation were balanced in the derived lines (**Fig. 3B**), indicating that cell volume homeostasis has been reached. Reconstruction of *pbp1A* mutations in SBW25 Δ*mreB* also restored this balance, suggesting that the mutations contribute to maintenance of volume homeostasis in the absence of *mreB*. In contrast, the Line 7 deletion reconstructed in SBW25 Δ*mreB* did not restore this balance (**Fig. 3B**,).

Another important feature for cell volume homeostasis is the fidelity of cell division: two daughter cells with equal volume must themselves generate four daughters of equal volume. Therefore, we measured the relative difference in elongation rates (C_g) and division time (C_T) between sister cells at generation F1 on agar pads, as well as the relative difference in precision of septum positioning (C_S) for all derived and reconstructed lines. All evolved lines regained symmetry both in elongation rate and in precision of septum positioning, but was most notable for the symmetry of elongation rate (**Fig. 3C** & **D**). These improvements correlate with an increase in proliferative capacity, while no significant correlation was observed in the relative difference of division time $C_T$between sister cells (**Supp. Fig. 18**). Reconstructions in ancestral SBW25, showed a slight alteration of symmetry, which is more pronounced in the case of Line 7 (five gene deletion). Reconstructions in evolved lines Line 1 and Line 4 showed improvement in growth asymmetry. Interestingly, recovery was only partial for Line 7, raising the possibility of additional effects due to a secondary mutation whose effects depend on the mechanics of the environment (liquid vs solid culture). Indeed, as indicated above and shown in **Supp. Table 1**, Line 7, at generation 1,000, also contains a non-synonymous mutation in *ftsE* (T188A) that had reached high frequency. Given that FtsE promotes elevated peptidoglycan synthesis at cell septa the possibility of synergistically beneficial effects with the OprD-inclusive deletion is not improbable (Mallik et al. 2023).

The mutations observed in Line 1 or Line 4 showed a significant improvement in the precision of septum position, suggesting that asymmetry in elongation rate between daughter cells has greater impacts on cell survival than septum positioning. This can also be seen from the shape of the curves (**Fig. 3C** & **D**), where *p* decreases linearly with growth asymmetry up to 50%, while precision of septum positioning saturates at 20% regardless of *p*, probably because nucleoid occlusion is able to achieve a sufficiently good level of septum positioning.

Investigating the molecular consequences of mutations

To obtain insight into the molecular mechanisms by which mutations in *pbp1A* compensate for loss of *mreB*, we performed a range of biochemical analyses on SBW25 and SBW25 Δ*mreB* engineered with the fitness-rescuing mutations in *pbp1A*: SBW25 / SBW25 Δ*mreB* Pbp1A (D484N) (mutation in the TPase domain); SBW25 / SBW25 Δ*mreB* Pbp1A (T362P) (mutation in the OB/ODD domain). SBW25 *ΔmreB Δpbp1A* was used as a negative control. In ancestral SBW25 36.34% (SD±2.26) of muropeptides are cross-linked (**Fig. 4A**). The Δ*mreB* mutant showed a marked increase in cross-linking (40.47% SD±1.50). The degree of cross-linking in the *pbp1A* mutants in either SBW25 or the Δ*mreB* genetic background were lower (31.28% SD±0.98 to 33.66% SD±0.52).

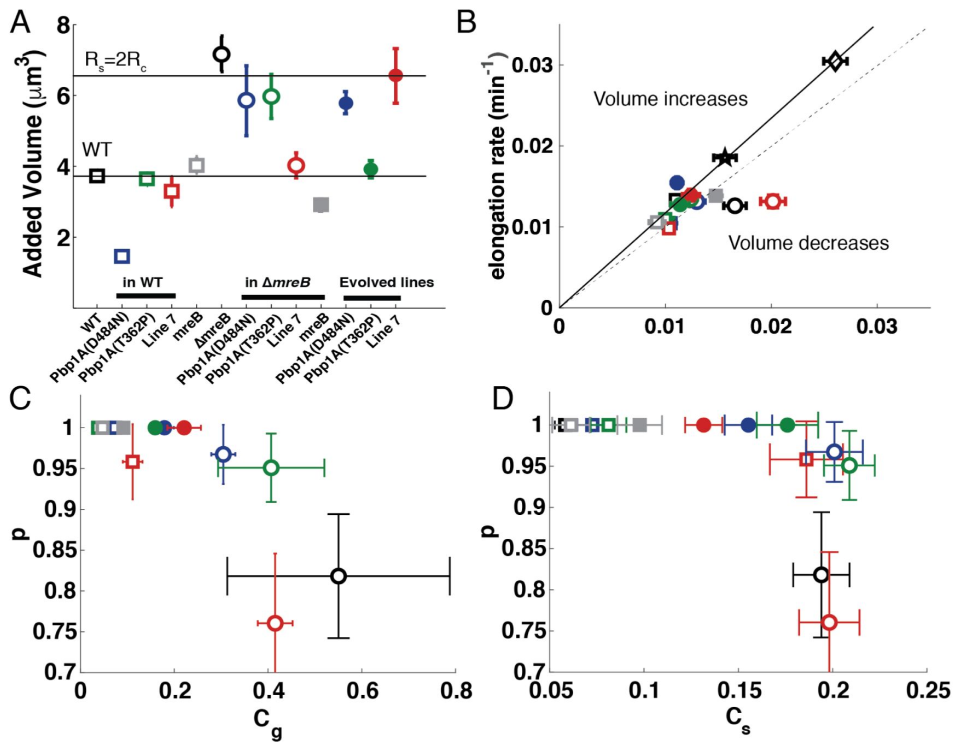

Figure 3.

Cell size homeostasis: A) Average added volume for the different genotypes, which is cell volume at septation less cell volume at birth. The horizontal lines correspond to the volume of ancestral SBW25 cells and to their spherical counterparts (R_S=2*R_C). B) Elongation rate plotted against septation rate. The black line corresponds to size homeostasis of wild type strains (square = *P. fluorescens*; star = *P. aeruginosa*; diamond = *E. coli*). Below this line, septation is faster than elongation and cell size decreases. The dashed line corresponds to equal septation and elongation rates. C) The probability *p* to pass to the next generation as a function of growth asymmetry C_g. D) The probability *p* to pass to the next generation as a function of error in septum positioning measured as the relative cell size between daughters at division C_s. In A) B), C) and D) square data points (both open and filled) represent rod-like cells, whereas circles (both open and filled) represent spherical cells. Ancestral genotypes are labeled in black. Open squares and open circles depict the reconstruction of mutations in the WT and Δ*mreB* backgrounds, respectively. Filled circles represent the evolved lines where the mutations have been identified. Colors as in **Fig 2**; blue is Line 1 or Pbp1A D484N, green is Line 4 or Pbp1a T362P, red is Line 7, grey is ectopic *mreB* expression. Error bars represent standard errors. In A), B), D) : (N_{WT} = 94; N_{L1_WT} = 52; N_{L4_WT} = 38; N_{L7_WT} = 72; N_{mreB_WT} = 36; $N_{\Delta mreB}$ = 99; $N_{L1\_\Delta mreB}$ = 92; $N_{L4\_\Delta mreB}$ = 102; $N_{L7\_\Delta mreB}$ = 96; $N_{mreB\_\Delta mreB}$ = 64; N_{L1} = 88; N_{L4} = 74; N_{L7} = 76); in C) (N_{WT} = 47; N_{L1_WT} = 26; N_{L4_WT} = 19; N_{L7_WT} = 38; N_{mreB_WT} = 18; $N_{\Delta mreB}$ = 55; $N_{L1\_\Delta mreB}$ = 56; $N_{L4\_\Delta mreB}$ = 62; $N_{L7\_\Delta mreB}$ = 53; $N_{mreB\_\Delta mreB}$ = 32; N_{L1} = 44; N_{L4} = 42; N_{L7} = 40)

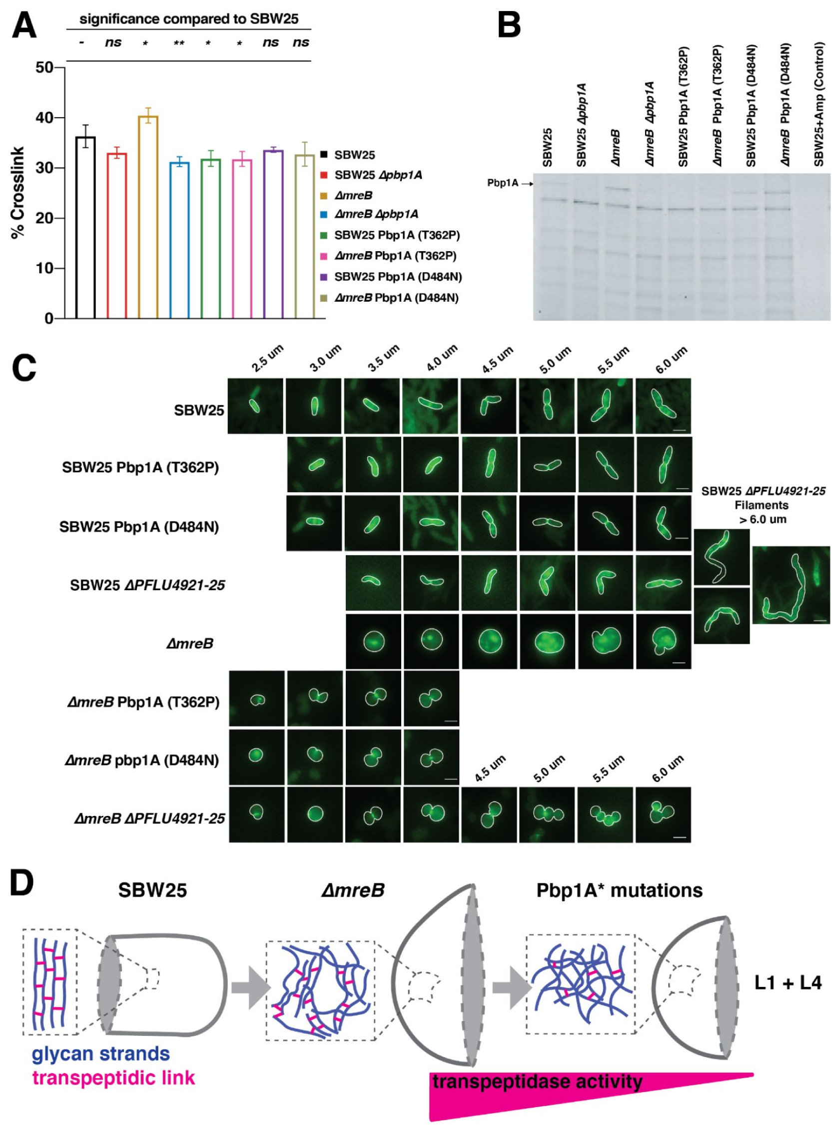

Figure 4.

Molecular investigation of adaptive mutations in Δ*mreB* evolution. A) Peptidoglycan cross-linking in the cell walls of mutants in SBW25 and Δ*mreB* genetic backgrounds. The error bars (SD) are the result of 3 biological replicates and significant differences (paired *t*-test between SBW25 and each mutant) are indicated by $*p< 0.05$, $**p < 0.01$ or $***p < 0.001$. B) Cell extracts subjected to polyacrylamide gel and SDS PAGE labelling with Bocillin-FL gel to demonstrate penicillin binding of Pbp1A (labelled) and other Pbps in *P. fluorescens* SBW25. See **Supp Fig 20** for quantification of band intensities. C) Fluorescent images of mutations in SBW25 and Δ*mreB* backgrounds subjected to short-pulse labelling with fluorescent D-amino acid BADA to reveal the location and organization of cell wall construction in the mutant backgrounds. As cells elongate in preparation to divide, septal peptidoglycan synthesis becomes more pronounced. Δ*mreB* cells have highly disorganized synthesis (see **Supp. Fig. 21** for quantitative analysis), which is alleviated in Δ*mreB* Pbp1A* and Δ*mreB* ΔPFLU4921-25 mutants. Cell outlines from phase contrast images show the cell boundaries. Scale bars = 2µm. D) Model of evolutionary rescue in Δ*mreB* mutant cells highlighting the organization of the predicted glycan meshwork in the rod and spherical geometries. In Pbp1A* mutants (Line 1 and Line 4), a decrease in transpeptidase activity favours disorganized cell wall architecture, which is better suited to the topology of a sphere.

Lower levels of cross-linking observed in the Δ*mreB* and SBW25 reconstructed lines suggests that mutations in Line 1 and 4 either decreased the amount of transpeptidation or increased the amount of transglycosylation. To discriminate between the two hypotheses, we utilised Bocillin-FL labelling, a fluorescent penicillin derivative that binds to the active site of PBPs, as a proxy to measure PBP1a TPase activity. We included SBW25 *Δpbp1A* as a control to specifically identify the band corresponding to Pbp1A in the SDS-PAGE gel (**Fig. 4B**). In agreement with higher levels of cross-linking activity measured in SBW25 Δ*mreB* (**Fig. 4A**), the intensity of the Pbp1A band was slightly higher in Δ*mreB* cells compared to the ancestral genotype. In comparison to the ancestor of the evolved lines (SBW25 Δ*mreB*), this signal was little changed in the Line 1 mutant (TP domain (SBW25 Pbp1A (D484N)) (**Fig. 4B**), but absent in the Line 4 mutant (OB/ODD domain (SBW25 Pbp1A (T362P))). The activity of the OB/ODD domain is not well characterised, but is thought to be an important regulatory domain that licences TPase domain function through interaction with LpoA (Sardis et al. 2021). The relative peptidoglycan density and UPLC traces are shown (**Supp. Fig. 19**).

In addition, we studied how the Δ*mreB* and *pbp1A* OB/ODD and TP domain mutations affect the location of active cell wall synthesis. This was achieved by incorporation of fluorescently labelled D-amino acids (FDAAs) via brief pulses of BADA into exponentially growing cells followed by microscopy to visualise locations of active cell wall synthesis (**Fig. 4C** and **Supp. Fig. 21**). In SBW25, BADA incorporation into the cell wall was homogeneous across the lateral surface during growth, but enriched at the mid-cell during septation. In SBW25 Δ*mreB* cells, BADA labelling was heterogeneous across the entire cell wall. This is contrary to expectation – at odds with observations of diffuse and homogeneous wall synthesis in MreB-depleted *E. coli* (Ursell et al. 2014) – and indicates that even in the absence of MreB, cell wall synthesis is active. SBW25 Δ*mreB* containing *pbp1A** mutations resulted in small spherical cells in which the prominence of cell wall synthesis in the septal region was restored as the primary location of peptidoglycan production, particularly in longer cells (**Fig. 4C**). Thus *pbp1A* mutations appear to restore cell homogeneity by eliminating disorganised patterns of cell wall synthesis (**Fig 4C**).

Discussion

Ability to generate a viable *mreB* deletion mutant of *P. fluorescens* SBW25 – albeit with significantly compromised fitness – has provided opportunity to investigate the phenotypic consequences of loss of MreB function without use of MreB-inhibiting drugs, and to explore mutational routes leading to fitness restoration.

After 250 generations of selection, fitness of all derived lines was restored to wild type levels (**Fig. 2A**). Measurement of fitness at earlier time points showed that compensation occurred early in the selection regime and in single steps (**Fig 2A**, **Supp. Fig. 8**), consistent with compensation occurring via one or a small number of mutations. Sequencing and genetic reconstruction experiments confirmed, particularly for the class of mutant with defects in *pbp1A*, that single point mutations reducing or eliminating gene function were sufficient. In all derived lines, cell shape remained spherical. This sits in accord with evidence suggesting that there are no genetic routes or growth conditions able to restore rod-like shape in the absence of MreB (Shi et al. 2018). This stated, we recognise that rod-shape bacteria belonging to the *Actinobacteria* and *Rhizobiales* lack MreB (Margolin 2009; Zhao et al. 2021) suggesting that in principle it is possible to restore rod-shape in the absence of MreB.

In experiments where selection is used to find mutations that compensate for deleterious effects arising from deletion of a gene that compromises fitness (Laan et al. 2015; Lind et al. 2015; Rainey et al. 2017), a central issue concerns the focus of selection: the defect that selection might stand to correct. In this work, where deletion of *mreB* provided the compromised starting genotype, the most obvious difference between SBW25 Δ*mreB* and each of the ten derived lines was cell volume. It is therefore tempting to suggest that volume is a target of selection, and especially so given known adaptive changes in cell volume in the long-term Lenski experiment (Lenski and Travisano 1994; Monds et al. 2014). However, a closer examination of data, particularly from time-resolved microscopy, patterns of peptidoglycan synthesis and biophysical considerations, suggests a more complex set of effects and adaptive responses.

At first glance, the most notable distinction between cells of ancestral and derived types was shape and volume: SBW25 cells are rod shaped (thus with low compactness) with relatively little variance in the volume of daughter cells, whereas the shape of Δ*mreB* cells tends toward spherical with large variance in volume. While the rate of elongation is the same in SBW25 and Δ*mreB* cells, substantial variance was apparent in the rate of elongation among daughter (Δ*mreB*) cells (**Fig. 1D**). Also evident were significant differences in cell viability, with viability of daughter cells being high in SBW25, but much reduced in the Δ*mreB* mutant. With focus on Δ*mreB* cells, viability of daughter cells depends on cell volume and declines significantly in both very large and very small cells (**Fig. 1G**). This is understandable in terms of effects on DNA segregation, with small cells proving non-viable most likely through lack of DNA, and large cells being similarly non-viable as a consequence of multiple copies of intertwined chromosomes and imbalance of osmotic pressure.

A key issue concerns the causes of variability in cell volume, toward which both septation asymmetry (C_s) and differential elongation rate (C_g) between daughter cells are the primary contributory factors. As evident by comparing C_s and C_g for ancestral SBW25 (black open square in **Figs 3C** and **3D**) and Δ*mreB* (blue open circle in **Figs 3C** and **3D**) cells, respectively, the greatest difference is the relative difference in elongation rate among sister cells (C_g). This indicates that this is the primary cause of variability in cell volume in the *mreB* mutant. Moreover, if correct, it leads to the prediction that fitness restoration will depend on selection finding mutational routes to restore population-level homogeneity in elongation rate among offspring.

What then, underlies differences in elongation rate? Here, data on peptidoglycan cross-linking, TPase activity, and patterns of cell wall synthesis in SBW25 versus Δ*mreB* cells are suggestive. Relative to SBW25, cells of the *mreB* mutant showed increased peptidoglycan cross-linking (**Fig. 4A**) likely resulting from enhanced TPase activity of Pbp1A (**Fig. 4B**) and heterogeneous (uneven) patterns of cell wall synthesis (**Fig. 4C**). These data indicate that despite the absence of MreB, cell wall synthesis continues, but is dysregulated in space and time. This is in marked contrast to cell wall synthesis in MreB-impaired (by treatment with A22) *E. coli* where cell wall synthesis is diffuse and homogeneous (Ursell et al. 2014).

Just what contributes to cell wall synthesis on removal of MreB is unclear. The standard view is that on deletion (or depolymerisation) of MreB, the elongasome is registered non-functional (Park and Uehara 2008), but compelling evidence appears to be lacking (Cho et al. 2016). In *E. coli*, PBP2 can trigger elongasome initiation independently of MreB by promoting interaction with a hypothetical protein that in turn interacts with PBP2 and the cell wall (Özbaykal et al. 2020). Additionally, in *A. baumannii* high Pbp1A activity inhibits elongasome function (Simpson et al. 2021) with Δ*ponA* (*pbp1A*) mutants having enhanced elongasome function (Kang et al. 2021). Accordingly, in the following discussion we present two hypotheses to account for our findings: the first assumes that the elongasome is non-functional, whereas the second considers the possibility that the elongasome remains functional, but is stalled (or trapped).

Irrespective of whether the elongasome is rendered non-functional on removal of MreB, it is necessary to explain the observed heterogeneity of cell wall synthesis. Assuming, firstly, a non-functional elongasome, then obvious candidates for cell wall synthesis are the aPBPs, Pbp1A and Pbp1B, that can function independently of the elongasome (Cho et al. 2016) (although depend on activation by LpoA and LpoB respectively), with Pbp1B being particularly important in repair of cell wall lesions (Vigouroux et al. 2020). Perhaps heterogeneity of cell wall synthesis in Δ*mreB* arises from attempts by Pbp1B to repair large scale lesions, but there being insufficient bound (active) Pbp1B to fully accomplish this.

Assuming that the peptidoglycan-synthesising activity of the elongasome remains active on deletion of *mreB*, then heterogeneity of cell wall synthesis indicates that the multi-protein complex has become either stalled or trapped. This is reminiscent of observations in *B. subtilis* where inactivation of MreB causes failure in the dynamic redistribution of PBP1 (Kawai et al. 2009). Stalling – and concomitant heterogeneity of synthesis – could arise from elevated TPase activity that could in turn stem from altered interactions between Pbp2 and Pbp1A, which are known to mutually affect the catalytic activity of the other (Banzhaf et al. 2012; Egan et al. 2020). In support of this hypothesis, the TPase activity of Pbp1A in SBW25 Δ*mreB* is elevated compared to the same protein in ancestral SBW25 (**Fig. 4B**).

In the populations derived from the 1,000 generation selection experiment, solutions to variability in cell volume stemmed largely from reduction (or complete abolition) of Pbp1A function. As predicted above and shown in **Fig. 3D**, derived lines showed greatest improvement in the degree to which daughter cells differ in elongation rate (C_g). Such improvement correlates directly with significant increases in the probability that daughter cells are viable. By way of comparison changes in the relative positioning of the septum among daughter cells in derived lines (C_S) were relatively minor. Selection thus appears to have restored fitness by changes that reduced variation in the rate of elongation among daughter cells. Assays of TPase and peptidoglycan cross-linking indicate that this is linked to reduction of TPase function of Pbp1A and a reduction in peptidoglycan cross-linking, with the two together likely favouring a disorganised cell wall architecture suited to the topology of spherical cells (**Fig. 4D**)

That selection compensated for the maladapted effects of *mreB* deletion by mutations in *pbp1A* can now be viewed in light of the two hypotheses for heterogeneity in cell wall synthesis. In the case of heterogeneity stemming from absence of elongasome function and localised activity of Pbp1B – and drawing upon the findings that inactivation of Pbp1A in *E. coli* results in a four-fold elevation of the amount of bound (and thus active) Pbp1B (Vigouroux et al. 2020) – inactivation of Pbp1A is expected to significantly increase Pbp1B activity perhaps to a level that would allow for wall repair uniformly throughout the cell (Vigouroux et al. 2020). Should heterogeneity in cell wall synthesis arise from a stalled, but otherwise functional elongasome, then elimination of Pbp1A might serve to correct the balance between TPase and GTase activity and thus allow the elongasome to move more diffusively along the inner membrane (Simpson et al. 2021). Future work is planned to test these competing hypotheses aided by the fact that deletion of *mreB* in SBW25 is not lethal.

The reason that compromised, albeit viable Δ*mreB* cells, can be generated in SBW25 under standard laboratory conditions is unknown, but answers may shed new light on the complexities of elongasome function and particularly the connection between elongasome function and MreB. Deletion of *mreB* in many bacteria is lethal including the closely related *P. aeruginosa* (Robertson et al. 2007), although in *E. coli* the defect can be rescued by slow growth or by increased activity of the cell division protein FtsZ (Kruse et al. 2005; Bendezú and de Boer 2008). In *B. subtilis* lethality can be ameliorated by growth in the presence of magnesium (Formstone and Errington 2005). This suggests that factors contributing to lethality involve subtleties in interactions among elongasome components (including associated factors such as *pbp1A* and *pbp1B*), which are dysregulated in SBW25 Δ*mreB*, but are insufficiently out of kilter to result in a lethal phenotype.

In this context there exists possible explanations for the repeated and independent transitions from ancestral rod-shape cells to cocci (Siefert and Fox 1998). Recent work on the rod to coccus transition in nasopharyngeal pathogens of the *Neisseriaceae* family, where deletion of *mreB* and other determinants of the Rod complex, while not lethal, result in a significant fitness decrease

(Veyrier et al. 2015). Such reductions in fitness were not apparent when coccoid cells arose from deletion of the FtsZ ring assembly-promoting protein YacF (ZapD), suggesting the requirement for a series of events beginning with loss of *yacF* followed by evolutionary compensation with eventual loss of the elongasome machinery (Veyrier et al. 2015). Our work here shows that the evolutionary route from rod to sphere is readily achievable under laboratory conditions via loss of *mreB* followed by compensatory mutation in *pbp1A* and reorganisation of peptidoglycan structure. Whether such a route is ever realised in nature will depend on the initial fitness effects of the *mreB* mutation, which in turn will depend on environmental context, but it is not implausible that inactivation of *mreB* may confer a fitness advantage under some conditions. This argument is further elaborated by Yulo and Hendrickson (2019).

Given the complexity of the cell wall synthesis machinery that defines rod-shape in bacteria, it is hard to imagine how rods could have evolved prior to cocci. However, the cylindrical shape offers a number of advantages. For a given biomass (or cell volume), shape determines surface area of the cell envelope, which is the smallest surface area associated with the spherical shape. As shape sets the surface/volume ratio, it also determines the ratio between supply (proportional to the surface) and demand (proportional to cell volume). From this point of view, it is more efficient to be cylindrical (Young 2006). This also holds for surface attachment and biofilm formation (Young 2006). But above all, for growing cells, the ratio between supply and demand is constant in rod shaped bacteria, whereas it decreases for cocci. This requires that spherical cells evolve complex regulatory networks capable of maintaining the correct concentration of cellular proteins despite changes in surface/volume ratio. From this point of view, rod-shaped bacteria offer opportunities to develop unsophisticated regulatory networks.

While fitness in the majority of experimental lines was restored by mutations in *pbp1A*, one line, Line 5, contained a mutation in *ftsZ*, but oddly this mutation, while apparent at generation 500, was undetected by population sequencing at generation 1,000 (**Supp Table 1**). Interestingly, the FtsZ D176A mutation occurred at precisely the same position as was found in a separate selection experiment in which compensatory mutations ameliorating harmful fitness effects associated with deletion of *rodA* were sought (D. W. Rogers, E. Franceschini & P. B. Rainey, unpublished). The prevalence of *pbp1A* mutations in the work reported here likely reflects the fact that loss-of-function mutations in *pbp1A* are more readily achieved compared to gain-of-function mutations in *ftsZ*. This is evident in screening of mutations in *pbp1A* at generation 50, where all lines carried at least one *pbp1A* mutation (**Supp Table 2**). It will be of interest to determine whether *ftsZ* mutations that restore fitness to Δ*rodA* cells also compensate for defects due to elimination of *mreB*.

Derived Line 7, which carries a five gene deletion spanning the outer membrane porin OprD – and with the possibility of additional effects via mutation in *ftsE* – provides a contrasting route for compensatory evolution. Although *oprD* has not been specifically deleted, it seems reasonable to predict that in the absence of the porin, influx of water is likely limited, thus damping changes in turgor pressure and reducing cell swelling. Curiously, deletion of the focal five genes from SBW25 led to incomplete septation (**Fig. 4C** and **Supp. Fig. 15F**), with an indication from time-resolved movies of Δ*mreB* ΔPFLU4921-4925 (Supp. Movie 3) that there is also fault with septation in this background. This suggests that while ΔPFLU4921-4925 contributed to rescue of Δ*mreB*, it came at a cost to septum formation, which was subsequently rescued by mutation in the transmembrane divisome protein FtsE.

In conclusion, we return to the possibilities for new understanding of complex cellular processes arising from use of natural selection to rescue genetically perturbed cells (Lind et al. 2019; Remigi et al. 2019; LaBar et al. 2020). Here, a fitness-compromised, but viable, *mreB* deletion mutant of *P. fluorescens* SBW25 provided opportunity to explore consequences of both the initial genetic lesion and the bases of fitness compensation. In part attributable to the fact that Δ*mreB* SBW25 is viable, it was possible to glimpse functional effects arising from deletion of MreB, namely, uneven rates of growth among daughter cells caused by heterogeneity in patterns of cell wall synthesis. Restoration of fitness was readily achieved by mutations that reduced (or eliminated) Pbp1A function or increased septation. The primary effect was to reduce variance in growth rate among daughter cells, which is connected to resumption in homogeneity of cell wall synthesis. Disentangling two explanatory hypotheses – one invoking a role for Pbp1B and the other that elongasome function in SBW25 depends on a correct balance of among cell wall synthesising enzymes – awaits future work.

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Author contributions

PRY: Construction of SBW25 GFP, phase contrast and fluorescence (BADA) microscopy, flow cytometry, growth assays, data collection, protein modelling, manuscript review. ND: microscopy, data collection / analysis, manuscript preparation. MG: Construction of SBW25 Δ*mreB*, selection experiment and fitness measures. BRR: Peptidoglycan analysis, Bocillin-FL PBP labelling assays, data analysis and manuscript review. ADF: Sequencing and data analysis, manuscript review. XXZ & YH: Genetics and mutation construction, manuscript review. MM: Mutation construction, aztreonam analysis. FC: Supervision, funding acquisition, data analysis, manuscript review. PBR: Experimental conception and design, funding acquisition, microscopy, data analysis, manuscript preparation. HH: DNA extraction and sequencing, genomic analysis, supervision of PRY, manuscript preparation.

Supplementary Material

	Mutation			
	Gene	Mutation	Freq @ 500	Freq @ 1,000
Line 1	pbp1A	D484N (GAT->AAT)	74% (n = 118)[1]	78% (n = 129)
Line 2	ftsA	H346R (CAC->CGC)	71% (n = 105)	72% (n = 114)
	ctpA	T174P (ACC->CCC)	66% (n = 93)	60% (n = 106)
Line 3	pbp1A	D721A (GAC->GCC)	99% (n = 75)	87% (n = 78)
Line 4	pbp1A	G719D (GGT->GAT)	60% (n = 90)	ND[2]
	pbp1A	T362P (ACC->CCC)	ND	98% (n = 100)
Line 5	ftsZ	D176A (GAC->GCC)	48% (n = 147)	ND
Line 6	pbp1A	N698K (AAC->AAG)	100% (n = 103)	100% (n = 52)
Line 7	del	5399934-5403214	30% (n = 34)	96% (n = 112)
	ftsE	T188A (ACC->GCC)	ND	63% (n = 125)
Line 8	pbp1A	del A at 450,515	39% (n = 94)	ND
	ctpA	T174P (ACC->CCC)	ND	73% (n = 105)
Line 9	pbp1A	G580D (GGC->GAC)	ND	42% (n = 86)
	pbp1A	W322* (TGG->TGA)	ND	40% (n = 111)
Line 10	pbp1A	T362P (ACC->CCC)	32% (n = 111)	ND
	pbp1A	G580D (GGC->GAC)	ND	36% (n = 100)

[1]Percentage of reads, n = number of reads.

[2]ND: no mutation detected.

Supplementary Table 1.

Mutations identified in derived lines at generations 500 and 1,000

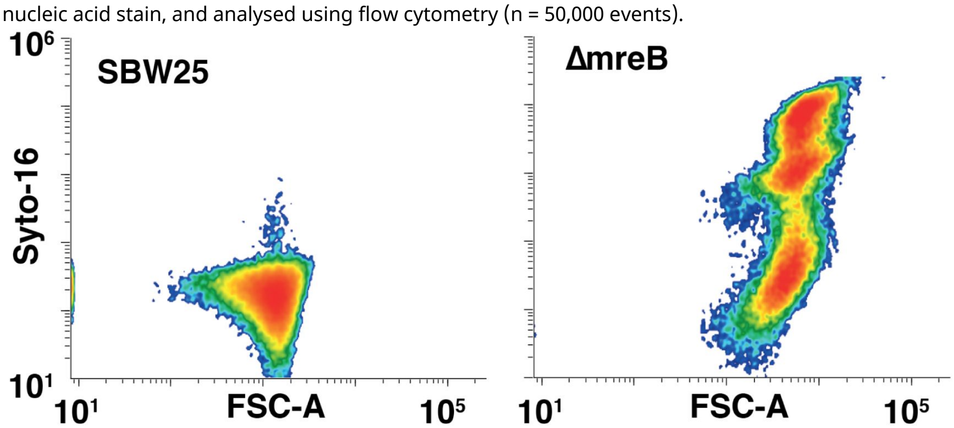
Supplementary Table 2.

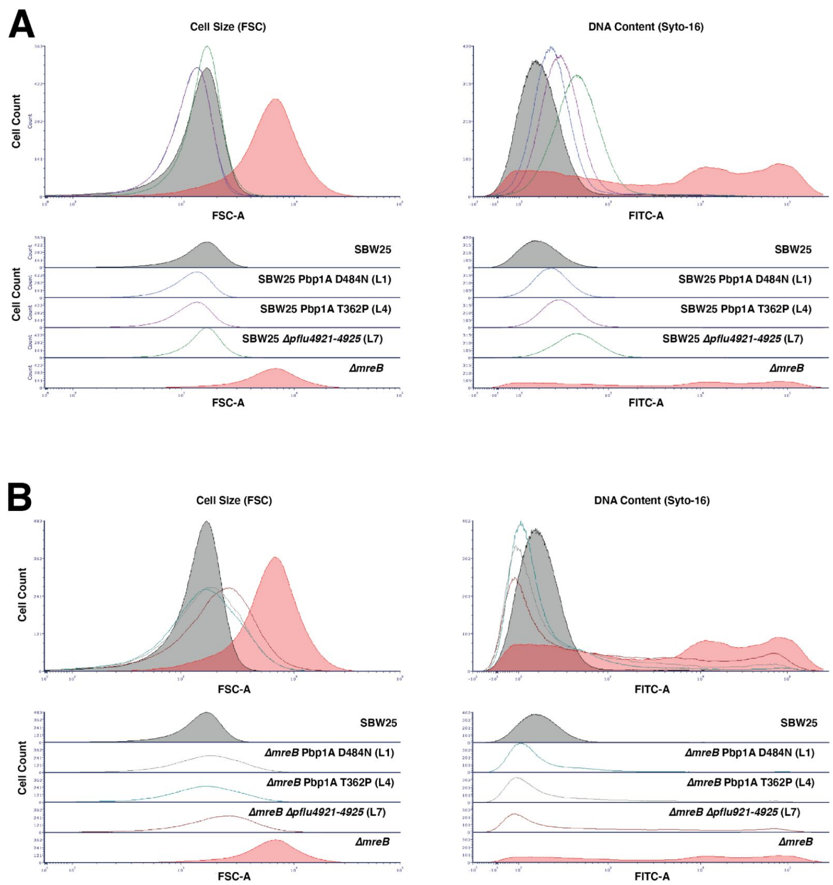
Mutations in *pbp1A* at generation 50

Line	Colonies with mutation (of 16 colonies)	Nucleotide position in PBP1a	Nucleotide change	Genomic Position	Protein-level change
1	16	1450	G>A	449754	D484N
2	1	2114	T>C	449090	F705S
2	1	1814 - 1815	Insertion	449389 - 449390	fs 605
3	3	1814 - 1815	Insertion	449389 - 449390	fs 605
4	2	1148 - 1149	Insertion	450055 - 450056	fs 383
4	1	1818 - 1819	Deletion	449385 - 449386	fs 606
6	4	2032	A>C	449172	T678P
6	1	1084	A>C	450120	T362P
7	1	922 - 923	Insertion	450281 - 450282	L308P
8	2	689	Deletion	450515	fs 230
8	1	1339	A>C	449865	S447R
9	2	996	A>G	450208	W>STOP
10	1	1739	G>A	449465	G580D

Supplementary Figure 1.

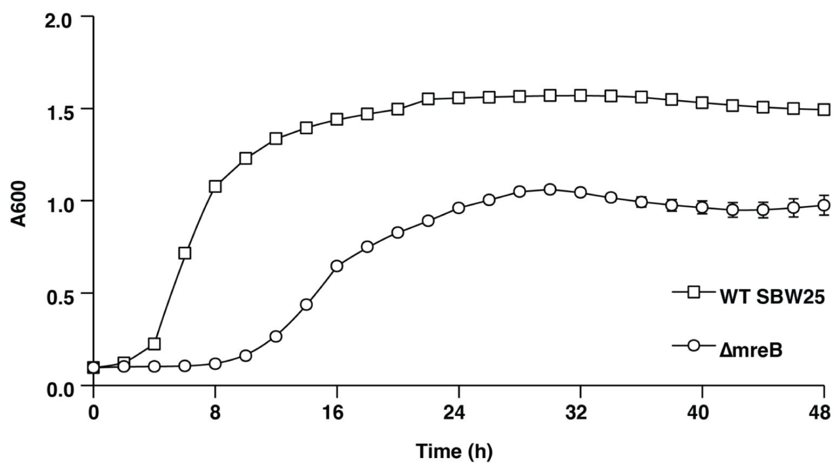
Correlation between cell size and DNA content in SBW25 and Δ*mreB* cells. DNA per cell does not vary in SBW25 cells to the degree that it varies in the Δ*mreB* cells as they grow larger. Cells were stained with SYTO 16, a cell-permeant green-fluorescent nucleic acid stain, and analysed using flow cytometry (n = 50,000 events).





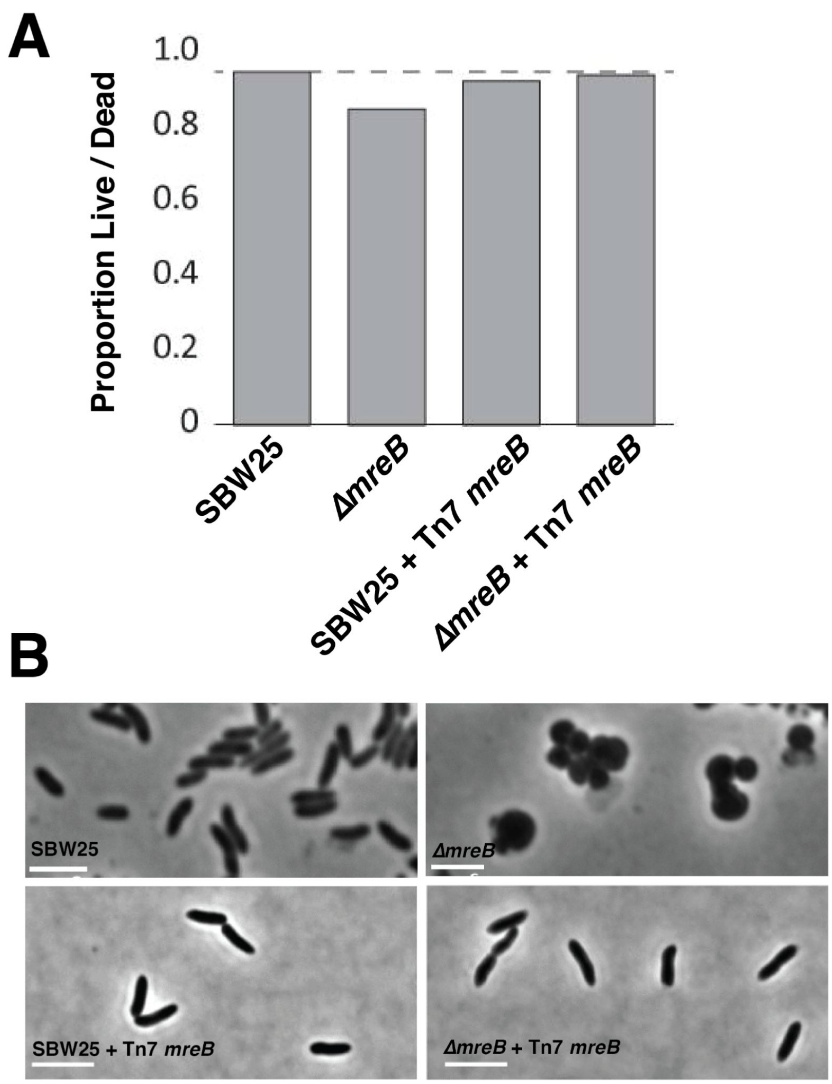
Supplementary Figure 2.

Genomic content and cell size of cells with mutations of interest A) Flow cytometry reveals an increase in DNA content coupled with an increase in size of SBW25 Δ*mreB*. In the SBW25 background *pbp1A* Line 1 and Line 4 mutants are slightly smaller than SBW25 cells and have comparable DNA content. The Line 7 reconstruction cells are larger by FSC than SBW25 and contain more DNA, reflective of the filamenting phenotype seen via microscopy. B) In the Δ*mreB* background, all three reconstructions have smaller cells than Δ*mreB*, with DNA content that is similar to SBW25. A small proportion of cells with large amounts of DNA are seen in the Line 7 reconstruction; these are also observed via microscopy as clumped spherical cells forming incomplete septa.



Supplementary Figure 3.

Growth curves for ancestral SBW25 and Δ*mreB* strains as measured by OD 600 in LB. NB: All growth curves were produced simultaneously. Data are means and standard deviations of three biological replicates.

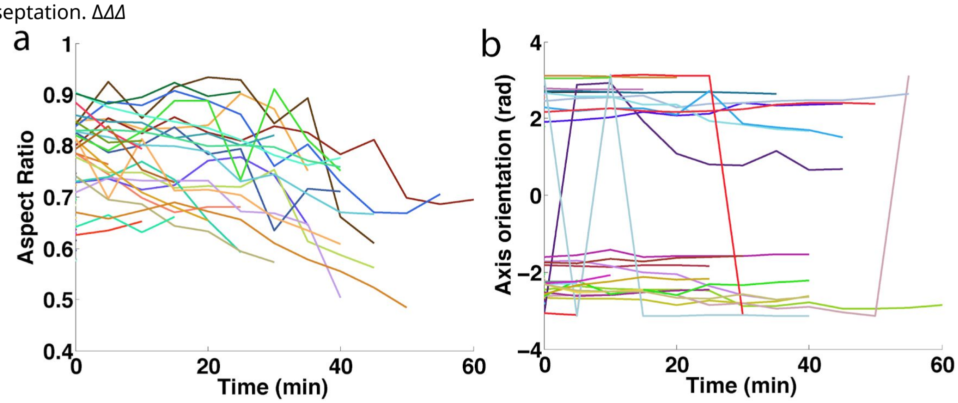


Supplementary Figure 4.

Ancestral SBW25 and Δ*mreB* cells, with ectopically added MreB expressed from the *glmS* region of the chromosome. A) Viability of respective strains via live-dead cell staining. B) Phase contrast micrographs of SBW25, Δ*mreB,* SBW25 + Tn*7 mreB*, and Δ*mreB* + Tn*7 mreB*. Scale bars = 3 μm.

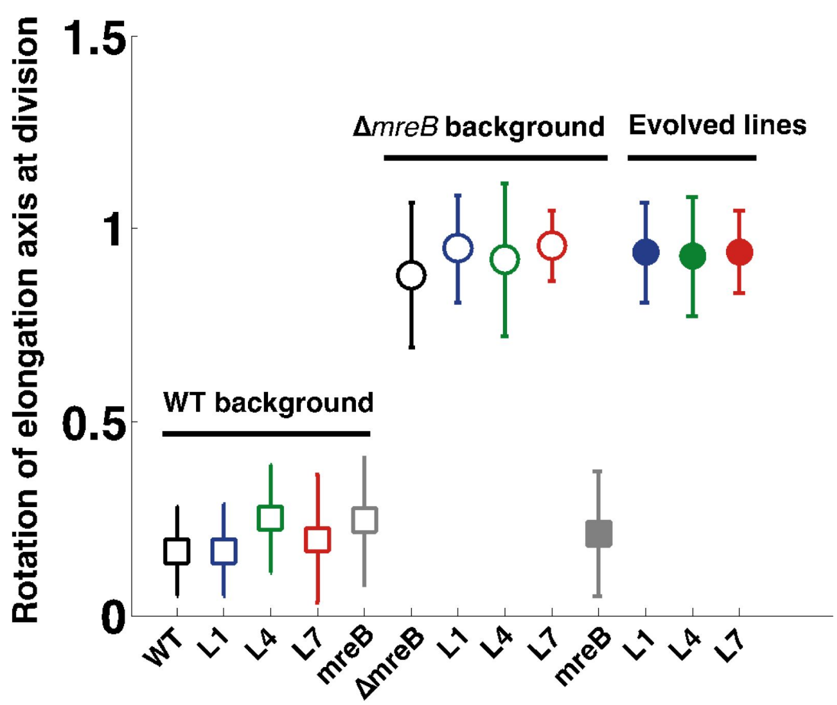
Supplementary Figure 5.

a) Aspect ratio for Δ*mreB* cells. Aspect ratio is defined as the ratio between the short and the long axis of the cell mask fitted with an ellipse. The decrease of the aspect ratio indicates that the long axis grows faster than the short axis. b) Orientation of the elongation axis in Δ*mreB* cells is constant during the division. Angles are in radians. Sudden changes correspond to phase shift (angles are expressed within the interval [-π;π] with periodic boundaries). In a) and b), time traces are plotted from cell birth until septation. ΔΔΔ



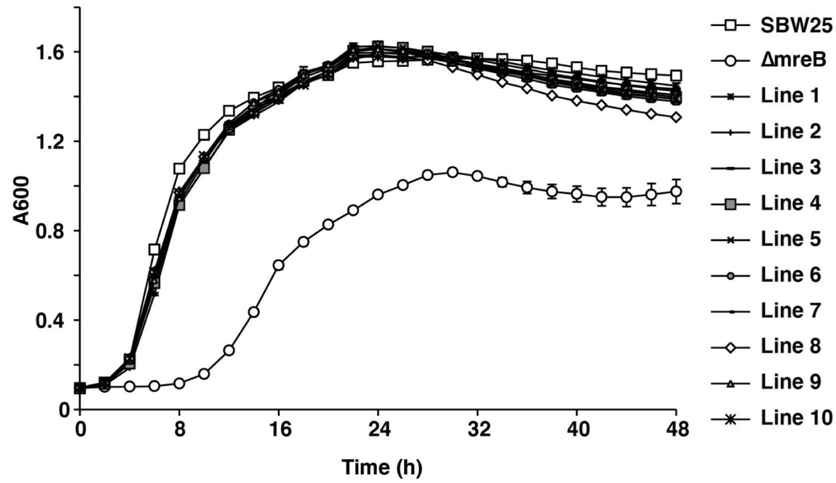
Supplementary Figure 6.

From the angle, θ, between mother and sister cells, we compute the relative orientation between mother and daughter using |sin θ | in order to get average in populations. |sin θ |=0 means that mother and daughter cells are aligned, |sin θ |=1 means that they are perpendicular. Filled or open square data points represent rod-like cells and filled or open circles represent spherical cells. WT strains are labelled in black. Open squares and open circles represent the reconstruction of the different observed mutations in the WT and Δ*mreB* backgrounds, respectively. Filled circles represent the evolved lines where the mutations have been identified. Colors as in **Fig 3**; blue is Line 1 or PBP1a D484N, green is Line 4 or PBP1a T362P, red is Line 7, grey is ectopic *mreB* expression. Data are means and standard deviation (N_{WT} = 141; N_{L1_WT} = 78; N_{L4_WT} = 57; N_{L7_WT} = 110; N_{mreB_WT} = 54; $N_{\Delta mreB}$ = 154; $N_{L1\_\Delta mreB}$ = 148; $N_{L4\_\Delta mreB}$ = 164; $N_{L7\_\Delta mreB}$ = 149; $N_{mreB\_\Delta mreB}$ = 96; N_{L1} = 132; N_{L4} = 116; N_{L7} = 116).



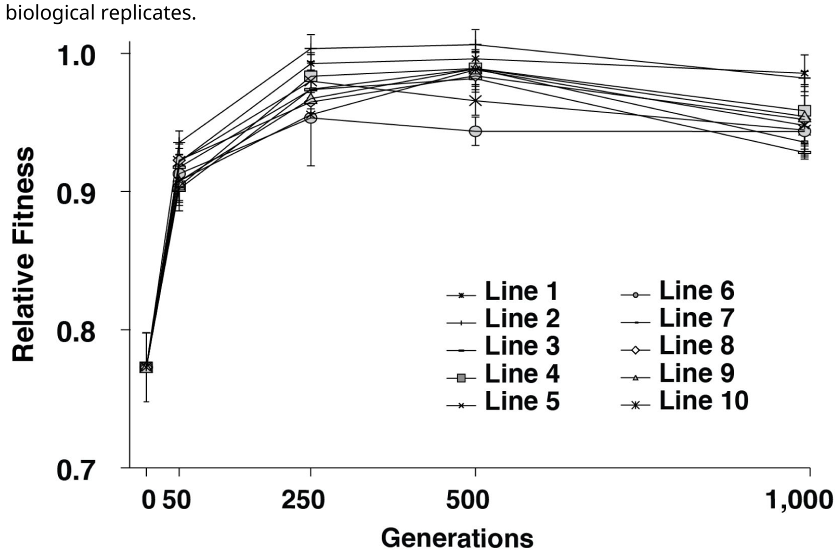
Supplementary Figure 7.

Growth curves of evolved Δ*mreB* lineages after 1,000 generations of evolution. Ancestral SBW25 and Δ*mreB* are shown for comparison. NB: All growth curves were produced simultaneously as described in Figure Supp. 1, SBW25 (WT) and Δ*mreB* are shown for comparison.



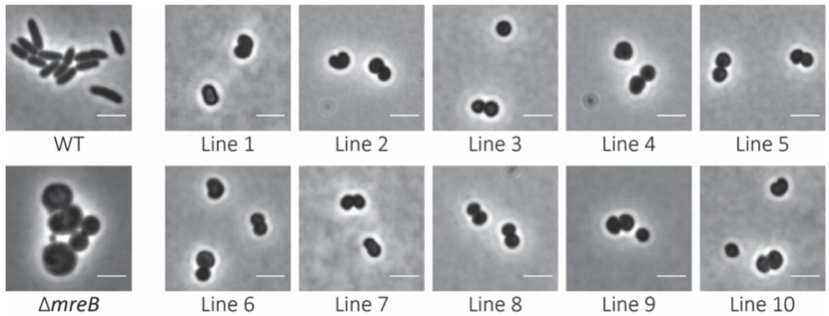
Supplementary Figure 8.

Relative fitness of the evolved lines at distinct points during the 1,000 generations of evolution. Data are means and standard deviation of three biological replicates.



Supplementary Figure 9.

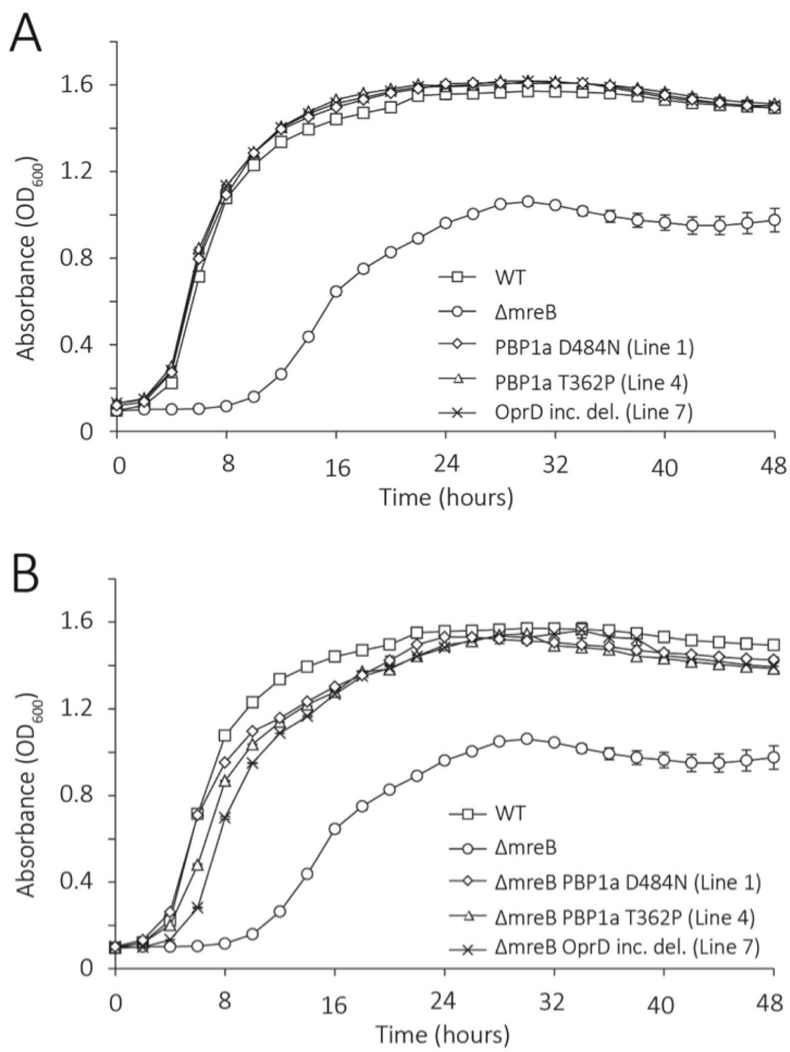
Phase contrast images of the 10 independent evolved lines. Cells were harvested at log phase (OD_{600} = 0.4). Scale bars = 3 um.



Supplementary Figure 10.

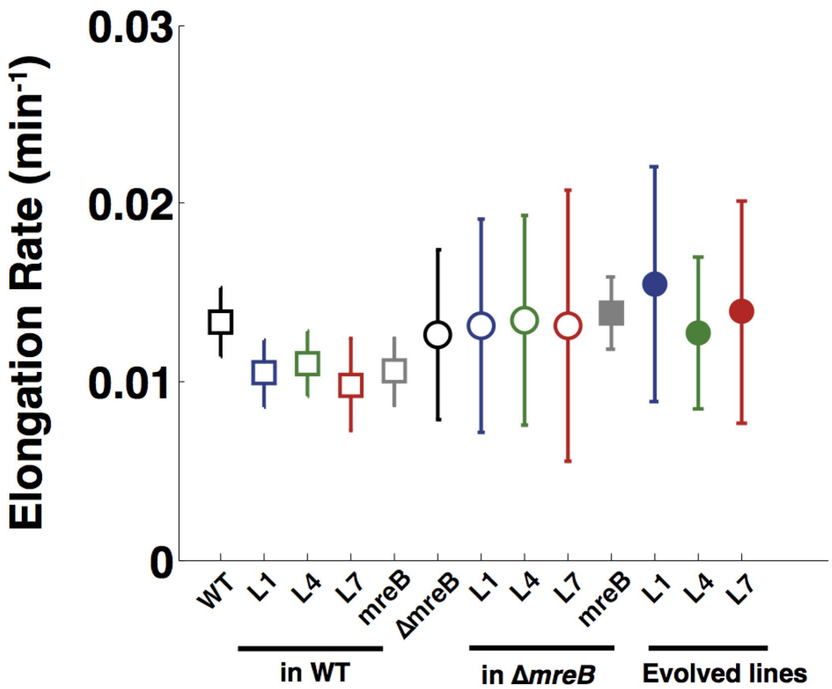
A Clustal Omega sequence alignment of a segment of Pbp1A amino acid sequences from seven bacterial species, including *A. baumannii*, which was used for the construction of the protein model showing the location of the evolved line mutations. NB: *Streptococcus* does not contain the OB/ODD domain. Regions of interest containing the evolved Line 1 and 4 mutations are shown. Mutated residues are in bold. The mutations are shaded in red and green respectively and labeled accordingly. Strictly conserved residues are labelled below the alignment with asterisks.

```
Strep_agala|WP_000628365.1|         TRRNTVLQQMYQDKNISKKEYDQAVATPVTDGLKELKQKSTYPKYMDNYL-KQVISEVKQ     288
Escher_coli|CAP77843.1|             ARRNVVLSRMLDEGYITQQQFDQTRTEAINANYHAPEIAFSA-----PYLSEMVRQEMYN     282
Salmon_ent_Typhi|CAD08121.1|        ARRNVVLSRMLSEGYITQAQYDQARSEPIDANYHAPEIAFSA-----PYLSEMVRQEMYN     282
Pseudo_fluor|YP_002870086.1|        ERRDWILGRMYKLGKIDQAAYESAVAEPLNASYHVPTPEVNA-----PYIAEMARAEMVG     275
Pseudo_aeri_PA01|AAB39710.1|        ERRNWILERMLKLGFIDQQRYQAAVEEPINASYHVQTPELNA-----PYIAEMARAEMVG     274
Acineto_baum|ACJ59085.1|            ERRNWILGRMLQLGYISQTEYQKAVAEPINLNMPNRDLNNIH-----PYAGEMVRSELVK     250
Morax_cata|WP_003659066.1           ERRNWIIGRMHQEGFITASEKDAAIAEPMGLNIYQEKLDLNM-----PYVAEMVRSTLVD     288
                                     **: :: :* .   *     : :    :  .                *  : .   :

Strep_agala                         KTGKDIFTAGLKVYTNINTDAQKQLYDIYN-----SDTYIAYPNNE--------------     329
Escher_coli                         RYGESAYEDGYRIYTTITRKVQQAAQQAVRNNVLDYDMRHGYRGPANVLWKVGESAWDNN     342
Salmon_ent_Typhi                    RYGESAYEDGYRIYTTITRKVQQAAQQAVRNNVLDYDMRHGYSGPANVLWKVGETAWDSK     342
Pseudo_fluor                        RYGSEAYTEGFRVTTTVPSNLQDIANEAVHSGLITYDQRHGYRGPESRLPGKTLSAWTT-     334
Pseudo_aeri_PA01                    RYGSEAYTEGYKVITTVRSDLQNAASQSVRDGLIDYDQRHGYRGPETRLPGQTRDAWLK-     333
Acineto_baum                        HFGEQAIDSGYKVYTTINAKRQAIAEKAVQDGLEAYDRRHGWRGAEAHD---------K-     300
Morax_cata                          RYGEAVMDSGWRVQTTINSSSQLAANAALVGGLRDYDRRHGWRGVEAES---------G-     338
                                    : *.     * :: *.:  . *              *   .: .
                                                         Line 4 T363P
Strep_agala                         ------------------------------------------------------------     329
Escher_coli                         KITDTLKALPTYGPLLPAAVTSANPQEATAMLADGSTVVLSMEGVRWARPYRSDTQQGPT     402
Salmon_ent_Typhi                    KITDTLKALPTYGPLLPAVVTSANPQEATAALADGTSVSLHMEGMRWARPYRSDTQQGPT     402
Pseudo_fluor                        ----ELGKQRPISGLEPAIVTQVKKDGVQVLTRTGE-AHVAWDSMKWARPFLNTNSMGAM     389
Pseudo_aeri_PA01                    ----HLGQQRSIGGLEPAIVTQVEKSGIMVMTRDGKEEAVTWDSMKWARPFLSNNSMGPM     389
Acineto_baum                        ----PLSEFRAYANTYPAQVTKVNSSSFEALMQDGSTVTVQWSGMSWARPYRNANSVGAA     356
Morax_cata                          ----SLEGRKNFDHIYPAKVTKVHNQSFEAKLQSGETVTIGWSGMNWARRYYDANRVGGG     394


Strep_agala                         ----------------------------------LQIASTIMDATNGKVIAQLGGRHQNEN     356
Escher_coli                         PRKVTDVLQTGQQIWVRQ---VGDAWWLAQVPEVNSALVSINPQNGAVMALVGGFDFNQS     459
Salmon_ent_Typhi                    PRKVTDVVQTGQQIWVRQ---VDNDWWLAQVPEVNSALVSLNPQTGAVLALVGGFDFNQS     459
Pseudo_fluor                        PKQPSDVAQVGDLIRVQRQ--KDDSLKFSQVPLAQGALVSLDPQNGAIRALVGGFAFEQS     447
Pseudo_aeri_PA01                    PRQPADVAQAGDQIRVQRQ--EDGTLRFVQIPAAQSALISLDPKDGAIRSLVGGFSFEQS     447
Acineto_baum                        PSRASQIVKVKDIVRLRP-NEAKTAWSLVQVPKVQGQLIAINPNDGSIEAIVGGYNFYQS     415
Morax_cata                          YSNAHQMVKVGNIVRISPINEAKTAWRMESIPKVQGALVSLDPENGALRAVVGGFHFNHS     454
                                                                      :     ::   * : : :**    ..

Strep_agala                         ISFGTNQSVLTDRDWGSTMKPISAYAPAIDSGVYNSTGQSLNDSVYYWPGTS---TQLYD     413
Escher_coli                         ---KFNRATQALRQVGSNIKPF-LYTAAMDKG-LTLASMLNDVPISRWDAGAGSDWQPKN     514
Salmon_ent_Typhi                    ---KFNRATQALRQVGSNIKPF-LYTAAMDKG-LTLASMLNDVPISRWDAGAGSDWRPKN     514
Pseudo_fluor                        ---NYNRATQAKRQPGSSFKPF-IYSAALDNG-YTAASLVNDAPIVFVDEYLDKVWRPKN     502
Pseudo_aeri_PA01                    ---NYNRAIQAKRQPGSSFKPF-IYSAALDNG-FTAASLVNDAPIVFVDEYLDKVWRPKN     502
Acineto_baum                        ---KFNRALQGWRQPGSTIKPF-LYALALERG-MTPYSMVNDSPITI------GKWTPKN     464
Morax_cata                          ---KFNRAIQGYRQPGSIIKPF-IFAAALESGQYTPDSLISDAAIRV------GRWQPKN     504
                                         *::    *: ** :**:  :: *:: *  .  .   :  :              :
                                                                     Line 1 D484N
Strep_agala                         WDRQYMGWMSMQTAIQQSRNVPAVRALEAAGLDEAKSFLEKLGIY-YPEMNYSNAISSNN     472
Escher_coli                         SPPQYAGPIRLRQGLGQSKNVVMVRAMRAMGVDYAAEYLQRF-GFPAQNIVHTESL----     569
Salmon_ent_Typhi                    SPPQYAGPIRLRQGLGQSKNVVMVRAMRAMGVDYAAEYLQRF-GFPAQNIVHTESL----     569
Pseudo_fluor                        DTNTFLGPIRIREALYKSRNLVSIRLLQAMGVGKTIDYMTRF-GFAKSDLPPNLSL----     557
Pseudo_aeri_PA01                    DTNTFLGPIPLREALYKSRNMVSIRVLQGLGIERAISYITKF-GFQRDELPRNFSL----     557
Acineto_baum                        SDGRYLGMIPLRRALYLSRNTVSVRLLQTVGIERTRQLFMDF-GLQEDQIPRNYTI----     519
Morax_cata                          ADGRYTGDMTLRRALTLSRNTPAIRLLRSAGVDESRHLMNAMGGLEIERMPSTLAL----     560
                                        : * : :: .:  *:*   :* :.  *:  :   :  :       :  . ::
```



Supplementary Figure 11.

Growth curves of the reconstructions in A) SBW25 and B) Δ*mreB* backgrounds. NB: All growth curves were produced simultaneously as described in **Fig Supp. 1**. Ancestral SBW25 (WT) and Δ*mreB* are shown for comparison.

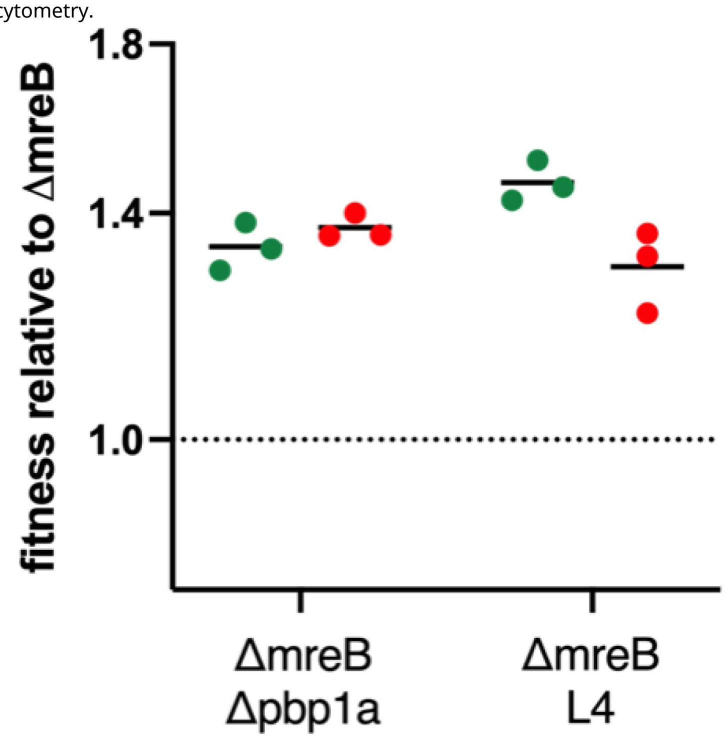


Supplementary Figure 12.

Elongation rate measures the rate, r, at which cell volume increases $V(t)=V_0e^{rt}$. Filled or open square data points represent rod-like cells and filled or open circles represent spherical cells. Ancestral SBW25 (WT) strains are labelled in black. Open squares and open circles represent the reconstruction of the different observed mutations in the WT and Δ*mreB* backgrounds, respectively. Filled circles represent the evolved lines where the mutations have been identified. Colors as in **Fig 3**; blue is Line 1 or PBP1a D484N, green is Line 4 or PBP1a T362P, red is Line 7, grey is ectopic *mreB* expression. Error bars represent standard deviations. (N_{WT} = 94; N_{L1_WT} = 52; N_{L4_WT} = 38; N_{L7_WT} = 72; N_{mreB_WT} = 36; $N_{\Delta mreB}$ = 99; $N_{L1\_\Delta mreB}$ = 92; $N_{L4\_\Delta mreB}$ = 102; $N_{L7\_\Delta mreB}$ = 96; $N_{mreB\_\Delta mreB}$ = 64; N_{L1} = 88; N_{L4} = 74; N_{L7} = 76)

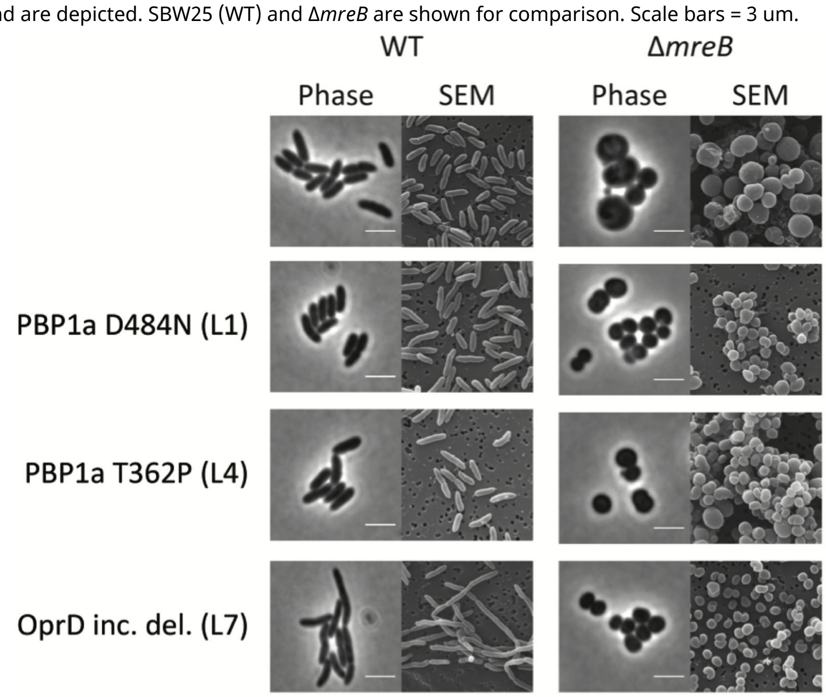
Supplementary Figure 13.

Fitness of SBW25 Δ*mreB* Δ*pbp1A* and Δ*mreB pbp1A* (T362P) relative to SBW25 Δ*mreB* and. Each genotype was marked separately with green and red fluorescent protein allowing reciprocal fitness measures to control for any marker effect. Data were collected by flow cytometry.



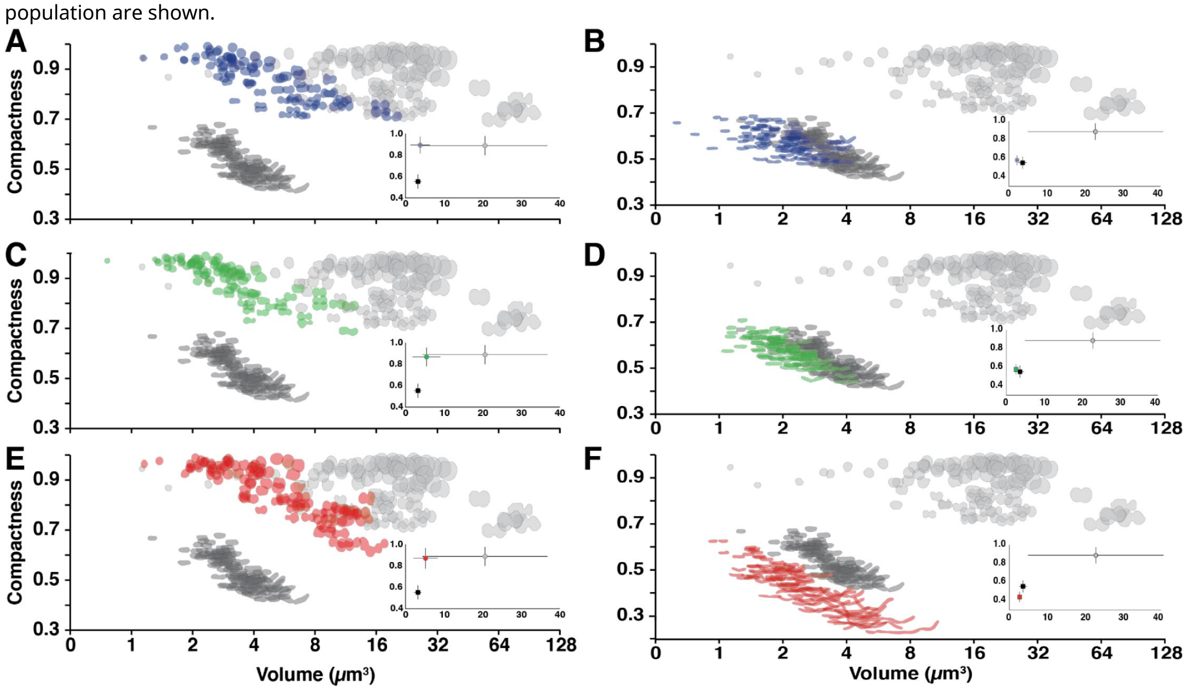
Supplementary Figure 14.

Cell morphologies. Phase contrast and SEM images of reconstruction strains. Reconstructions in either the SBW25 or the Δ*mreB* background are depicted. SBW25 (WT) and Δ*mreB* are shown for comparison. Scale bars = 3 um.



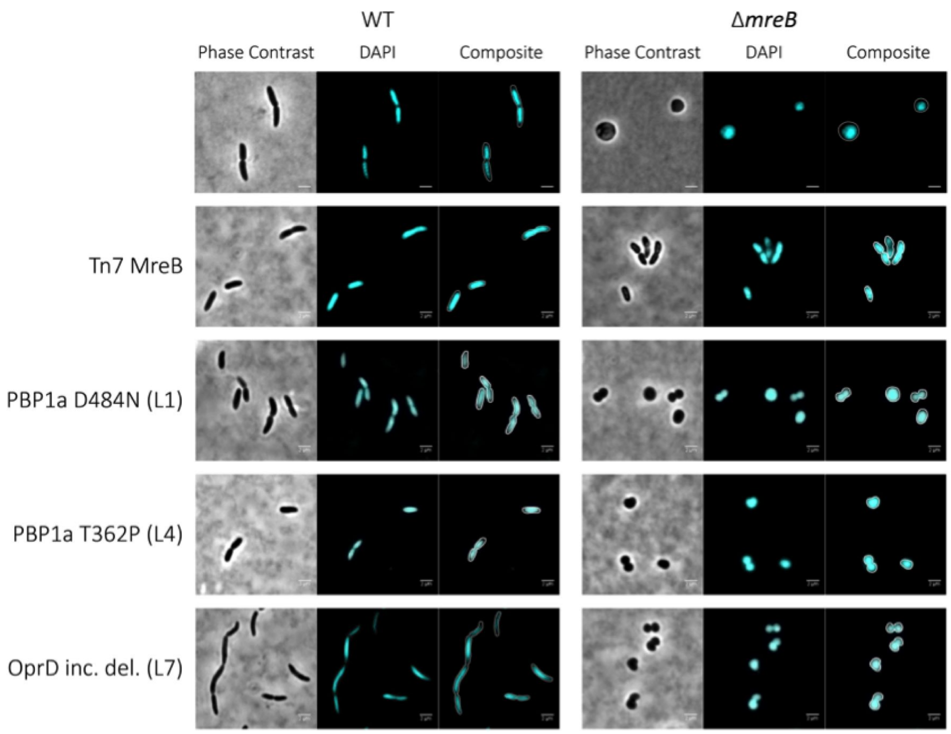
Supplementary Figure 15.

The cell morphologies and relationship between cell shape and estimated volume (V_e) represented using compactness as in **Fig 1B** of mutation reconstructions. Cell shape for each mutation of interest in A) the Line 1 or PBP1a D484N in Δ*mreB* and B) in SBW25. C) The Line 4 or PBP1a T362P in Δ*mreB* and D) in SBW25. E) The Line 7 PFLU4921-4925 Δ5399934-5403214 in Δ*mreB* and F) in SBW25. All populations are shown alongside the Δ*mreB* and SBW25 morphologies for reference. Colours are as **Fig 2**. Insets as in **Fig 2B** reflecting the Stfor the mutant populations. One hundred representative cells from each population are shown.



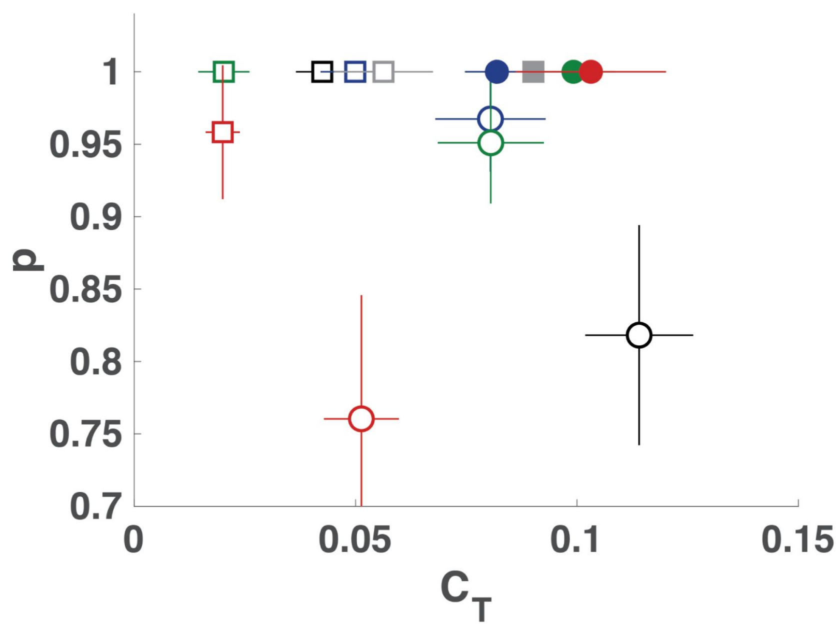
Supplementary Figure 16.

Genome map of the *oprD* inclusive deletion, (PFLU4921-4925 Δ5399934-5403214) and surrounding region. Genes with function calls are noted.



Supplementary Figure 17.

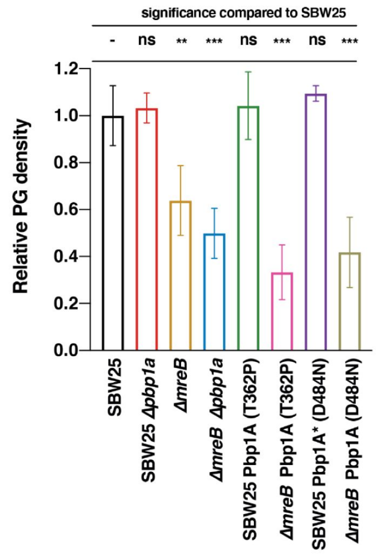
Reconstruction of Line 1, 4 and 7 mutations in ancestral SBW25 (WT) and SBW25 Δ*mreB* background stained with DAPI. SBW25 and Δ*mreB* cells shown for comparison. DNA segregation defects appear to be common at septation in this population. Scale bars = 2 um.



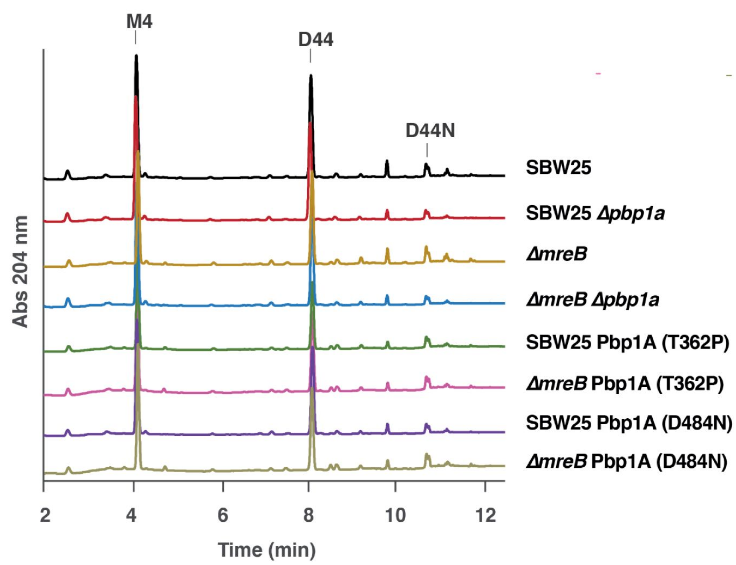
Supplementary Figure 18.

The probability p to pass to the next generation as a function of division time asymmetry C_T. Division time asymmetry is measured as the relative time elapsed between cell birth and division. Error bars represent standard errors (N_{WT} = 94; N_{L1_WT} = 52; N_{L4_WT} = 38; N_{L7_WT} = 72; N_{mreB_WT} = 36; $N_{\Delta mreB}$ = 99; $N_{L1\_\Delta mreB}$ = 92; $N_{L4\_\Delta mreB}$ = 102; $N_{L7\_\Delta mreB}$ = 96; $N_{mreB\_\Delta mreB}$ = 64; N_{L1} = 88; N_{L4} = 74; N_{L7} = 76). Filled or open square data points represent rod-like cells and filled or open circles represent spherical cells. Ancestral SBW25 genotypes are labelled in black. Open squares and open circles represent the reconstruction of the different observed mutations in the ancestral and Δ*mreB* backgrounds, respectively. Filled circles represent the evolved lines where the mutations have been identified. Colors as in **Fig 3**; blue is Line 1 or PBP1a D484N, green is Line 4 or PBP1a T362P, red is Line 7, grey is ectopic *mreB* expression.

A

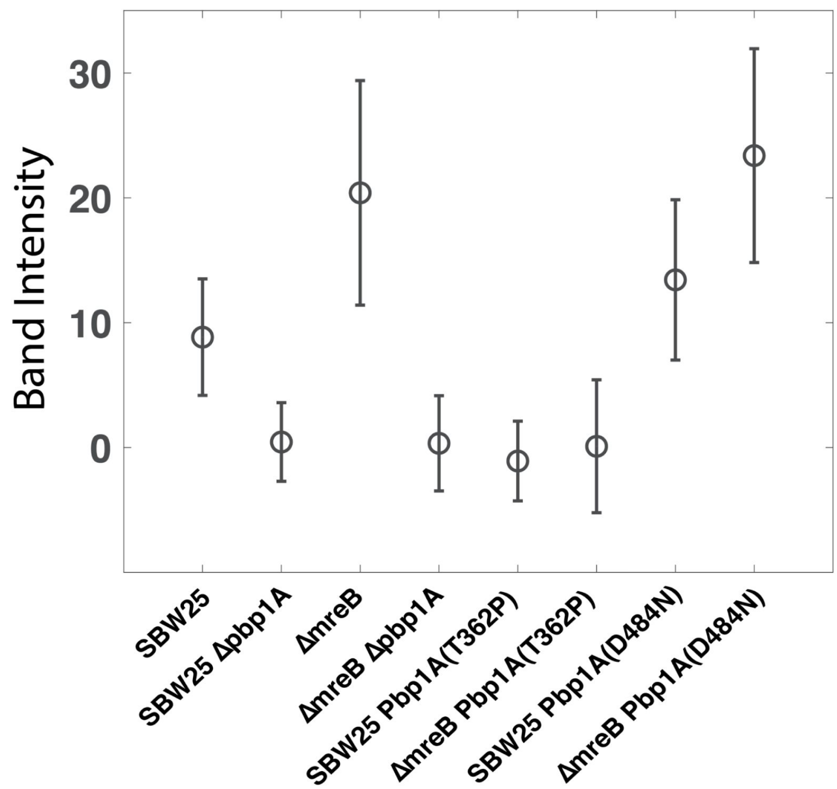


B



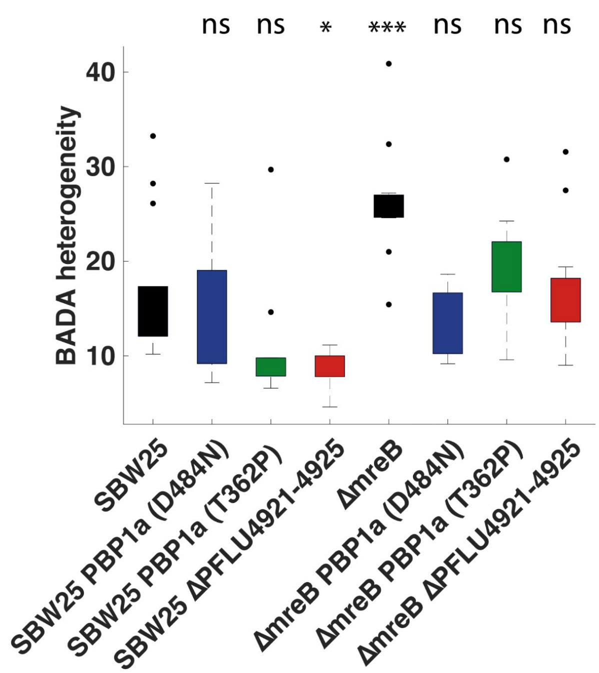
Supplementary Figure 19.

A. Peptidoglycan analysis showing the relative PG density of different *P. fluorescens* strains relative to SBW25 (wild type). Error bars (SD) are the result of 3 biological replicates. Significant differences (paired *t*-test between SBW25 and each mutant) are indicated by *p< 0.05, **p < 0.01, or ***p < 0.001. B. UPLC spectra of the peptidoglycan profiles of the different *P. fluorescens* strains. Major muropeptide peaks were labelled as M4 (monomer disaccharide tetrapeptide), D44 (dimer disaccharide tetra-tetrapeptide) and D44N (anhydrous dimer disaccharide tetra-tetrapeptide).



Supplementary Figure 20.

Quantification of band intensity corresponding to PBP1A TPase activity as presented in the gel shown in **Fig 4B**. Band intensity was measured from the raw image of the gel. For each condition, we measured the mean background level and subtracted the mean grey level of the band measured in ROIs of the same size.



Supplementary Figure 21.

Quantification of heterogeneity in cell wall synthesis as determined by BADA-labelling (see **Fig 4C**). Data are standard deviations divided by the square root of the mean pixel intensity (y-axis) of between 10 and 14 cells for each cell lineage. To rule out possible artefacts associated with variation of the mean intensity, we calculated BADA heterogeneity as the ratio of the standard deviation divided by the square root of the mean. Significant differences (paired *t*-test between SBW25 and each mutant) are indicated by *p< 0.05, **p < 0.01, or ***p < 0.001 or ns when p>0.05.

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Reviewer #1 (Public review):

Summary:

The authors performed experimental evolution of MreB mutants that have a slow growing round phenotype and studied the subsequent evolutionary trajectory using analysis tool from molecular biology. It was remarkable and interesting that they found that the original phenotype was not restored (most common in these studies) but that the round phenotype was maintained.

Strengths:

The finding that the round phenotype was maintained during evolution rather than that the original phenotype, rod shape cells, was recovered is interesting. The paper extensively investigates what happens during adaptation with various different techniques. Also the extensive discussion of the findings at the end of the paper is well thought through and insightful.

Weaknesses:

I find there are three general weaknesses
(1) Although the paper states in the abstract that it emphasizes "new knowledge to be gained" it remains unclear what this concretely is. At page 4 they state 3 three research questions, these could be more extensively discussed in the abstract. Also these questions read more like genetics questions while the paper is a lot about cell biological findings.
(2) It is not clear to me from the text what we already know about restoration of MreB loss from suppressors studies (in the literature). Are there supressor screens in the literature and which part of the findings is consistent with suppressor screens and which parts are new knowledge?
(3) The clarity of the figures, captions and data quantification need to be improved.

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Reviewer #3 (Public review):

This paper addresses a long-standing problem in microbiology: the evolution of bacterial cell shape. Bacterial cells can take a range of forms, among the most common being rods and spheres. The consensus view is that rods are the ancestral form and spheres the derived form. The molecular machinery governing these different shapes is fairly well understood but the evolutionary drivers responsible for the transition between rods and spheres is not. Enter Yulo et al.'s work. The authors start by noting that deletion of a highly conserved gene called MreB in the Gram-negative bacterium Pseudomonas fluorescens reduces fitness but does not kill the cell (as happens in other species like E. coli and B. subtilis) and causes cells to become spherical rather than their normal rod shape. They then ask whether evolution for 1000 generations restores the rod shape of these cells when propagated in a rich, benign medium.

The answer is no. The evolved lineages recovered fitness by the end of the experiment, growing just as well as the unevolved rod-shaped ancestor, but remained spherical. The authors provide an impressively detailed investigation of the genetic and molecular changes that evolved. Their leading results are:

(1) the loss of fitness associated with MreB deletion causes high variation in cell volume among sibling cells after cell division;
(2) fitness recovery is largely driven by a single, loss-of-function point mutation that evolves within the first ~250 generations that reduces the variability in cell volume among siblings;
(3) the main route to restoring fitness and reducing variability involves loss of function mutations causing a reduction of TPase and peptidoglycan cross-linking, leading to a disorganized cell wall architecture characteristic of spherical cells.

The inferences made in this paper are on the whole well supported by the data. The authors provide a uniquely comprehensive account of how a key genetic change leads to gains in fitness and the spectrum of phenotypes that are impacted and provide insight into the molecular mechanisms underlying models of cell shape.

Suggested improvements and clarifications include:
(1) A schematic of the molecular interactions governing cell wall formation could be useful in the introduction to help orient readers less familiar with the current state of knowledge and key molecular players;
(2) It remains unclear whether corrections for multiple comparisons are needed when more than one construct or strain is compared to the common ancestor, as in Supp Fig 19A (relative PG density of different constructs versus the SBW25 ancestor). The author's response did not clarify matters: was data for the WT obtained independently alongside each each strain/construct (justifying a paired t-test) or was a single set of data for the WT obtained and used to compare against all other strains/constructs (which would demand a correction for multiple comparisons)?
(3) The authors refrain from making strong claims about the nature of selection on cell shape, perhaps because their main interest is the molecular mechanisms responsible. They identify sources of stabilizing selection favouring an intermediate cell size (lack of DNA in small cells and disorganized DNA in large cells). Their interpretation of stabilizing selection in the review is correct and entirely consistent with the mechanistic causes identified here. I think this is valuable and interesting, although I recognize it is not the focus of the paper.

Comments on revisions:

Please further clarify the experimental design and replication for the contrast between mutants and WT to address the issue of multiple comparisons.

https://doi.org/10.7554/eLife.98218.2.sa1

Author response:

The following is the authors’ response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The authors performed experimental evolution of MreB mutants that have a slow-growing round phenotype and studied the subsequent evolutionary trajectory using analysis tools from molecular biology. It was remarkable and interesting that they found that the original phenotype was not restored (most common in these studies) but that the round phenotype was maintained.*
>
> *Strengths:*
>
> *The finding that the round phenotype was maintained during evolution rather than that the original phenotype, rod-shaped cells, was recovered is interesting. The paper extensively investigates what happens during adaptation with various different techniques. Also, the extensive discussion of the findings at the end of the paper is well thought through and insighXul.*
>
> *Weaknesses:*
>
> *I find there are three general weaknesses:*
>
> *(1) Although the paper states in the abstract that it emphasizes "new knowledge to be gained" it remains unclear what this concretely is. On page 4 they state 3 three research questions, these could be more extensively discussed in the abstract. Also, these questions read more like genetics questions while the paper is a lot about cell biological findings.*

Thank you for drawing attention to the unnecessary and gratuitous nature of the last sentence of the Abstract. We are in agreement. It has been modified, and we have taken advantage of additional word space to draw attention to the importance of the two competing (testable) hypotheses laid out in the Discussion.

As to new knowledge, please see the Results and particularly the Discussion. But beyond this, and as recognised by others, there is real value for cell biology in seeing how (and whether) selection can compensate for effects that are deleterious to fitness. The results will very o_en depart from those delivered from, for example, suppressor analyses, or bottom up engineering.

In the work recounted in our paper, we chose to focus – by way of proof-of principle – on the most commonly observed mutations, namely, those within *pbp1A*. But beyond this gene, we detected mutations in other components of the cell shape / division machinery whose connections are not yet understood and which are the focus of on-going investigation.

As to the three questions posed at the end of the Introduction, the first concerns whether selection can compensate for deleterious effects of deleting *mreB* (a question that pertains to evolutionary aspects); the second seeks understanding of genetic factors; the third aims to shed light on the genotype-to-phenotype map (which is where the cell biology comes into play). Given space restrictions, we cannot see how we could usefully expand, let alone discuss, the three questions raised at the end of the Introduction in restrictive space available in the Abstract.

> *(2) It is not clear to me from the text what we already know about the restoration of MreB loss from suppressors studies (in the literature). Are there suppressor screens in the literature and which part of the findings is consistent with suppressor screens and which parts are new knowledge?*

As stated in the Introduction, a previous study with *B. subtilis* (which harbours three MreB isoforms and where the isoform named "MreB" is essential for growth under normal conditions), suppressors of MreB lethality were found to occur in *ponA*, a class A penicillin binding protein (Kawai et al., 2009). This led to recognition that MreB plays a role in recruiting Pbp1A to the lateral cell wall. On the other hand, Patel et al. (2020) have shown that deletion of classA PBPs leads to an up-regulation of rod complex activity. Although there is a connection between rod complex and class A PBPs, a further study has shown that the two systems work semi-autonomously (Cho et al., 2016).

Our work confirms a connection between MreB and Pbp1A, and has shed new light on how this interaction is established by means of natural selection, which targets the integrity of cell wall. Indeed, the Rod complex and class A PBPs have complementary activities in the building of the cell wall with each of the two systems able to compensate for the other in order to maintain cell wall integrity. Please see the major part of the Discussion. In terms of specifics, the connection between *mreB* and *pbp1A* (shown by Kawai et al (2009)) is indirect because it is based on extragenic transposon insertions. In our study, the genetic connection is mechanistically demonstrated. In addition, we capture that the evolutionary dynamics is rapid and we finally enriched understanding of the genotype-to-phenotype map.

> *(3) The clarity of the figures, captions, and data quantification need to be improved.*

Modifications have been implemented. Please see responses to specific queries listed below.

> ***Reviewer #2 (Public Review):***
>
> *Yulo et al. show that deletion of MreB causes reduced fitness in P. fluorescens SBW25 and that this reduction in fitness may be primarily caused by alterations in cell volume. To understand the effect of cell volume on proliferation, they performed an evolution experiment through which they predominantly obtained mutations in pbp1A that decreased cell volume and increased viability. Furthermore, they provide evidence to propose that the pbp1A mutants may have decreased PG cross-linking which might have helped in restoring the fitness by rectifying the disorganised PG synthesis caused by the absence of MreB. Overall this is an interesting study.*
>
> *Queries:*
>
> *Do the small cells of mreB null background indeed have have no DNA? It is not apparent from the DAPI images presented in Supplementary Figure 17. A more detailed analysis will help to support this claim.*

It is entirely possible that small cells have no DNA, because if cell division is aberrant then division can occur prior to DNA segregation resulting in cells with no DNA. It is clear from microscopic observation that both small and large cells do not divide. It is, however, true, that we are unable to state – given our measures of DNA content – that small cells have no DNA. We have made this clear on page 13, paragraph 2.

> *What happens to viability and cell morphology when pbp1A is removed in the mreB null background? If it is actually a decrease in pbp1A activity that leads to the rescue, then pbp1A- mreB- cells should have better viability, reduced cell volume and organised PG synthesis. Especially as the PG cross-linking is almost at the same level as the T362 or D484 mutant.*

Please see fitness data in Supp. Fig. 13. Fitness of Δ*mreB* Δ*pbp1A* is no different to that caused by a point mutation. Cells remain round.

> *What is the status of PG cross-linking in ΔmreB Δpflu4921-4925 (Line 7)?*

This was not analysed as the focus of this experiment was PBPs. *A priori*, there is no obvious reason to suspect that Δ4921-25 (which lacks *oprD*) would be affected in PBP activity.

> *What is the morphology of the cells in Line 2 and Line 5? It may be interesting to see if PG cross-linking and cell wall synthesis is also altered in the cells from these lines.*

The focus of investigation was restricted to L1, L4 and L7. Indeed, it would be interesting to look at the mutants harbouring mutations in *:sZ*, but this is beyond scope of the present investigation (but is on-going). The morphology of L2 and L5 are shown in Supp. Fig. 9.

> *The data presented in 4B should be quantified with appropriate input controls.*

Band intensity has now been quantified (see new Supp. Fig .20). The controls are SBW25, SBW25Δ*pbp1A*, SBW25 Δ*mreB* and SBW25 Δ*mreBpbp1A* as explained in the paper.

> *What are the statistical analyses used in 4A and what is the significance value?*

Our oversight. These were reported in Supp. Fig. 19, but should also have been presented in Fig. 4A. Data are means of three biological replicates. The statistical tests are comparisons between each mutant and SBW25, and assessed by paired *t*-tests.

> *A more rigorous statistical analysis indicating the number of replicates should be done throughout.*

We have checked and made additions where necessary and where previously lacking. In particular, details are provided in Fig. 1E, Fig. 4A and Fig. 4B. For Fig. 4C we have produced quantitative measures of heterogeneity in new cell wall insertion. These are reported in Supp. Fig. 21 (and referred to in the text and figure caption) and show that patterns of cell wall insertion in Δ*mreB* are highly heterogeneous.

> ***Reviewer #3 (Public Review):***
>
> *This paper addresses an understudied problem in microbiology: the evolution of bacterial cell shape. Bacterial cells can take a range of forms, among the most common being rods and spheres. The consensus view is that rods are the ancestral form and spheres the derived form. The molecular machinery governing these different shapes is fairly well understood but the evolutionary drivers responsible for the transition between rods and spheres are not. Enter Yulo et al.'s work. The authors start by noting that deletion of a highly conserved gene called MreB in the Gram-negative bacterium Pseudomonas fluorescens reduces fitness but does not kill the cell (as happens in other species like E. coli and B. subtilis) and causes cells to become spherical rather than their*

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>
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>
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>
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>
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>
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>
> *Suggested improvements and clarifications include:*
>
> *(1) A schematic of the molecular interactions governing cell wall formation could be useful in the introduction to help orient readers less familiar with the current state of knowledge and key molecular players.*

We understand that this would be desirable, but there are numerous recent reviews with detailed schematics that we think the interested reader would be better consulting. These are referenced in the text.

> *(2) More detail on the bioinformatics approaches to assembling genomes and identifying the key compensatory mutations are needed, particularly in the methods section. This whole subject remains something of an art, with many different tools used. Specifying these tools, and the parameter sesngs used, will improve transparency and reproducibility, should it be needed.*

We overlooked providing this detail, which has now been corrected by provision of more information in the Materials and Methods. In short we used Breseq, the clonal option, with default parameters. Additional analyses were conducted using Genieous. The BreSeq output files are provided https://doi.org/10.17617/3.CU5SX1 (which include all read data).

> *(3) Corrections for multiple comparisons should be used and reported whenever more than one construct or strain is compared to the common ancestor, as in Supplementary Figure 19A (relative PG density of different constructs versus the SBW25 ancestor).*

The data presented in Supp Fig 19A (and Fig 4A) do not involve multiple comparisons. In each instance the comparison is between SBW25 and each of the different mutants. A paired *t*-test is thus appropriate.

> *(4) The authors refrain from making strong claims about the nature of selection on cell shape, perhaps because their main interest is the molecular mechanisms responsible. However, I think more can be said on the evolutionary side, along two lines. First, they*

> *have good evidence that cell volume is a trait under strong stabilizing selection, with cells of intermediate volume having the highest fitness. This is notable because there are rather few examples of stabilizing selection where the underlying mechanisms responsible are so well characterized. Second, this paper succeeds in providing an explanation for how spherical cells can readily evolve from a rod-shaped ancestor but leaves open how rods evolved in the first place. Can the authors speculate as to how the complex, coordinated system leading to rods first evolved? Or why not all cells have lost rod shape and become spherical, if it is so easy to achieve? These are important evolutionary questions that remain unaddressed. The manuscript could be improved by at least flagging these as unanswered questions deserving of further attention.*

These are interesting points, but our capacity to comment is entirely speculative. Nonetheless, we have added an additional paragraph to the Discussion that expresses an opinion that has yet to receive attention:

"Given the complexity of the cell wall synthesis machinery that defines rod-shape in bacteria, it is hard to imagine how rods could have evolved prior to cocci. However, the cylindrical shape offers a number of advantages. For a given biomass (or cell volume), shape determines surface area of the cell envelope, which is the smallest surface area associated with the spherical shape. As shape sets the surface/volume ratio, it also determines the ratio between supply (proportional to the surface) and demand (proportional to cell volume). From this point of view, it is more efficient to be cylindrical (Young 2006). This also holds for surface attachment and biofilm formation (Young 2006). But above all, for growing cells, the ratio between supply and demand is constant in rod shaped bacteria, whereas it decreases for cocci. This requires that spherical cells evolve complex regulatory networks capable of maintaining the correct concentration of cellular proteins despite changes in surface/volume ratio. From this point of view, rod-shaped bacteria offer opportunities to develop unsophisticated regulatory networks."

> *why not all cells have lost rod shape and become spherical.*

Please see Kevin Young's 2006 review on the adaptive significance of cell shape

> *The value of this paper stems both from the insight it provides on the underlying molecular model for cell shape and from what it reveals about some key features of the evolutionary process. The paper, as it currently stands, provides more on which to chew for the molecular side than the evolutionary side. It provides valuable insights into the molecular architecture of how cells grow and what governs their shape. The evolutionary phenomena emphasized by the authors - the importance of loss-of-function mutations in driving rapid compensatory fitness gains and that multiple genetic and molecular routes to high fitness are o_en available, even in the relatively short time frame of a few hundred generations - are wellunderstood phenomena and so arguably of less broad interest. The more compelling evolutionary questions concern the nature and cause of stabilizing selection (in this case cell volume) and the evolution of complexity. The paper misses an opportunity to highlight the former and, while claiming to shed light on the latter, provides rather little useful insight.*

Thank you for these thoughts and comments. However, we disagree that the experimental results are an overlooked opportunity to discuss stabilising selection. Stabilising selection occurs when selection favours a particular phenotype causing a reduction in underpinning population-level genetic diversity. This is not happening when selection acts on SBW25 Δ*mreB* leading to a restoration of fitness. Driving the response are biophysical factors, primarily the critical need to balance elongation rate with rate of septation. This occurs without any change in underlying genetic diversity.

> ***Recommendations for the authors:***
>
> ***Reviewer 1 (Recommendations for the Authors):***
>
> *Hereby my suggestion for improvement of the quantification of the data, the figures, and the text.*
>
> *- p 14, what is the unit of elongation rate?*

At first mention we have made clear that the unit is given in minutes^-1

> *- p 14, please give an error bar for both p=0.85 and f=0.77, to be able to conclude they are different*
>
> *Error on the probability p is estimated at the 95% confidence interval by the formula:1.96*
>
> $\sqrt{(p(1-p)/N)}$*, where N is the total number of cells. This has been added in the paragraph*
>
> *p »probability » of the Image Analysis section in the Material and Methods.*
>
> *We also added errors on p measurement in the main text.*
>
> *- p 14, all the % differences need an errorbar*

The error bars and means are given in Fig 3C and 3D.

> *- Figure 1B adds units to compactness, and what does it represent? Is the cell size the estimated volume (that is mentioned in the caption)? Shouldn't the datapoints have error bars?*

Compactness is defined in the “Image Analysis” section of the Material and Methods. It is a dimensionless parameter. The distribution of individual cell shapes / sizes are depicted in Fig 1B. Error does arise from segmentation, but the degree of variance (few pixels) is much smaller than the representations of individual cells shown.

> *- Figure 1C caption, are the 50.000 cells?*

Correct. Figure caption has been altered.

> *- Figure 1D, first the elongation rate is described as a volume per minute, but now, looking at the units it is a rate, how is it normalized?*

Elongation rate is explained in the Materials and Methods (see the image analysis section) and is not volume per minute. It is dV/dt = r*V (the unit of r is min^-1). Page 9 includes specific mention of the unit of *r*.

> *- Figure 1E, how many cells (n) per replicate?*

Our apologies. We have corrected the figure caption that now reads:

“Proportion of live cells in ancestral SBW25 (black bar) and *ΔmreB* (grey bar) based on LIVE/DEAD BacLight Bacterial Viability Kit protocol. Cells were pelleted at 2,000 x g for 2 minutes to preserve *ΔmreB* cell integrity. Error bars are means and standard deviation of three biological replicates (n>100).”

> *- Figure 1G, how does this compare to the wildtype*

The volume for wild type SBW25 is 3.27μm^3 (within the "white zone"). This is mentioned in the text.

> *- Figure 2B, is this really volume, not size? And can you add microscopy images?*

The x-axis is volume (see Materials and Methods, subsection image analysis). Images are available in Supp. Fig. 9.

> *- Figure 3A what does L1, L4 and L7 refer too? Is it correct that these same lines are picked for WT and delta_mreB*

Thank you for pointing this out. This was an earlier nomenclature. It was shorthand for the mutants that are specified everywhere else by genotype and has now been corrected.

> *- Figure 3c: either way write out p, so which probability, or you need a simple cartoon that is plotted.*

The value *p* is the probability to proceed to the next generation and is explained in Materials and Methods subsection image analysis. We feel this is intuitive and does not require a cartoon. We nonetheless added a sentence to the Materials and Methods to aid clarity.

> *- Figure 4B can you add a ladder to the gel?*

No ladder was included, but the controls provide all the necessary information. The band corresponding to PBP1A is defined by presence in SBW25, but absence in SBW25 Δ*pbp1A*.

> *- Figure 4c, can you improve the quantification of these images? How were these selected and how well do they represent the community?*

We apologise for the lack of quantitative description for data presented in Fig 4C. This has now been corrected. In brief, we measured the intensity of fluorescent signal from between 10 and 14 cells and computed the mean and standard deviation of pixel intensity for each cell. To rule out possible artifacts associated with variation of the mean intensity, we calculated the ratio of the standard deviation divided by the square root of the mean. These data reveal heterogeneity in cell wall synthesis and provide strong statistical support for the claim that cell wall synthesis in Δ*mreB* is significantly more heterogeneous than the control. The data are provided in new Supp. Fig. 21.

> *Minor comments:*
>
> *- It would be interesting if the findings of this experimental evolution study could be related to comparative studies (if these have ever been executed).*

Little is possible, but Hendrickson and Yulo published a portion of the originally posted preprint separately. We include a citation to that paper.

> *- p 13, halfway through the page, the second paragraph lacks a conclusion, why do we care about DNA content?*

It is a minor observation that was included by way of providing a complete description of cell phenotype.

> *- p 17, "suggesting that ... loss-of-function", I do no not understand what this is based upon.*

We show that the fitness of a *pbp1A* deletion is indistinguishable from the fitness of one of the *pbp1A* point mutants. This fact establishes that the point mutation had the same effects as a gene deletion thus supporting the claim that the point mutations identified during the course of the selection experiment decrease (or destroy) PBP1A function.

> *- p 25, at the top of the page: do you have a reference for the statement that a disorganized cell wall architecture is suited to the topology of spherical cells?*

The statement is a conclusion that comes from our reasoning. It stems from the fact that it is impossible to entirely map the surface of a sphere with parallel strands.

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