

# 1 Experimental Evolution of Cell Shape in Bacteria

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21  
22 **Cell shape is a fundamental property in bacterial kingdom. MreB is a protein**  
23 **that determines rod-like shape, and its deletion is generally lethal. Here, we**  
24 **deleted the *mreB* homolog from rod-shaped bacterium *Pseudomonas fluorescens***  
25 **SBW25 and found that *ΔmreB* cells are viable, spherical cells with a 20%**  
26 **reduction in competitive fitness and high variability in cell size. We show that**  
27 **cell death, correlated with increased levels of elongation asymmetry between**  
28 **sister cells, accounts for the large fitness reduction. After a thousand generations**  
29 **in rich media, the fitness of evolved *ΔmreB* lines was restored to ancestral levels**  
30 **and cells regained symmetry and ancestral size, while maintaining spherical**  
31 **shape. Using population sequencing, we identified *pbp1A*, coding for a protein**  
32 **involved in cell wall synthesis, as the primary target for compensatory mutations**  
33 **of the *ΔmreB* genotype. Our findings suggest that reducing elongasome**  
34 **associated PBPs aids in the production of symmetric cells when MreB is absent.**

35  
36 **Keywords:** Cell shape, Evolution, Experimental Evolution, Coccoid, MreB, Pbp1A,  
37 OprD, Single cell analysis, Cell wall synthesis, Asymmetry.

38

## 39 **1 Introduction**

40 Bacterial cell shape is the result of the coordinated action of a suite of enzymes  
41 involved in cell wall construction, DNA segregation and cell division<sup>1-6</sup>. These are  
42 highly interdependent processes that can be difficult to genetically disentangle<sup>7</sup>. Cell  
43 shape is far from fixed on evolutionary time scales and is a key trait mediating  
44 bacterial fitness and adaptation<sup>1</sup>. Rod-like shape is hypothesized to be ancestral in  
45 bacteria but a myriad of shapes have successfully developed<sup>5,8-10</sup>. One of the key  
46 determinants of rod-like cell shape is MreB, the prokaryotic structural homolog of  
47 actin<sup>11,12</sup>. MreB is the molecular linchpin of rod-like shape and its loss is hypothesized  
48 to be either a primary or very early event in the transition between rod-like and  
49 spherical cell shape in bacteria<sup>5,8-10,13-17</sup>.

50

51 MreB acts as a dynamic platform that directs the timing and location of a complex of  
52 cell wall elongation enzymes, the ‘elongasome’<sup>18</sup> including the bi-functional lateral  
53 cell wall synthesis enzyme, Penicillin Binding Protein 1a (PBP1a)<sup>19</sup>. PBP1a and the  
54 other members of the elongasome complex move along the inner membrane of rod-  
55 like cells, manufacturing the growing peptidoglycan cell wall<sup>20-23,24</sup>. There is also  
56 growing evidence that MreB actively straightens cells during growth by associating  
57 with and directing the elongasome to regions of negative curvature in cell walls<sup>25,26</sup>.

58 In addition, MreB disrupting studies, some using A22, demonstrate that the bundled  
59 MreB filaments participate in establishing the width and stiffness of the cell while  
60 exerting an inward force on the cell wall<sup>26-30</sup>. MreB is also known to have other  
61 pleiotropic effects on a range of cellular functions and its loss is frequently lethal in  
62 model microbial systems<sup>12</sup>. Some  $\Delta mreB$  mutants can be grown for short periods in  
63 heavily supplemented media<sup>15</sup>. In A22 treated cells and in transiently viable  $\Delta mreB$   
64 strains the loss of *mreB* function leads to spherical shape and continuous volume  
65 increase, lysis and a loss of cell membrane potential<sup>5,20,31-33</sup>.

66

67 Previous work has demonstrated the viability of *mreB*-defective transposon-generated  
68 mutants of otherwise rod-shaped *Pseudomonas fluorescens* SBW25<sup>34</sup>. These mutants  
69 produce spherical cells in standard Lysogeny Broth (LB) media. The discovery of a  
70 nascent spherical phenotype in the absence of MreB in a rod-like bacterium provides

71 the opportunity to investigate the consequences of MreB loss and the range of  
72 compensatory mutations that might restore fitness.

73

74 Here we demonstrate that deletion of *mreB* ( $\Delta mreB$ ) in *P. fluorescens* SBW25 results  
75 in viable spherical cells with decreased fitness and highly variable cell size. Evolving  
76 this mutant for 1,000 generations in ten independent lineages led to recovery of both  
77 WT fitness and cell volume whilst retaining spherical cell shape. Three primary  
78 compensatory mutations are studied, two mutations in a PBP (Pencillin Binding  
79 Protein) and a separate five-gene deletion. Morphological and single cell time-lapse  
80 analysis of strains carrying these mutations demonstrate that these mutations affect  
81 lateral cell wall synthesis and septation frequency, reducing sister cell growth  
82 asymmetry and proliferation arrest in these cells. Finally, we use comparative  
83 genomics of rod-like and spherical cells to infer that PBP loss is a common  
84 phenomenon in the evolution of spherical species. Together, our results highlight  
85 possible mutational routes by which rod-like cells can adapt their genetic machinery  
86 to cope with MreB loss and spherical cell shape.

87

88

## 89 **2 Methods**

90

### 91 **Bacterial strains and culture conditions**

92 *Escherichia coli*, *Neisseria lactamica*, and *Staphylococcus aureus* were grown at  
93 37°C, whilst *Lactococcus lactis* cremoris was grown at 30°C, and *P. fluorescens*  
94 SBW25 at 28°C. Antibiotics were used at the following concentrations for *E. coli*  
95 and/or *P. fluorescens* SBW25: 12  $\mu\text{g ml}^{-1}$  tetracycline; 30  $\mu\text{g ml}^{-1}$  kanamycin; 100  $\mu\text{g}$   
96  $\text{ml}^{-1}$  ampicillin. Bacteria were propagated in LB.

97

### 98 **Strain construction**

99 The  $\Delta mreB$  strain was constructed using SOE-PCR (splicing by overlapping extension  
100 using the polymerase chain reaction), followed by a two-step allelic exchange  
101 protocol<sup>1</sup>. Genome sequencing confirmed the absence of suppressor mutations. The  
102 same procedure was used to reconstruct the mutations from the evolved lines (PBP1a  
103 G1450A, PBP1a A1084C,  $\Delta\text{PFLU4921-4925}$ ) into WT-SBW25 and the  $\Delta mreB$   
104 backgrounds. DNA fragments flanking the gene of interest were amplified using two

105 primer pairs. The internal primers were designed to have overlapping complementary  
106 sequences which allowed the resulting fragments to be joined together in a subsequent  
107 PCR reaction. The resulting DNA product was TA-cloned into pCR8/GW/TOPO  
108 (Invitrogen). This was then subcloned into the pUIC3 vector, which was mobilized  
109 via conjugation into SBW25 using pRK2013. Transconjugants were selected on LB  
110 plates supplemented with nitrofurantoin, tetracycline and X-gal. Allelic exchange  
111 mutants identified as white colonies were obtained from cycloserine enrichment to  
112 select against tetracycline resistant cells, and tetracycline sensitive clones were  
113 examined for the deletion or mutations using PCR and DNA sequencing.

114

### 115 **Evolution Experiment**

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

122

### 123 **Competitive fitness assay**

124 Competitive fitness was determined relative to SBW25 marked with GFP. This strain  
125 was constructed using the mini-Tn7 transposon system, expressing GFP and a  
126 gentamicin resistance marker in the chromosome (mini-Tn7(Gm)PrnB P1 *gfp-a*)<sup>2</sup>.

127

128 Strains were brought to exponential phase in shaken LB at 28°C before beginning the  
129 competition. Competing strains were mixed with SBW25-GFP at a 1:1 ratio by  
130 adding 150 μL of each strain to 5 mL LB, then grown under the same conditions for 3  
131 hours. Initial ratios were determined by counting 100,000 cells using flow cytometry  
132 (BD FACS Diva II). Suitable dilutions of the initial population were plated on LBA  
133 plates to determine viable counts. The mixed culture was diluted 1,000-fold in LB,  
134 then incubated at 28°C for 24 hours. Final viable counts and ratios were determined as  
135 described above. The number of generations over 24 hours of growth were  
136 determined using the formula  $\ln(\text{final population}/\text{initial population})/\ln(2)$ , as  
137 previously described<sup>3</sup>. Selection coefficients were calculated using the regression  
138 model  $s = [\ln(R(t)/R(0))]/[t]$ , where R is the ratio of the competing strain to SBW25-

139 GFP, and  $t$  is the number of generations. Control experiments were conducted to  
140 determine the fitness cost of the GFP marker in SBW25. For each strain, the  
141 competition assay was performed with a minimum of 3 replications. WT SBW25 had  
142 a relative fitness of 1.0 when compared to the marked strain, indicating that the GFP  
143 insert is neutral, and that the SBW25-GFP strain was a suitable reference strain for  
144 this assay.

145

## 146 **Microscopy**

147 **Cells from liquid culture.** Cells were routinely grown in LB, and harvested at log  
148 phase ( $OD_{600}$  0.4). Viability assays were conducted using the LIVE/DEAD BacLight  
149 Bacterial Viability Kit (Thermo Fisher). Viability was measured as the proportion of  
150 live cells in the total population (live/(live + dead)). Nucleoid staining was done using  
151 the DAPI nucleic acid stain (Thermo Fisher) following the manufacturer's protocols.

152

153 **Time-lapse on agarose pads.** Strains were inoculated in LB from glycerol stocks and  
154 shaken overnight at 28°C. The next day, cultures were diluted  $10^2$  times in fresh LB  
155 and seeded on a gel pad (1% agarose in LB). The preparation was sealed on a glass  
156 coverslip with double-sided tape (Gene Frame, Fischer Scientific). A duct was cut  
157 through the center of the pad to allow for oxygen diffusion into the gel. Temperature  
158 was maintained at 30°C using a custom-made temperature controller<sup>35</sup>. (Bacteria were  
159 imaged on a custom built microscope using a 100X/NA 1.4 objective lens (Apo-ph3,  
160 Olympus) and an Orca-Flash4.0 CMOS camera (Hamamatsu). Image acquisition and  
161 microscope control were actuated with a LabView interface (National Instruments).  
162 Typically, we monitored 10 different locations; images were taken every 5 min in  
163 correlation mode<sup>36</sup>. Segmentation and cell lineage were computed using a MatLab  
164 code implemented from Schnitzcell<sup>37</sup>. Bacteria were tracked for 3 generations.

165

166 **Scanning Electron Microscopy (SEM).** Cells were grown in LB, and harvested at  
167 log phase. Cells were fixed in modified Karnovsky's fixative then placed between two  
168 membrane filters (0.4 $\mu$ m, Isopore, Merck Millipore LTD) in an aluminum clamp.  
169 Following three washes of phosphate buffer, the cells were dehydrated in a graded-  
170 ethanol series, placed in liquid CO<sub>2</sub>, then dried in a critical-point drying chamber. The  
171 samples were mounted onto aluminum stubs and sputter coated with gold (BAL-TEC

172 SCD 005 sputter coater) and viewed in a FEI Quanta 200 scanning electron  
173 microscope at an accelerating voltage of 20kV.

174

### 175 **Image analysis**

#### 176 **Compactness and estimated volume measurements of cells from liquid culture.**

177 The main measure of cell shape, compactness or  $C$ , was computed by the CMEIAS  
178 software as:  $(\sqrt{4\text{Area}/\pi})/\text{length}$ . Estimated volume or  $V_e$  was estimated with different  
179 formula, according to cell compactness, for spherical cells that have a compactness  $\geq$   
180 0.7,  $V_e$  was computed using the general formula for spheroids:  $v=4/3\pi(L/2)(W/2)^2$ ,  
181 where  $L$ =length and  $W$ =width.  $V_e$  of rod-shaped cells, defined as having a  
182 compactness value  $\leq 0.7$ , were computed using the combined formulas for cylinders  
183 and spheres:  $V_e = (\pi(W/2)^2(L-W))+(4/3\pi(W/2)^3)$ .

184

#### 185 **Cell size, elongation rate, and division axis of cells on agarose pads.**

186 Cell size was computed as the area of the mask retrieved after image segmentation. The elongation  
187 axis is given by the major axis of the ellipse that fits the mask of the cell. Division  
188 axis is the computed by comparing the elongation axis between mother and sister cell,  
189 through the following formula:  $|\sin \theta|$ , where  $\theta$  is the angle between mother and  
190 sister cell. We measured the elongation rate of individual bacteria by fitting the  
191 temporal dynamics of cell area with a mono-exponential function. The elongation rate  
192 is then given by the rate of the exponential. To obtain the intrinsic cell size and  
193 disentangle it from the variability associated to asynchrony in the cell cycle, cell size  
194 was measured at cell birth, i.e. right after septation. Cell size was then normalized to  
195 the size of the WT strain.

196

#### 197 **Proliferation probability.**

198 For the first and second generations, we computed the proliferation probability as the capability of progressing through the cell cycle and  
199 dividing. Bacteria that do not grow or stop elongating before dividing are classified as  
200 non-proliferating. For all non-proliferating bacteria, we confirmed that no division  
201 occurs for the next 5 hours.

202

#### 203 **Growth asymmetry.**

204 For all sister cell pairs, we computed the asymmetry as the contrast in cell elongation given by:  $\left| \frac{r_2 - r_1}{r_1 + r_2} \right|$ , where  $r_{1,2}$  are the elongation rate of the

205 two sisters measured for the second generation. We then computed the population  
206 average on the sub-population that proliferates in order to avoid trivial bias due to cell  
207 proliferation arrest of one of the two sister cells.

208

### 209 **Protein sequence alignment and modeling**

210 Protein sequences were obtained from NCBI BLAST (<http://blast.ncbi.nlm.nih.gov>)  
211 and The Pseudomonas Genome Database<sup>38</sup>, and aligned using MEGA7<sup>39</sup>. The  
212 sequence alignment was visualised using ESPript 'Easy Sequencing in PostScript'<sup>40</sup>.

213

214 Protein visualisation was done on Visual Molecular Dynamics (VMD)<sup>41</sup> using the  
215 crystal structure of *Acinetobacter baumannii* PBP1a in complex with Aztreonam as  
216 the base model, which shares a 73% sequence identity (E value = 0.0) to the PBP1a of  
217 *P. fluorescens* SBW25. Sequences were aligned, and locations of the mutations in the  
218 evolved lines were mapped in the corresponding regions. The PDB file was  
219 downloaded from the RCSB Protein Data Bank ([www.rcsb.org](http://www.rcsb.org)) using PDB ID 3UE0.

220

## 221 **3 Results**

### 222 **MreB deletion in *P. fluorescens* SBW25 generates viable spherical cells**

223  $\Delta mreB$  cells to be spherical and display a highly variable cell size and shape  
224 compared to the WT strain in phase contrast and SEM (Fig. 1A). The  $\Delta mreB$  strain is  
225 viable with approximately 82.5% ( $\pm 7.9\%$ ) live cells compared to WT at 95.2%  
226 ( $\pm 1.2\%$ )(Fig. 1B). Relative fitness in pairwise competition assays demonstrates that  
227 the  $\Delta mreB$  strain has a markedly lower relative fitness of 0.78 ( $\pm 0.02$ ) compared to  
228 the WT (Fig. 1C)<sup>42</sup>. The  $\Delta mreB$  strain had a slower generation time of 65 min (WT,  
229 45 min), prolonged lag phase, and lower maximum yield (Supp. Fig. 1).

230

231 The *mreB* gene was ectopically expressed from the Tn7 site near the *glmS* region of  
232 the  $\Delta mreB$  strain completely restored WT morphology, viability, and relative fitness  
233 in the  $\Delta mreB$  cells with slightly delayed growth (longer lag) (Supp. Fig. 2).  
234 Therefore the morphological effects seen in  $\Delta mreB$  are considered to be due solely to  
235 loss of MreB.

236

237 To quantify variability in size and shape we performed a principle components  
238 analysis of the shape metrics (CMEIAS software package)<sup>43,44</sup> which motivated a  
239 focus on a metric called compactness<sup>4</sup>, a measure of the circularity of the cell's  
240 outline. A compactness of 1.0 is circular whilst values below 0.7 are more typical of  
241 rod-shaped cells. For our purposes, cells with an average compactness of 1.0 to 0.8,  
242 before visible septation initiation are considered to be “spherical”. The projected cell  
243 outlines were used to estimate volume, ( $V_e$ ) (see Material and Methods) and plotted  
244 each cell's  $V_e$  vs compactness for both WT and  $\Delta mreB$  cells (Fig. 1D).

245

246 WT cells have a small  $V_e$  range and a negative correlation between  $V_e$  and  
247 compactness, reflecting the linear elongation and regular cell division of rod-shaped  
248 cells. In contrast, the  $\Delta mreB$  strain exhibits large spherical to ovoid cells (, with a  
249 wide distribution of  $V_e$  ranging from  $1.12 \text{ um}^3$  to  $\sim 90 \text{ um}^3$ , averaging  $20.65 \text{ um}^3$   
250 ( $\pm 16.17 \text{ um}^3$ ). Spherical  $\Delta mreB$  cells initiate septation at a wide range of volumes  
251 from  $10 \text{ um}^3$  to  $90 \text{ um}^3$ , indicating that the relationship between cell size and division  
252 is lost in  $\Delta mreB$  cells (see lower compactness cells in Fig. 1D).

253

254 As cell volume increases, DNA content might also be expected to increase if DNA  
255 replication continues irrespective of division frequency. Increased DNA content and  
256 spherical cell shape are both predicted to further perturb cell division<sup>45,46</sup>. WT and  
257  $\Delta mreB$  cells were stained with a nucleic acid stain (FITC) to label DNA and subjected  
258 to flow cytometry. In both strains DNA content scaled with cell size as measured by  
259 Forward Scatter Area (FSC-A). The largest  $\Delta mreB$  cells have many times the DNA  
260 content of WT cells, scaling roughly with volume (Fig. 1E, Supp. Fig. 3) indicating  
261 that DNA replication continues irrespective of cell size. In addition, WT cells  
262 observed by time-lapse, orientation of the division plane is consistent across divisions  
263 ( $|\sin(\phi)| = 0$ ). In contrast, in the  $\Delta mreB$  population, septa positioned perpendicularly  
264 relative to the last plane at each generation ( $|\sin(\phi)| = 1$ ) (Fig. 1F, Supp. Fig. 4B). The  
265 change from maintaining septation angles to alternating septation suggests that DNA  
266 segregation (prior to septation) is perturbed in cells that have lost rod-like shape. This  
267 is consistent with similar results obtained from *E. coli* treated with the MreB inhibitor,  
268 A22<sup>47</sup>.

269

270 **Experimentally evolving spherical cells**



271 Having established that we have a viable *ΔmreB* in *P. fluorescens* SBW25 an  
272 investigation into how this strain adapts to the challenge of MreB loss, was conducted  
273 using an experimental evolution approach to select for mutants that restore fitness.  
274 After 1,000 generations of evolution (Fig. 2A) the final evolved populations displayed  
275 both relative fitness (Fig. 2B) and growth dynamics that were similar to the WT  
276 (Supp. Fig. 5). However, the evolved cells remained spherical in shape (Fig. 2D,  
277 Supp. Fig. 6). The size, however, as measured by  $V_e$  had decreased to roughly that of  
278 the ancestral cells (Fig. 2D). The  $V_e$  of the evolved lines does not overlap with the  
279 *ΔmreB* population (Fig. 1D), evidence that these evolved cells present a new  
280 phenotype and are not a subset of the spherical *ΔmreB* ancestor. These newly evolved  
281 spherical cells are most similar in cell shape, particularly at septation, to species like  
282 *Lactococcus lactis* cremoris or *Neisseria lactamica* and other spherical bacterial  
283 species that still undergo some elongation prior to division, not like *Staphylococcus*  
284 *aureus* (Supp. Fig 11)<sup>48</sup>. The latter experience rapid division as nearly perfect spheres  
285 (Supp. Fig.11)<sup>49</sup>.

286

287 In order to understand the dynamics of the fitness recovery the frozen evolved  
288 populations were resuscitated at various time points and competed these pairwise  
289 against a GFP labeled WT ancestor (Fig. 2C). The fitness increase during evolution  
290 occurred rapidly: after only 50 generations of growth, the evolved lines had an  
291 average competitive fitness score of 0.92 ( $\pm 0.01$ ). This increased to an average fitness  
292 of 0.97 ( $\pm 0.02$ ) by the end of the experiment.

293

#### 294 **Identifying mutations compensating for costs arising from deletion of *mreB*.**

295 The rapid fitness increase observed indicates that a small number of mutations arose  
296 early and swept through the populations of poorly competing *ΔmreB* cells. In order to  
297 identify these mutations, we conducted population sequencing at 500 and 1,000  
298 generations and reference mapped these reads to the *P. fluorescens* SBW25 genome  
299 (GCA\_000009225.1) to an average read depth of 100 fold. We identified several  
300 mutations affecting open reading frames that were found in over 75% of the sequence  
301 reads in several evolved lines (detailed, Supp. Table 1). A single gene, *pbp1a* had  
302 independent mutations in multiple lines. Representative *pbp1a* mutations from lines 1,  
303 4 were chosen for further study. Line 7 had a five-gene deletion that included the  
304 *oprD* homolog which was also chosen for further analysis.

305

306 The *pbp1A* gene (PFLU0406) encodes the major Class A penicillin-binding protein  
307 responsible for the final steps of peptidoglycan synthesis. PBP1a proteins are key  
308 components of peptidoglycan synthesis machinery in the cell wall elongation  
309 complexes and are associated with the MreB cytoskeleton in rod-like cells<sup>5</sup>.

310

311 This PBP1a contains three known domains (Fig. 3A). Structure mapping of the  
312 mutations demonstrates that the mutation in Line 1 occurred in a well-conserved  
313 region in the transpeptidase (TP) domain, proximal to the active site (Supp. Fig. 7).  
314 Similar mutations in *Streptococcus pneumoniae*<sup>50</sup> cause a loss of function in this  
315 domain. The mutation in Line 4 took place in the oligonucleotide/oligosaccharide  
316 binding (OB) domain<sup>6</sup>. These will be referred to from hereon as the *pbp1a* Line 1 and  
317 Line 4 mutations respectively. In order to determine the effects of these mutations on  
318 cell shape and growth, these separately reconstructed in the WT and  $\Delta mreB$   
319 backgrounds.

320

321 The  $\Delta mreB$  *pbp1A* mutation strains remained spherical to ovoid, near WT volume and  
322 DNA content (Fig. 3D, Supp. Fig. 9). These cells also retained the shorter generation  
323 times (48 min), growth dynamics (Supp. Fig. 8) and relative fitness of the evolved  
324 line populations (Fig. 3C), suggesting that the *pbp1A* mutations are each sufficient to  
325 both restore WT fitness and to recapitulate the major phenotypes of evolved lines 1  
326 and 4.

327 The function of PBP1A in the ancestral strain and therefore the presence of MreB, is  
328 not well studied. The same mutations were therefore reconstructed in the WT  
329 background. The WT *pbp1a* mutations also had generation times, growth curves  
330 (Supp. Fig. 7) and relative fitness measures similar to WT (Fig. 3C).

331

332 The major phenotypic difference in the presence of the *pbp1a* mutations was that both  
333 the *Pbp1a* TP and OB mutation reconstructions had rod-like cells that are significantly  
334 narrower in cell widths ( $0.89 \text{ um} \pm 0.07$ ,  $0.94 \text{ um} \pm 0.05$  respectively) compared to  
335 WT ( $1.00 \text{ um} \pm 0.06$ ) ( $p = <0.001$ ). This resulted in smaller cell volumes (Fig. 3E and  
336 Supp. Fig. 9). This decrease in cell width as a result of an amino acid change near the  
337 transpeptidase domain of an elongasome-component is evidence that this mutation  
338 decreases the function of PBP1a, likely by interfering with transpeptidase function

339 (Fig. 3E in Blue). The similar phenotype conferred by the OB domain indicates that  
340 these domains act similarly in contributing to cell width (Fig. 3E in Green). The cell  
341 size decrease also corresponded with a slight decrease in DNA content (Supp. Fig. 3).  
342 The production of thinner cells, is consistent with previous work on the effects of  
343 PBP1a function loss in both *B. subtilis* and in *E. coli*<sup>51-54</sup>. Based on the positions of  
344 the respective mutations, and their resulting phenotypes in WT cells, we interpret  
345 these results to indicate that either of these *pbp1A* mutations can reduce lateral cell  
346 wall synthesis, resulting in smaller cells when MreB is present.

347

348 The other major mutation identified in the evolved lines was a five-gene deletion  
349 (PFLU4921-4925) in evolved Line 7 (Fig. 3B, Supp. Table 1). The deletion contains  
350 three hypothetical proteins, a cold shock protein (PFLU4922, encoding CspC), and an  
351 outer membrane porin, PFLU4925 which encodes OprD. The latter is responsible for  
352 the influx of basic amino acids and some antibiotics into the bacterial cell<sup>7</sup>. This  
353 deletion was constructed and characterised in the  $\Delta mreB$  and WT backgrounds.

354

355 The  $\Delta mreB$  five-gene deletion strain had a generation time and growth dynamics  
356 similar to WT, with an additional extended lag time (Supp. Fig. 8). The viability and  
357 relative fitness were also highly similar to WT (Fig. 3C). The cells were spherical  
358 with an average  $V_e$  of  $5.32 \text{ um}^3 (\pm 3.18)$  (Fig. 3D and Supp. Fig. 8). As in the *pbp1A*  
359 Line 1 and Line 4 mutations, DNA content was also decreased compared to the  
360  $\Delta mreB$  ancestor (Supp. Fig. 3). In addition, the five-gene deletion produces cell  
361 division defects in 25.61% ( $\pm 6.42\%$ ) of these cells, manifesting as septation defects  
362 and connected clumps of spherical cells (Fig 3D, Supp. Fig. 9).

363

364 In the WT strain, the five-gene deletion produced rod shaped cells with growth  
365 characteristics similar to the WT strain (Supp. Fig 8). These cells were however  
366 significantly thinner than WT (width =  $0.74 \text{ um} \pm 0.06$ ,  $p = < 0.001$ ) and had a smaller  
367 average  $V_e$  of  $2.47 \text{ um}^3 (\pm 1.18)$  (Fig. 3E and Supp. Fig. 8). As in the  $\Delta mreB$   
368 background, a sub-population exhibits a filamenting phenotype occurring in 20% ( $\pm$   
369 4%) of the population. The five-gene deletion strains were the only ones that showed  
370 evidence of dispersed DNA between incomplete septa in DAPI staining (Supp. Fig  
371 S10). Intriguingly, clinically isolated *Pseudomonas* with *oprD* deletions have  
372 significant changes in the regulation of the MinCD system<sup>55</sup>. In closely related model

373 systems MinCD, acts to negatively affect septal placement by poles and accumulating  
374 as a result of cell shape asymmetry<sup>17,56-58</sup>. The connection between OprD and MinCD  
375 in *Pseudomonas* merits further investigation but *oprD* loss may mitigate large cell  
376 size and increase fitness in  $\Delta mreB$  by retuning septation frequencies. This would also  
377 imply that the viable  $\Delta mreB$  cells lack proper the geometry required to support  
378 MinCD oscillations<sup>59</sup>, resulting in erratic septation and driving large cell size.

379

### 380 **Sister Cell Asymmetry at the Single Cell Level**

381 In order to determine the basis of the fitness cost of the *mreB* deletion, we, we  
382 conducted single cell experiments in the reconstructed mutants and representative  
383 evolved clones from lines 1, 4 & 7. Time-lapse microscopy was used to track  
384 individual cells through subsequent generations to measure size, elongation rate,  
385 division axis and shape for each cell as well as their capacity to produce two  
386 daughters<sup>36,60</sup>.

387

388 All reconstructed strains except the strain that ectopically expresses *mreB* (closed  
389 grey square), have a reduced rate of cell wall synthesis relative to the WT (Fig. 4A)  
390 but all are higher than the ancestral  $\Delta mreB$  strain. Cell elongation rates are higher in  
391 the presence of MreB in the *pbp1A* Line 4 mutant, but not the Line 1 mutant,  
392 suggesting that the transpeptidase domain mutation may affect the degree to which  
393 MreB stimulates synthesis<sup>61</sup>.

394

395 In addition, single cell experiments measured that a fraction of cells underwent  
396 persistent proliferation arrest on solid media, even after five hours of observation (Fig.  
397 4B). Tracking pairs of dividing cells coming from the same mother revealed that they  
398 experience unequal rates of cell wall synthesis, or ‘growth asymmetry’ (Fig. 4B). A  
399 strong correlation is observed between proliferation arrest and growth asymmetry in  
400 our reconstructed mutation strains and representative clones. In strains that had higher  
401 growth asymmetry, more proliferation arrest was observed (Fig 4D). This increased  
402 growth asymmetry might either initiate proliferation arrest or both features may be  
403 symptoms of another attribute of these cells such as cell size or defects in DNA  
404 segregation driven by cell shape and septum aberrations<sup>46,62</sup>.

405

406 Accordingly, cells that have lost MreB are able to find a new equilibrium by  
407 decreasing elongation synthesis (*pbp1A* mutations) or possibly modulating septation  
408 associated synthesis (the *oprD* inclusive deletion). Either serves to increase the  
409 relative proportion of synthesis at the septum, and decrease elongasome associated  
410 synthesis. The advantage gained through these adjustments in response to MreB loss  
411 hint at a previously unrecognized role of MreB in ensuring the equal partitioning of  
412 the elongasome components before and after cell division.

413

#### 414 **Recapitulating spherical shape evolution**

415 These experiments demonstrate that either a decrease in activity in a PBP in the  
416 elongasome or a five-gene deletion that includes *oprD* allow a rebound in fitness  
417 when *mreB* is lost. It was previously reported that coccoid bacterial species have  
418 lower estimated numbers of PBPs based on estimates from a biochemical function  
419 assay<sup>63</sup>. We were therefore interested in whether comparative genomics of completely  
420 sequenced bacteria bore this pattern out as well. We therefore selected 26 bacterial  
421 species pairs in which one member has maintained rod-like shape and the other has  
422 become spherical and compared the abundance of the homologues of the genes  
423 implicated in our evolution experiment; MreB, PBPs and OprD homologs. OprD  
424 homologs were too rare across species to analyze. However, we observed a significant  
425 relationship between coccoid lineages that had lost MreB and a decrease in the  
426 number of PBP homologs (avg. PBPs in rods =  $9.22 \pm 5.15$ ; spheres =  $3.89 \pm 2.65$ ;  
427 difference:  $p = <0.001$ ). From this we infer that species that have naturally evolved  
428 from rod-like to spherical shape tend to have lost both *mreB* and approximately half  
429 of their PBP genes.

430

#### 431 **Reshaping a rod-like pseudomonad to be a spherical cell**

432 *P. fluorescens* SBW25 is a rod-like bacterium that can be reshaped into a rapidly  
433 growing spherical cell in as little as two mutational steps, the deletion of *mreB* and  
434 either a single amino acid changing mutation in *pbp1A* or an *oprD* inclusive deletion.  
435 The reason that this strain is tolerant of MreB loss is not currently known but a  
436 separate paralog does not exist in this strain.

437

438 The loss of MreB from the ancestral SBW25 causes extremely large cells with  
439 multiple chromosomes (Fig. 1D,E) with highly irregular septation. In addition, sister

440 cells elongate perpendicularly to mother cells, across cell divisions (Fig 1E-F),  
441 consistent with MreB disruption experiments using *ili* and *P. aeruginosa*<sup>31,64</sup>. Both cell  
442 wall synthesis and DNA replication are continuous in these cells (Fig. 1E and Fig.  
443 4A) meaning that large cell size is the result of a reduction in septation frequency,  
444 maybe due to the loss of the ordered relationship between septation and DNA  
445 segregation in spherical cells<sup>46</sup>.

446

447 The  $\Delta mreB$  population had high levels of cell wall synthesis asymmetry and while  
448 either of the *pbp1A* mutations increased this symmetry, the five-gene deletion did not  
449 (Fig. 4D). This increase in symmetry suggests that the distribution of active  
450 elongasomes may be disorganised in cells lacking MreB and that this disorganization  
451 is reduced when *pbp1A* is mutated<sup>52</sup>(Fig 4C). This raises the possibility that symmetry  
452 in cell synthesis is maintained in these cells by continued septal cell wall synthesis.  
453 While this is consistent with models of other spherically shaped cells in which much  
454 of the cell wall synthesis further investigation of cell wall synthesis is required to  
455 support or refute this hypothesis<sup>65-67</sup>.

456

#### 457 **Implications for the evolution of spherical cell shape**

458 The wide array of cell shapes and sizes observed in the eubacteria have arisen from an  
459 ancestral rod-like cell shape<sup>2,68-70</sup>. Coccoid or spherical cells are the product of a  
460 degradation of this shape<sup>14,71</sup>. The transition to spherical cell shape has taken place  
461 independently many times<sup>8,72-74</sup> and is associated with *mreB* loss, possibly as an early  
462 event<sup>48,49</sup>.

463

464 This study uncovers separate compensatory mutations that allow rapid fitness  
465 recovery after MreB loss. If MreB loss is a common early event in coccus evolution  
466 then there are likely to be both genetic and environmental contexts that favor this  
467 state<sup>2,9,14,71</sup>. One possibility is that the transient increase in cell size observed in  
468  $\Delta mreB$  cells is advantageous in some settings. This hypothesis compels further  
469 investigation<sup>16</sup>.

470

471

#### 472 **4 Conclusions**

473 Cell shape is a fundamental property of cells that defines motility, DNA segregation,

474 replication, nutrient acquisition, waste elimination and predator evasion<sup>16</sup>. *P.*  
475 *fluorescens* SBW25 is a rod-shaped bacterium that is amenable to MreB deletion.  
476 The loss of MreB is a non-lethal but deleterious event that leads to irregular, large-  
477 sized spherical cells. Further, separate mutations can restore fitness and volume whilst  
478 retaining spherical cell shape and these are likely decrease-of-function mutations in  
479 the gene encoding elongasome member PBP1A, or a five-gene deletion that includes  
480 *oprD*. These mutations are able to restore symmetry in cell growth between sister  
481 cells and decrease cell death. We therefore propose a model of molecular change  
482 when MreB is lost, essentially re-storing symmetric cell wall synthesis by relying  
483 more heavily on synthesis at the septum. Last, our study implicates a decrease of PBP  
484 function, as a general strategy in cells recovering from the loss of MreB and refining  
485 spherical cell shape in bacteria.

486

#### 487 **Acknowledgements**

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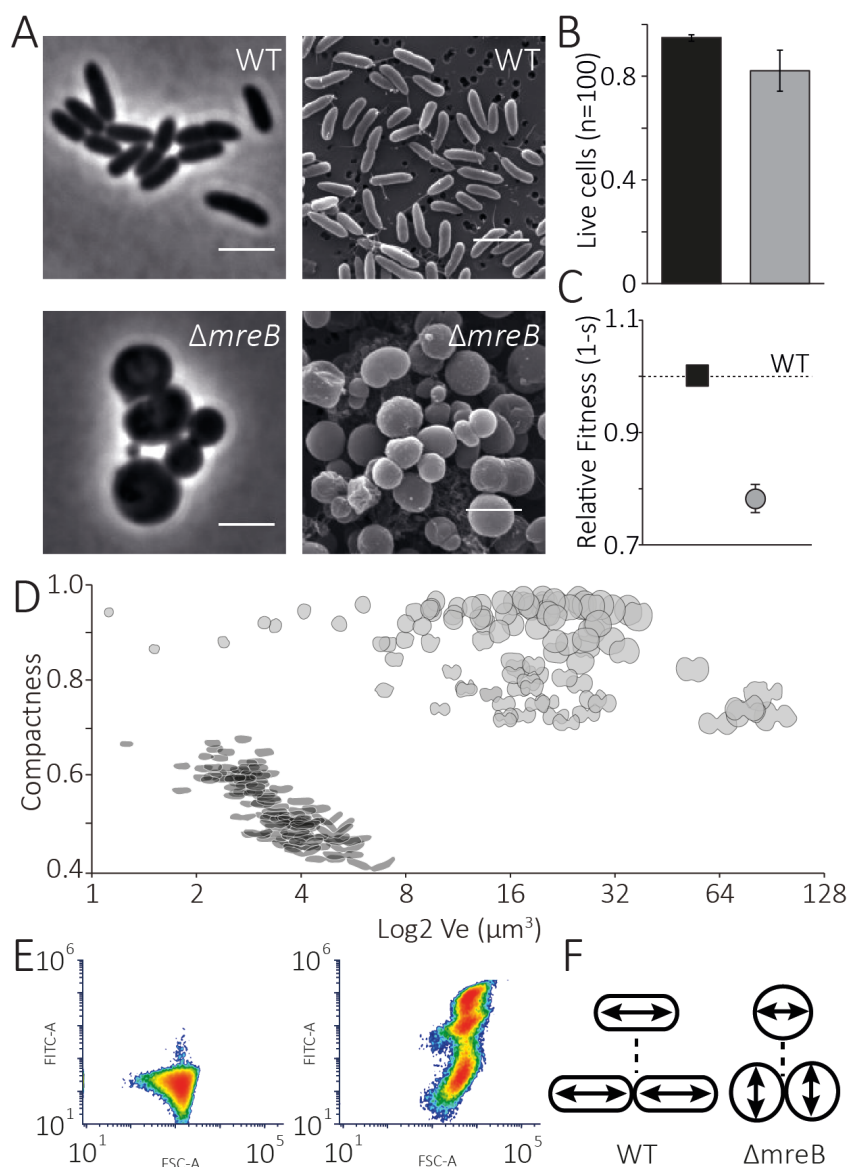
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495

#### 496 **Figures**

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520 Figure 1. Characterisation of WT and  $\Delta mreB$  strains. Figure shows (A)  
521 photomicrographs of WT and  $\Delta mreB$ . Scale bars, 3  $\mu\text{m}$ . B) Proportion of live cells in  
522 WT (black bar) and  $\Delta mreB$  (grey bar). Error bars represent standard error (n = 3). C)  
523 Fitness of WT GFP and the ancestral  $\Delta mreB$  mutant relative to WT when both are in  
524 exponential phase during pairwise competition assays. Error bars as in 1B. D) The  
525 relationship between cell shape and estimated volume ( $V_e$ ) is represented using  
526 compactness, a measure of roundness. One hundred representative cells from each  
527 WT and  $\Delta mreB$  are shown as cell outlines. E) DNA content (FITC-A) is highly  
528 correlated with increased cell sizes (FSC-A) in  $\Delta mreB$  but both are limited in WT  
529 cells (n=50,000 events). F) Diagram of WT cells maintaining a single consistent  
530 division plane whilst  $\Delta mreB$  cells alternate division planes by 90° from one division  
531 to the next.



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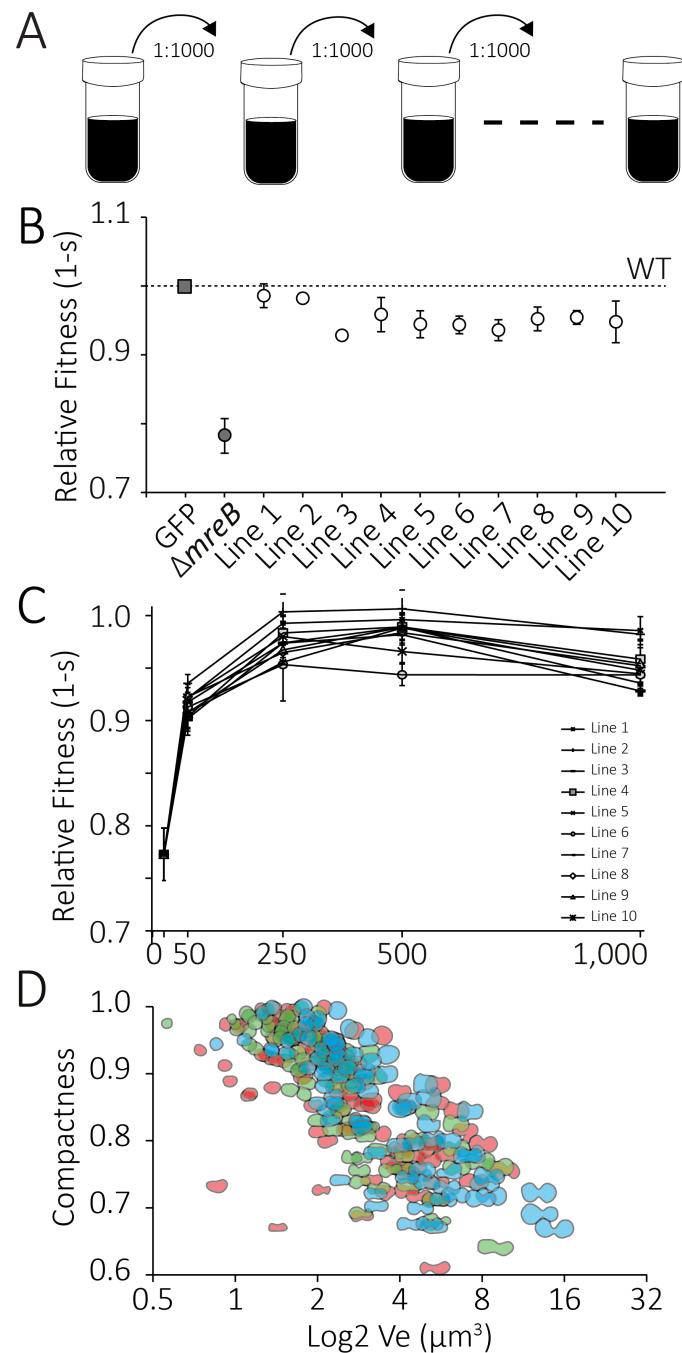
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558 Figure 2. Characterisation of Evolved Lines at 1,000 generations. A) Diagram of

559 evolution experiment protocol. 1:1000 transfer each 24 hours. B) Relative fitness of

560 the  $\Delta mreB$  mutant and evolved lines after  $\sim 1,000$  generations relative to WT (dashed

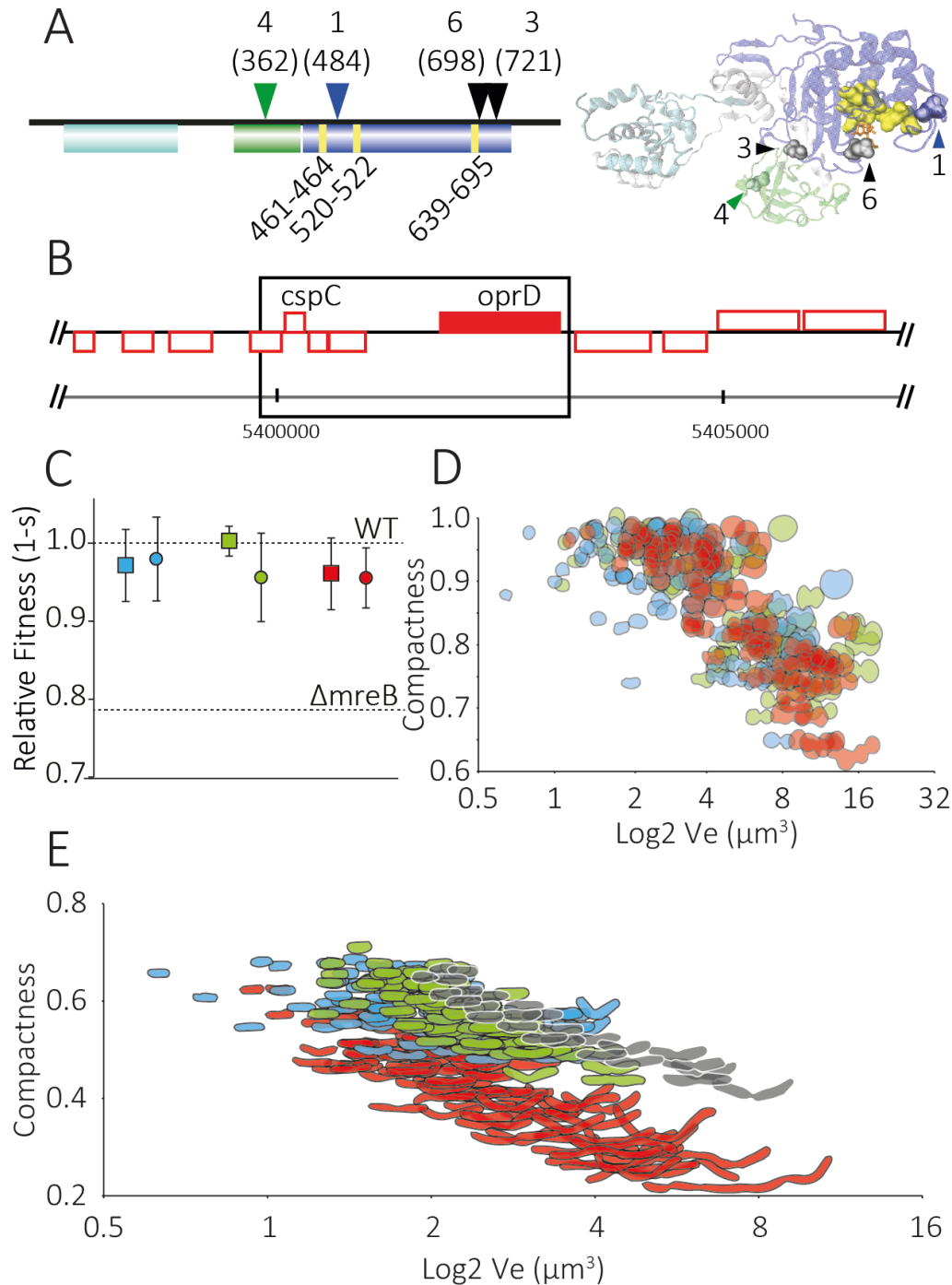
561 line) in pairwise competition experiments. Error bars represent standard error (n = 3).

562 C) Relative fitness of the evolved lines during 1,000 generations of growth. Error bars

563 as in B. D) Cell outlines of three representative evolved lines Line 1 in blue, Line 4 in

564 green and line 7 in red. One-hundred randomly chosen cell outlines from each line are

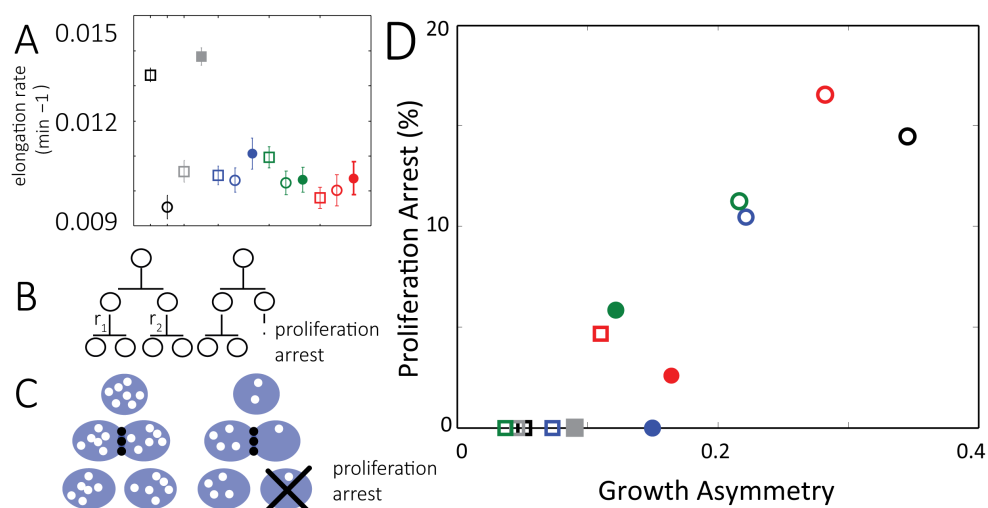
565 depicted.



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567 Figure 3. Characterisation of the reconstructed mutations in WT and  $\Delta mreB$   
 568 background. A) Domain map and model of PBP1a (PFLU0406) showing the 2 major  
 569 active sites; the glycosyltransferase (GT) domain (cyan) and the transpeptidase (TP)  
 570 domain (blue). The oligonucleotide/oligosaccharide binding (OB) domain is shown in  
 571 green. The active site of the TP domain is also shown (yellow). The mutations  
 572 identified are indicated above the map. B) Genome map of *oprD* inclusive deletion,  
 573 (PFLU4921-PFLU4925) and surrounding region. Genes with function calls are noted.  
 574 C) Relative fitness of the three reconstructed mutants in the  $\Delta mreB$  (circles) and WT

575 (squares) backgrounds. Line 1 reconstruction, PBP1a D484N is shown in blue, Line 4  
576 PBP1a T362P is shown in green and the OprD containing deletion reconstruction is  
577 shown in red. D) Compactness versus estimated volume ( $V_e$ ) for reconstruction  
578 strains in the  $\Delta mreB$  background, colors as in 1C (N=100). E) Compactness versus  
579 estimated volume ( $V_e$ ) of the mutants in the WT background colours and N as in C  
580 and D. A subset of WT cells are shown in light grey for comparison.  
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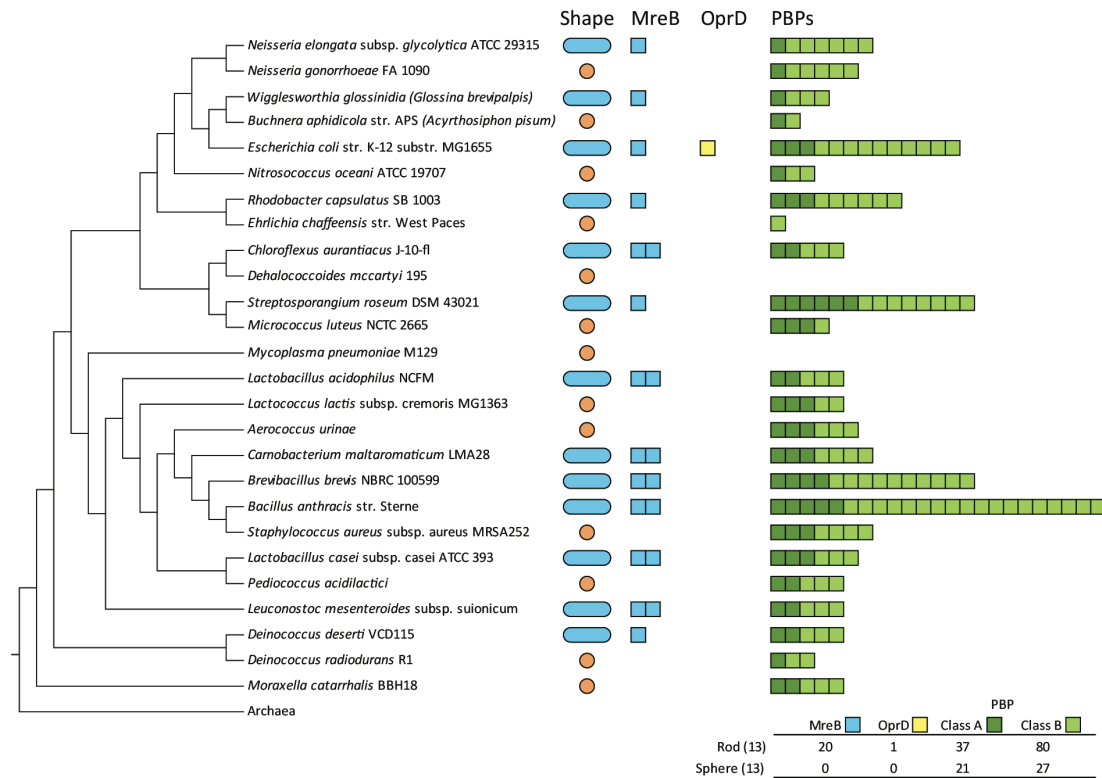
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585 Figure 4. Single cell time-lapse measurements of reconstructed mutations. A) Average  
 586 population elongation rate for WT, reconstructions and evolved cells. Open square  
 587 data points represent WT or WT and respective mutations (open grey square =  $\Delta mreB$   
 588 with ectopic *mreB*), open circles are  $\Delta mreB$  or  $\Delta mreB$  and respective mutations and  
 589 closed symbols are evolved cell lines. Colors as in Fig 3; blue is Line 1 or PBP1a  
 590 D484N, green is Line 4 or PBP1a T362P, red is Line 7 or OprD inclusive deletion. B)  
 591 Strategy for analyzing single cell measurements during growth on agarose pads  
 592 including  $r_1$  and  $r_2$ . C) Model for relationship between growth asymmetry and  
 593 proliferation arrest driven by disordered acquisition of cell wall synthesis machinery  
 594 (white dots). The septum associated PBPs (black circles) at the division plane provide  
 595 symmetric cell wall synthesis to respective daughter cells. D) Relationship between  
 596 paired-sister cell proliferation arrest and growth asymmetry in all reconstructions and  
 597 representative evolved cells (N=100 for each).

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601 Figure 5. Penicillin-Binding Proteins (PBPs) is observed in extant spherically-shaped  
 602 cells. A selection of 26 paired rod-shaped and spherical cells were analyzed for their  
 603 PBP and OprD homologs. The genomes of naturally evolved spherical cells have  
 604 fewer PBPs than rod-shaped species. OprD homologs were rare.  
 605

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