Supplemental information

Supplemental Materials and Methods

Plasmid constructions

Plasmid pLPZ (*ftsZp-ftsZ,* TetR) is derived from pZAQ and expresses only *ftsZ* from its native promoters. The *ftsQ* and *ftsA* genes were deleted from pZAQ to construct pLPZ, keeping all the *ftsZ* promoters intact. In pZAQ, *ftsZ* is present in an operon consisting of *ftsQ*, *ftsA* and *ftsZ* genes and has six promoters: four promoters (*ftsZ1p* - *ftsZ4p*) in the *ftsA* coding sequence and two promoters (*ftsQ1p* and *ftsQ2p*) in the *ddlB* coding sequence, which is upstream of *ftsQ* (Aldea *et al.*, 1990). Of these, *ftsZ3p* and *ftsZ4p* are responsible for 80 % of overall FtsZ production (Navarro *et al.*, 1998). To maintain FtsZ expression at levels similar to that of pZAQ, all six *ftsZ* promoters were preserved during pLPZ construction. To create pLPZ, the entire pZAQ plasmid was amplified by PCR, except for $ftsQ^{1\text{-}828}$ and $ftsA^{1\text{-}534}$, by using the forward primer (5' – CTCTCTCTCT**GAGGAG**ATTGGCTTCC*AGCAGATAAAAC – 3') and the reverse primers (5' – TCTCTCTCTCT**GAGGAG**AACGTTGT*GGGCTGAAAGTTGA – 3'). *BseR*I restriction sites (sequences in bold) were incorporated into both the primers. *BseR*I makes a staggered cut in the DNA, outside the restriction enzyme recognition site (10 bases after the recognition site in the 5' -> 3' direction and 8 bases after the recognition site in the opposite strand; cut sites are represented by asterisks), leaving sticky ends with two overhanging bases (underlined in the primer sequence). The MasterAmp Extra-Long PCR kit (Epicentre) was used according to the manufacturer's instructions to amplify the PCR fragment. The resulting PCR product was digested with *BseR*I and self ligated by using the Quick Ligation kit (NEB). The ligated product was electroporated into DH5-α electro-competent cells and tetracycline resistant colonies were selected. One of the clones was designated as pLPZ, and the sequences of the *ftsZ* gene and ligation regions were confirmed by DNA sequencing. We found two silent mutations in the *ftsZ* gene of pLPZ (A101A – GCT to GCC and P114P – CCA to CCG) that did not affect the amino acid composition. FtsZ protein levels produced

from pLPZ and pZAQ were similar and were two-to-three-fold higher than native FtsZ levels, as measured by the western blot (Fig. S1).

Plasmid pLP18-Kan (p_{BAD}, KanR) is a derivative of pBAD18-Kan. pBAD18-Kan has two *Hind*III restriction sites, one in the multiple cloning site (MCS) and the other in the Kanamycin resistance (KanR) gene. The *Hind*III site in the Kan gene was modified with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), by using the primer 5'-GGAAAGAAATGCATAAACTTTTGCCATTCTCACC-3' and its reverse complement. The resulting plasmid (pLP18-Kan) carried the *KanR* gene with a silent mutation (underlined) at codon 184 (lysine), which eliminated the internal *Hind*III site so that a single *Hind*III sequence remained in the MCS.

Plasmid pLP322 (pBR322Δ*bla,* TetR) is a derivative of pBR322. pZAQ and pLPZ plasmids do not possess the *bla* gene because it was replaced with the *ftsZAQ* operon (Ward & Lutkenhaus, 1985). The presence of the *bla* gene affects pentapeptide levels in *E. coli* (R. Priyadarshini, unpublished). In order to avoid this problem, the *bla* gene was removed from pBR322, so we could compare the PG composition of FtsZ overexpressing strains with strains not overexpressing FtsZ. pBR322 was digested with *ClaI* and *PstI* to release the *bla* fragment, and the vector backbone was treated with DNA polymerase I, Klenow fragment (NEB), to blunt the sticky ends. The resulting DNA fragment was ligated using the Quick Ligation kit (NEB), electroporated into DH5-α electro-competent cells, and plated onto LB-Tet plates. The resulting transformants were checked for ampicillin sensitivity, and plasmid size was confirmed by gel shift. One of the clones was designated pLP322.

Plasmid pLP402 (P_{lac}-*dsbA(SS)-sfgfp-dacB, lacI*^{q}, KanR) was created by cloning the wild type *dacB* gene between the *BamH*I and *Hind*III sites of pLP9. The *dacB* gene was amplified from *E. coli* MG1655 genomic DNA, by using primers (upstream) 5'- GCTCGGATCC**GCAAATGTTGATGAGTAC**-3' and (downstream) 5'- CTGCAAGCTT**CTAATTGTTCTGATAAAT**-3' (*BamH*I and *Hind*III sites are underlined, bolded sequences represent portions of the amplified gene). The resulting DNA fragment does not encode the original 30 amino acid signal sequence (SS) of DacB.

Plasmid pLPKC403 (*p_{BAD}-dacB,* KanR) was created by cloning wild type *dacB* from pPJ4 (Nelson and Young 2001) into the *Nhe*I and *Hind*III sites of pLP18-Kan.

Plasmid pLPKC404 (*P_{BAD}-dsbA(SS)-sfgfp-dacB,* KanR) was created by cloning the *dsbA(SS)-sfgfp-dacB* gene fusion from pLP402 into the *EcoR*I and *Hind*III sites of pLP18-Kan.

Plasmid pLP405 (*P_{BAD}-dacB^{S62A},* KanR) was created by PCR amplifying the pLPKC403 plasmid with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), by using the primer 5'-CAGATGGCGCTGCCTGCCGCTACCCAGAAAGTGATTACTGCG-3' and its reverse complement. The resulting plasmid carries the *dacB^{S62A}* gene, in which serine 62 (codon AGT) in the active site was replaced with alanine (GCT - underlined).

Plasmid pLP406 (*P_{lac}-dsbA(SS)-sfgfp-dacB^{S62A}, lacI^{q},* KanR) was created the same way as pLP405, except that pLP402 was used as a DNA template. The resulting plasmid carries the *dsbA(SS)-sfgfp-dacB^{S62A}* fusion, in which the serine 62 in the active site was replaced with alanine.

Plasmid pLPKC407 (*P_{lac}-dsbA(SS)-sfgfp-dacBΔC17, lacI^{q},* KanR) was created by PCR amplifying a DNA fragment lacking the final 51 nucleotides of the *dacB* gene (*dacBΔC17*) from *E. coli* MG1655 genomic DNA, by using the primers (upstream) 5'-GCTCGGATCC**GCAAATGTTGATGAGTAC**-3' and (downstream) 5'-ATCAAGCTT*CTA* **AATACGGCGATTACGCTG**-3' (*Bam*HI and *Hind*III sites are underlined, bolded sequences represent portions of the amplified gene, and a stop codon (*CTA*) was inserted in front of the *Hind*III site). The PCR fragment was digested and cloned into the *Bam*HI and *Hind*III sites of pLP9. The resulting gene fusion does not encode the last 17 amino acids of PBP4, so that the expressed fusion protein lacks the carboxy terminal amphipathic helix.

Plasmid pLP513 (*P_{BAD}-dacA^{S44G},* CamR) was created as follows. During the construction of pLP514 (Potluri *et al.*, 2010), we discovered that the pPJ5S plasmid, which we previously reported as carrying an S44G mutation in PBP5 (Nelson & Young, 2001), also contained a deletion that

introduced a stop codon at amino acid position 45 of PBP5. Thus, instead of expressing a full length PBP5 with a mutation in the active site, pPJ5S encodes a truncated PBP5 fragment that terminates after the glycine located at amino acid position 44. In this work we corrected the error by re-introducing the missing nucleotide, cytosine (C), with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), by using the primer 5'-CGCGATCCTGCCGGCCTGACCAAAATGATGACC-3' (corrected C is underlined) and its reverse complement. The resulting plasmid, pPJ5S-SDM, carried dacA^{S44G} and eliminated the stop codon next to glycine 44. Next, the dacA^{S44G} gene was excised from pPJ5S-SDM and cloned between the *Nhe*I and *Hind*III sites of pBAD18-cam to create plasmid pLP513.

Plasmid pLP516 (*P_{BAD}-dacA,* KanR) was created by cloning the full length *dacA* gene from pPJ5 into the *Nhe*I and *Xba*I sites of pBAD18-Kan.

Plasmid pLP517 (*P_{BAD}-dacA^{S44G},* KanR) was created by cloning the *dacA^{S44G}* gene from pLP513 into the *Nhe*I and *Xba*I sites of pBAD18-Kan.

Plasmid pLP525 (*P_{BAD}-dsbA(SS)-sfgfp-dacA,* KanR) was created by cloning the *dsbA(SS)-sfgfp-dacA* gene fusion from pLP521 into the *EcoR*I and *Hind*III sites of pLP18-Kan.

Plasmid pLP602 (*P_{lac}-dsbA(SS)-sfgfp-dacC, lacI^{q},* KanR) was created by cloning the wild type *dacC* gene between the *BamH*I and *Hind*III sites of pLP9. The *dacC* gene was amplified from *E. coli* MG1655 genomic DNA, by using the primers (upstream) 5'-GCTCGGATCC**GCGGAACAAACCGTTGAA**-3' and (downstream) CTGCAAGCTT**TTAAGAGAACCAGCTGCC**-3' (*BamH*I and *Hind*III sites are underlined, bolded sequences represent portions of the amplified gene). The resulting DNA fragment does not encode the original 27 amino acid signal sequence (SS) of DacC. NOTE: even though there is an internal *BamH*I site in *dacC*, it was preserved during the construction process.

Plasmid pLPKC604 (p_{BAD}-*dacC,* KanR) was created by cloning wild type *dacC* from pAG6 (Nelson et al. 2002) into the *Nhe*I and *Hind*III sites of pLP18-Kan.

Plasmid pLPKC605 (P_{BAD}-*dsbA(SS)-sfgfp-dacC,* KanR) was created by cloning the *dsbA(SS)-sfgfp-dacC* gene from pLP602 into the *EcoR*I and *Hind*III sites of pLP18-Kan.

Plasmid pLP606 (P_{BAD}-$dacC^{S66A}$, KanR) was created by PCR amplifying the pLPKC603 plasmid with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), by using the primer 5'-GCGGATGAGAAACTGGACCCCGCG**GCC**CTGACTAAAATCATGACC-3' and its reverse complement. The resulting plasmid carries the $dacC^{S66A}$ gene, in which serine 66 (codon GAT) in the active site was replaced with alanine (GAC - underlined). The nearby *BamH*I site was removed by introducing a silent mutation at codon 68 (aspartic acid – GAT to GAC, bolded sequence).

Plasmid pLP607 (P_{lac}-*dsbA(SS)-sfgfp-*$dacC^{S66A}$, $lacI^{q}$, KanR) was created the same way as was pLP606, except that pLP602 was used as the DNA template. The resulting plasmid carries the *dsbA(SS)-sfgfp-*$dacC^{S66A}$ fusion, in which serine 66 in the active site was replaced with alanine.

Plasmid pLPKC608 (P_{lac}-*dsbA(SS)-sfgfp-dacCΔC22,* $lacI^{q}$, KanR) was created by PCR amplifying a DNA fragment lacking the final 66 nucleotides of the *dacC* gene (*dacCΔC22*) from pLP602, by using the primers (upstream) 5'-GTATGTTGTGTGGAATTGTG-3' (this sequence corresponds to pLP8 vector backbone) and (downstream) 5'- ATCAAGCTT*TTA***TCCGCCCTCTTCCACATTTTC**-3' (the *Hind*III site is underlined, bolded sequences represent portions of the amplified gene, and a stop codon (italicized *TTA*) was inserted in front of the *Hind*III site). The PCR fragment was digested and cloned into the *EcoRI* and *Hind*III sites of pLP8. The resulting gene fusion does not encode the last 22 amino acids of PBP6, so that the expressed fusion protein lacks the carboxy terminal amphipathic helix.

Plasmid pLP652 (P_{lac}-*dsbA(SS)-sfgfp-dacD,* $lacI^{q}$, KanR) was created by cloning the wild type *dacD* gene between the *BamH*I and *Hind*III sites of pLP9. The *dacD* gene was amplified from *E. coli*

MG1655 genomic DNA, using the primers (upstream) 5'- GCTCGGATCC**GCGGAAAACATTCCTTTTTC**-3' and (downstream) 5'- CTGCAAGCTT**TCAGGCCTTATGGTGGAA**-3' (*BamH*I and *Hind*III sites are underlined, bolded sequences represent portions of the amplified gene). The resulting DNA fragment does not encode the original 21 amino acid signal sequence (SS) of DacD.

Plasmid pLPKC653 (p_{BAD}-*dacD,* KanR) was created by cloning wild type *dacD* from pPJDacD (Nelson and Young 2001) into the *Nhe*I and *Hind*III sites of pLP18-Kan.

Plasmid pLPKC654 (P_{BAD}-*dsbA(SS)-sfgfp-dacD,* KanR) was created by cloning the *dsbA(SS)-sfgfp-dacD* gene fusion from pLP602 into the *EcoR*I and *Hind*III sites of pLP18-Kan.

Plasmid pLP655 (P_{BAD}-$dacD^{S63A}$, KanR) was created by PCR amplifying the pLPKC653 plasmid with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), by using the primer 5'-CAACAGCGCAATCCCGCCGCCCTGACAAAGCTGATGACG-3' and its reverse complement. The resulting plasmid carries the $dacD^{S63A}$ gene, in which serine 63 (codon AGC) in the active site was replaced with alanine (GCC, underlined).

Plasmid pLP656 (P_{lac}-*dsbA(SS)-sfgfp-*$dacD^{S63A}$, $lacI^{q}$, KanR) was created the same way as was pLP655, except that pLP652 was used as the DNA template. The resulting plasmid carries the *dsbA(SS)-sfgfp-*$dacD^{S63A}$ gene fusion, in which serine 63 in the active site was replaced with alanine.

Plasmid pLPKC657 (P_{lac}-*dsbA(SS)-sfgfp-dacDΔC13,* $lacI^{q}$, KanR) was created by PCR amplifying a DNA fragment lacking the final 39 nucleotides of the *dacD* gene (*dacDΔC13*) from pLP652, using the primers (upstream) 5'- GCTCGGATCC**GCGGAAAACATTCCTTTTTC**-3' and (downstream) 5'-ATCAAGCTT*TCA***GCTGCCTTCCCCGACAGATTC**-3' (*BamHI* and *Hind*III sites are underlined, bolded sequences represent portions of the amplified gene, and a stop codon (italicized *TCA*) was inserted in front of the *Hind*III site). The PCR fragment was digested and cloned into the *BamHI* and *Hind*III sites of pLP9. The resulting gene fusion does not encode the last 13 amino acids of DacD, so that the expressed fusion protein lacks the carboxy terminal amphipathic helix.

Plasmid pLP659 (P_{lac}-*dsbA(SS)-sfgfp-dacD*^{S63A}*ΔC13, lacI*^{q}, KanR) was created the same way as pLPKC657, except that pLP656 was used as the DNA template. The resulting plasmid carries the *dsbA(SS)-sfgfp-dacD*^{S63A}*ΔC13* gene fusion, in which serine 63 in the active site was replaced with alanine. Also, this construct does not encode the last 13 amino acids of DacD, so that the expressed fusion protein lacks the carboxy terminal amphipathic helix.

Plasmid pLP702 (P_{lac}-*dsbA(SS)-sfgfp-pbpG, lacI*^{q}, KanR) was created by cloning the wild type *pbpG* gene between the *BamH*I and *Hind*III sites of pLP9. The *pbpG* gene was amplified from *E. coli* MG1655 genomic DNA, by using the primers (upstream) 5'-GCTCGGATCC**AAAACGGCAGCCGCTACC**-3' and (downstream) 5'-CTGCAAGCTT**TTAATCGTTCTGTGCCGTC**-3' (*BamH*I and *Hind*III sites are underlined, bolded sequences represent portions of the amplified gene). The resulting DNA fragment does not encode the original 25 amino acid signal sequence (SS) of PBP7.

Plasmid pLPKC704 (p_{BAD}-*pbpG,* KanR) was created by cloning the wild type *pbpG* gene from pPJ7 (Nelson and Young 2001) into the *Nhe*I and *Hind*III sites of pLP18-Kan.

Plasmid pLPKC705 (P_{BAD}-*dsbA(SS)-sfgfp-pbpG,* KanR) was created by cloning the *dsbA(SS)-sfgfp-pbpG* gene fusion from pLP702 into *EcoR*I and *Hind*III sites of pLP18-Kan.

Plasmid pLP706 (P_{BAD}-*pbpG*^{S67A}, KanR) was created by PCR amplifying the pLPKC704 plasmid with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), by using the primer 5'-CTGGTGCGTCCGATTGCGGCTATCAGCAAATTAATGACC-3' and its reverse complement. The resulting plasmid carries the *pbpG*^{S67A} gene, in which the serine 67 (codon TCT) in the active site was replaced with alanine (GCT, underlined).

Plasmid pLP707 (*P_{lac}-dsbA(SS)-sfgfp-pbpG^{S67A}, lacI^{q},* KanR) was created the same way as was pLP706, except that pLP702 was used as the DNA template. The resulting plasmid carries the *dsbA(SS)-sfgfp-pbpG^{S67A}* gene fusion, in which serine 67 in the active site was replaced with alanine.

Plasmid pKC2 (*P_{BAD}-dsbA(SS)-sfgfp,* KanR) was created by cloning the *dsbA(SS)-sfgfp* gene fusion from pLP9 into the *Eco*RI and *Hind*III sites of pLP18-Kan.

PBP overexpression. *E. coli* CS109 harboring appropriate plasmids was grown overnight at 37^{o}C in LB-Kan or LB-Cam plus 0.4% glucose. Next morning, the cells were diluted 1:50 into 30 ml of LB-Kan or LB-Cam, and the flasks were incubated at 37^{o}C with shaking. The OD_{600} was measured every 30 min. When the cultures reached an OD_{600} of 0.2, L-arabinose was added (0.4% final concentration) to induce expression of PBPs.

Cell fractionation (periplasting). *E. coli* CS109 containing pLP9, pLPKC407, pLP523, pLPKC608, pLPKC657 or pLPKC702 were grown overnight in LB-Kan. The overnight cultures were diluted 1:250 into 5 ml of LB-Kan plus 0.2% maltose and incubated at 30^{o}C for 1.3 h, at which time IPTG was added (25 µM final concentration) and incubated for another 2.3 h. At this stage, all cultures had reached an OD_{600} of ~0.5. Each culture was split into two samples and the OD_{600} of each culture was normalized to 1.0 by centrifuging an appropriate amount of culture at 6,000 X g at room temperature for 2 min. The supernatant was removed and the cell pellet was dislodged by vortexing.

One set of the sample was selected to fractionate the cells into periplasmic and non-periplasmic components: This sample was resuspended gently into 100 µl of ice-cold periplasting buffer [50 mM Tris HCL (pH 8.0), 5 mM EDTA (pH 8.0), 20% sucrose and 62 U/µl Ready-Lyse Lysozyme (Epicentre Biotech)], and the samples were incubated at room temperature for 12 min, after which they were briefly placed on ice. The samples were pelleted at 16,000 X g for 3 min at room temperature, and the supernatant was collected without disturbing the pellet. This fraction was the periplasmic fraction. The pellet was resuspended in 100 µl of lysis buffer [50 mM Tris-HCL (pH

8.0), 5 mM EDTA (pH 8.0), 50 mM KCL, 1.25 mM $MgCl_2$, 0.1% deoxycholate and 400 U/ml of OmniCleave Endonuclease (Epicentre Biotech)], the samples were vortexed vigorously and incubated at room temperature for 5 min. This fraction was the non-periplasmic (spheroplasmic) fraction.

Whole cell lysates were prepared from the second sample, by resuspending the cell pellet in 50 µl of periplasting buffer and incubating at room temperature for 5 min, after which 50 µl of lysis buffer was added and the samples were incubated at room temperature for 5 min. All the samples were stored at -20^{o}C until further use. Since we were not able to see complete release of sfGFP-PBP fusions from the spheroplasmic fraction, we incubated the samples with 250 mM NaCl for 10 min on ice, after treatment with periplasting buffer, because this step enhances the release of peptidoglycan modifying enzymes from the periplasm (Bernhardt & de Boer, 2003). However, we did not see any significant increase in the release of sfGFP-PBP fusions from the spheroplasmic fraction (data not shown).

Separation and detection of proteins. To detect proteins, 12 µl of whole cell, periplasmic or spheroplasmic fractions were mixed with an equal volume of 2X Laemmli sample buffer (Bio-Rad, Hercules, CA), and the proteins were separated by electrophoresis through 12% SDS-PAGE gels (Pierce, Rockford, IL), at 80 V for 1.3 h. For "in-gel fluorescence", i.e. to detect GFP in the gel, samples were solubilized in Laemlli sample buffer for 30 min at 37^{o}C. After electrophoresis, GFP was visualized with a Typhoon Trio scanner (GE Healthcare, Piscataway, NJ), using an excitation wavelength of 488 nm and an emission filter of 530 nm. For immunoblotting, samples were boiled in a boiling water bath for 5 min, and proteins were transferred to PVDF membranes at 4^{o}C, at 100 V for 2 h or at 28 V overnight. For detection of GFP fusions by Western blot, blots were stained with primary antibody [mouse anti-GFP monoclonal antibodies (JL-8; from Clontech)] diluted 1:10,000, and secondary antibody [goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma)] diluted 1:10,000. The efficiency of the fractionation procedure was judged by probing the same blots for the presence of MalE, which served as a protein marker for periplasmic fraction, and for cytoplasmic fractions, GroEL or β-galactosidase were used. For detection of GroEL, the

antibodies were rabbit anti-GroEL (Sigma) diluted 1:50,000. For detection of β-galactosidase, primary antibodies were rabbit anti- β-gal (ab4761; abcam) diluted 1:20,000. For detection of GroEL and β-gal the secondary antibodies were goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) used at dilutions 1:50,000 and 1:20,000, respectively. For detection of MalE, primary antibodies were mouse anti-MalE monoclonal antibodies (Sigma) diluted 1:10,000, and secondary antibodies were goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma) diluted 1:10,000. Blots were developed with the Super Signal West Pico Chemiluminescent Substrate (Pierce) and exposed to the HyBlot CL autoradiography film (Denville Scientific Inc.). To detect multiple proteins on the same blot, blots were stripped with Restore PLUS Western Blot Stripping Buffer (Pierce) before restaining for the next protein.

Fluorescent labeling of PBPs. *E. coli* CS109 containing appropriate plasmids was grown and harvested as for the cell fractionation experiment, except that maltose was not added to the medium. The cell pellet was resuspended in 50 µl of periplasting buffer [50 mM Tris HCL (pH 8.0), 5 mM EDTA (pH 8.0), 20% sucrose and 62 U/µl Ready-Lyse Lysozyme (Epicentre)] and incubated on ice for 30 min. To this mixture was added 5 µl of protease inhibitor + endonuclease cocktail [stock – 50 µl of HALT protease inhibitor (Pierce) + 20 µl of 1:20 diluted Omnicleave endonuclease (Epicentre)], and the samples were frozen at -80^{o}C for 1 h, after which they were thawed at room temperature and vortexed vigorously. Bocillin-650/655, penicillin sodium salt (Invitrogen) was added to a final concentration of 20 µM (1.1 µl of 1mM stock), and the samples were incubated at 37^{o}C for 30 min. Laemelli sample buffer (Bio-Rad) (50 µl) was added to the sample and the sample was incubated either at 37^{o}C for 30 min or boiled in a water bath for 3 min. sfGFP is slightly resistant to boiling when compared to eGFP, under these conditions. Samples (20 µl) were loaded on a 12% SDS-PAGE gel (Pierce) and the gel was electrophoresed at 100 V until the dye-front reached the bottom of the gel, plus another 10 min. The gel was washed three times in deionized water and scanned with a Typhoon Trio scanner (GE Healthcare, Piscataway, NJ). GFP was visualized with an excitation wavelength of 488 nm and an emission filter of 530 nm. Bocillin-650 was visualized with an excitation wavelength of 633 nm and an emission filter of 670 nm. DacD did not bind to Bocillin-650, and PBP6 seems to bind to Bocillin-650 better than does PBP5. This

observation is consistent with the fact that PBP6 shows a higher rate of β-lactam binding when compared to PBP5 (Chowdhury *et al.*, 2010).

Peptidoglycan isolation and HPLC analysis. For muropeptide analysis of *E. coli* strains overexpressing FtsZ, strains CS109, CS12-7, CS203-1B and CS315-1 harboring pBR322, pZAQ or pLPZ were grown overnight at 37^{o}C in LB-Tet. The next morning the cultures were diluted 1:100 into LB-Tet and incubated at 37^{o}C until the OD_{600} ~0.6, after which cells were harvested for Peptidoglycan isolation. For assessing the D,D-CPase activity of the activesite mutated versions of PBP4, PBP6 and DacD, a pentapeptide-rich strain [that lacks all seven LMW PBPs (*E. coli* CS704-1)] harboring pLPKC403, pLP405, pLPKC604, pLP606, pLPKC653 and pLP655 plasmids were grown overnight in LB-Kan plus 0.4% glucose at 37^{o}C. The next morning, the cultures were diluted 1:100 into LB-Kan and incubated until reaching exponential phase (OD_{600} ~0.2 – 0.4), at which time gene expression was induced by adding 0.05% L-arabinose to the culture medium. Strains harboring pLP18-Kan or PBP6 allele plasmids were incubated with L-arabinose for 1 h. However, strains expressing the PBP4 or DacD alleles were incubated with L-arabinose for 1 min and 3 min, respectively, because longer expression times resulted in cell lysis. After induction, 0.4% glucose was added to repress the arabinose promoter, and the cultures were incubated for another 30 min and harvested. Muropeptide isolation and analysis was performed as described (Potluri et al., 2010, Caparros *et al.*, 1992).

Table S1. The PBP4 active site mutant (PBP4^{S62A}) does not have D,D-CPase activity.

Muropeptides	No clone	PBP4	PBP4^{S62A}
	pLP18-Kan	**pLPKC403**	**pLP405**
Monomers	57	71.0	56.2
Dimers	39.5	28.0	40.5
Trimers	3.5	1.0	3.33
Lipoprotein	2.6	7.4	2.9
Anhydro	4.8	1.3	4.5
DAP-DAP	0.31	2.7	0.3
Pentapeptide	**75.1**	**15.4**	**73.7**
Penta-glycine	5.8	1.1	7.2
Cross-Linkage	**46.5**	**30.0**	**47.1**
Average Length	20.8	75.0	22.2

Peptidoglycan from *E. coli* CS704-1 harboring pLP18-Kan (empty vector), pLPKC403 and pLP405 was isolated and the murein composition of each sample was analyzed by HPLC. The molecular percentage of each muropeptide is reported.

Supplemental References

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Supplemental Figure Legends

Fig. S1. Strains harboring pZAQ and pLPZ plasmids have three fold higher FtsZ levels. *E. coli* CS109 harboring pBR322 (lane 1), pZAQ (lane 2) or pLPZ (lane 3) were grown in LB-Tet medium until they reached an OD_{600} of ~0.5. Cells were harvested and the OD_{600} of all cultures was normalized to 1.0. The cell pellets were resuspended in 100 µl of Laemmli sample buffer and boiled for 5 min. Samples (12 µl) were loaded onto a 12% SDS-PAGE gel, separated , and the proteins transferred onto a Hybond-LFP PVDF membrane (Amersham). For detection of FtsZ, 1:20,000 diluted rabbit polyclonal anti-FtsZ antiserum (a gift from Dr. Piet de Boer) (Hale & de Boer, 1999) and 1:40,000 diluted Alexa Fluor 488 goat anti-rabbit IgG antibodies (Invitrogen) were used. The membrane was dried and scanned on a Typhoon Trio scanner using the GFP scanning settings described in Materials and Methods. The bands were quantified using ImageQuant TL software (GE Healthcare). The lane marked "M" represents the molecular weight marker (pre-stained All Blue protein standard marker, Bio-Rad). The arrowhead marks the FtsZ band.

Fig. S2. Branches arise from abnormal septation in cells lacking PBP5. *E. coli* CS12-7 (ΔPBP5) was grown and imaged as described in Materials and Methods. Black and white arrows represent abnormal cell constriction events. Black and white dots mark abnormal cell poles that were produced by abnormal cell constrictions and which gave rise to branches. The numbers in each panel indicate time in minutes. The scale bar equals 5 µm.

Fig. S3. Abnormal septation increases when FtsZ is overexpressed in LMW PBP mutants. *E. coli* CS315-1 (ΔPBPs 4, 5 and 7) without (A) or with (B) pLPZ. Cells were grown and imaged as described in Materials and Methods. Black and white arrows represent abnormal cell constriction events. Black and white dots mark abnormal cell poles that were produced by abnormal cell constrictions and which gave rise to branches. The numbers in each panel indicate time in minutes. The scale bar in each panel equals 5 µm (note that the magnification of planels A and B is different).

Fig. S4. Low levels of FtsZ-GFP are produced to image FtsZ ring. *E. coli* strains CS109 (lane 1), LP18-1 (lane 2), LP1 (lane 3), LP16-1 (lane 4), LP17-1 (lane 5) were grown in LB + 25 µM IPTG for 4h, and the cells were harvested and processed for western blot analysis as described in Fig. S1. Before the protein was transferred onto the membrane, the gel was scanned on a Typhoon Trio scanner to make sure that the FtsZ-GFP was denatured and that there was no fluorescence. The lane marked "M" represents the molecular weight marker (pre-stained All Blue protein standard marker, Bio-Rad). The arrowhead marks the native FtsZ protein, and the arrow marks the FtsZ-GFP fusion protein.

Fig. S5. Relationship between cell diameter and Z-ring orientation. The numbers of cells (Y axis) having the specified diameters (X axis) were determined for the parental strain (LP18-1) and for each of three PBP mutants, as indicated. Blue bars represent numbers of cells having normal (i.e., perpendicular) Z rings at the center of cells. Red bars represent numbers of cells having Z rings with abnormal orientations. Each cell contained only a single Z ring.

Fig. S6. Abnormal FtsZ polymers are responsible for abnormal cell constriction. *E. coli* strains LP18-1 (wild type) (A) and LP1 (ΔPBPs 4, 5 and 7) (B) were grown and imaged as described in Materials and Methods.

Fig. S7. PBPs 5, 6, 7 and DacD fused to DsbA-SS-sfGFP are exported to the periplasm. A and B. *E. coli* CS109 carrying appropriate plasmids were grown, harvested and periplasted as described in Materials and Methods. Protein samples were separated by electrophoresis through an SDS-PAGE gel. GFP, MalE, β-gal and GroEL proteins were detected as described in Materials and Methods. MalE was used as a marker for the periplasmic fraction. β-gal and GroEL were used as markers for the cytoplasmic fraction. C and D. *E. coli* CS109 carrying appropriate plasmids was grown in LB-Kan + 50 µM IPTG for 2.5 h at 30°C, harvested and the PBPs detected by the penicillin binding assay as described in Materials and Methods. C. PBPs detected by fluorescent penicillin (Bocillin 650). D.

GFP fluorescence from DsbA-sfGFP fusions. Rectangular boxes in panels C and D represent the corresponding sfGFP-PBP fusions.

Fig. S8. Expression of sfGFP-PBP fusion proteins. *E. coli* CS109 carrying appropriate plasmids were grown, harvested and detected by the penicillin binding assay as described in Materials and Methods. A. PBPs detected by fluorescent penicillin (Bocillin 650). B. GFP fluorescence from DsbA-sfGFP-PBP fusions. Rectangular boxes represent corresponding sfGFP fusions. A normal distribution of wild type PBPs (native, without sfGFP) is presented in lane 2 of panel A.

Fig. S9. sfGFP-PBP7 localizes evenly in the periplasm. *E. coli* CS9-19 (ΔPBP7) expressing sfGFP-PBP7 from pLP702 was grown and imaged as described in Materials and Methods. A. Untreated cells. B. Cells filamented by treating with aztreonam for 1 mass doubling. Each panel has a phase contrast image on the left and the corresponding fluorescence image on the right. All images have same magnification; the scale bar in panel A equals 5 μm.

Fig. S10. sfGFP localizes evenly in the periplasm. *E. coli* strains CS12-7 (ΔPBP5) (A and B), and CS703-1 (ΔPBPs 4, 5, 6, 7, AmpC and AmpH) (C and D) expressing DsbA-SS-sfGFP from pLP9, were grown in LB-Kan + 25 μm IPTG for 2.5 h at 30^{o}C, and the cells were imaged as described in Materials and Methods. Cells in panels B and D were filamented by treating with aztreonam for 1 mass doubling. Each panel has a phase contrast image on the left and the corresponding fluorescence image on the right. All images have same magnification; the scale bar in panel A equals 5 μm.

Fig. S11. Overexpression of active site mutants of PBPs 4, 5 and DacD triggers cell lysis, whereas expression of active site mutants of PBPs 6 and 7 does not. *E. coli* CS109 harboring appropriate plasmids were grown and the OD_{600} was measured as described in Materials and Methods. A. Growth curves when wild type proteins were overexpressed. B. Growth curves when active site mutated proteins were overexpressed. The arrow represents the time at which the proteins were induced.

Fig. S12. sfGFP-PBP fusions lacking their C-terminal amphipathic helices localize evenly in the periplasm. *E. coli* CS12-7 (ΔPBP5) harboring pLPKC608 (sfGFP-PBP6ΔC22) or pLPKC657 (sfGFP-DacDΔC13) were grown and imaged as described in Materials and Methods. A and C. Untreated cells. B and D. Cells filamented by treating with aztreonam for 1 mass doubling. Each panel has a phase contrast image on the left and the corresponding fluorescence image on the right. All images have same magnification; the scale bar in panel A equals 5 μm.

Figure S1

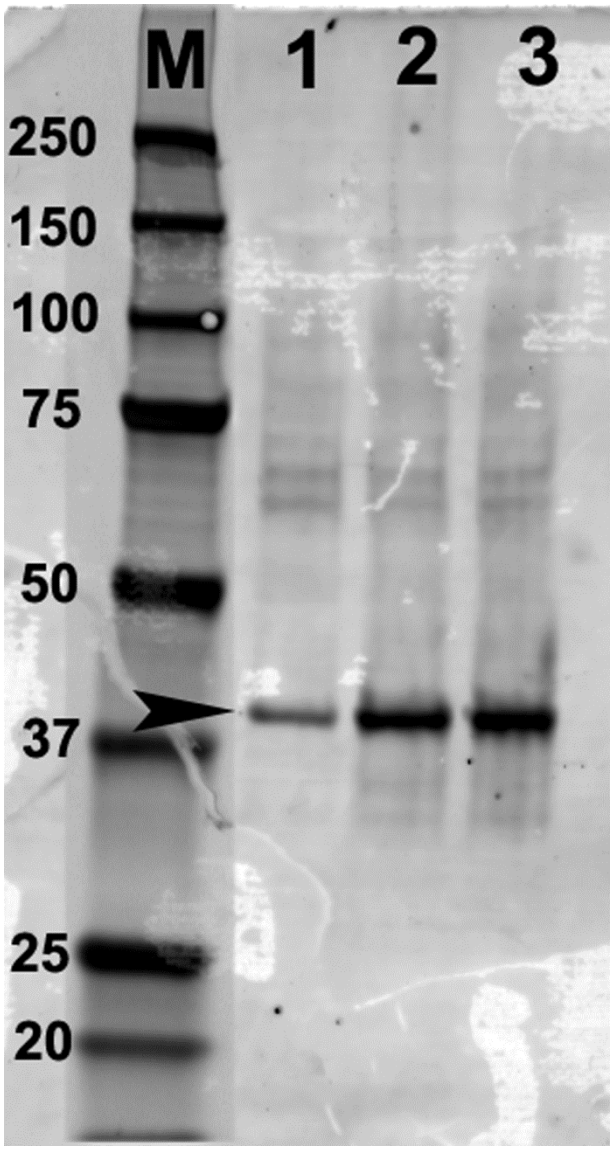

Figure S2

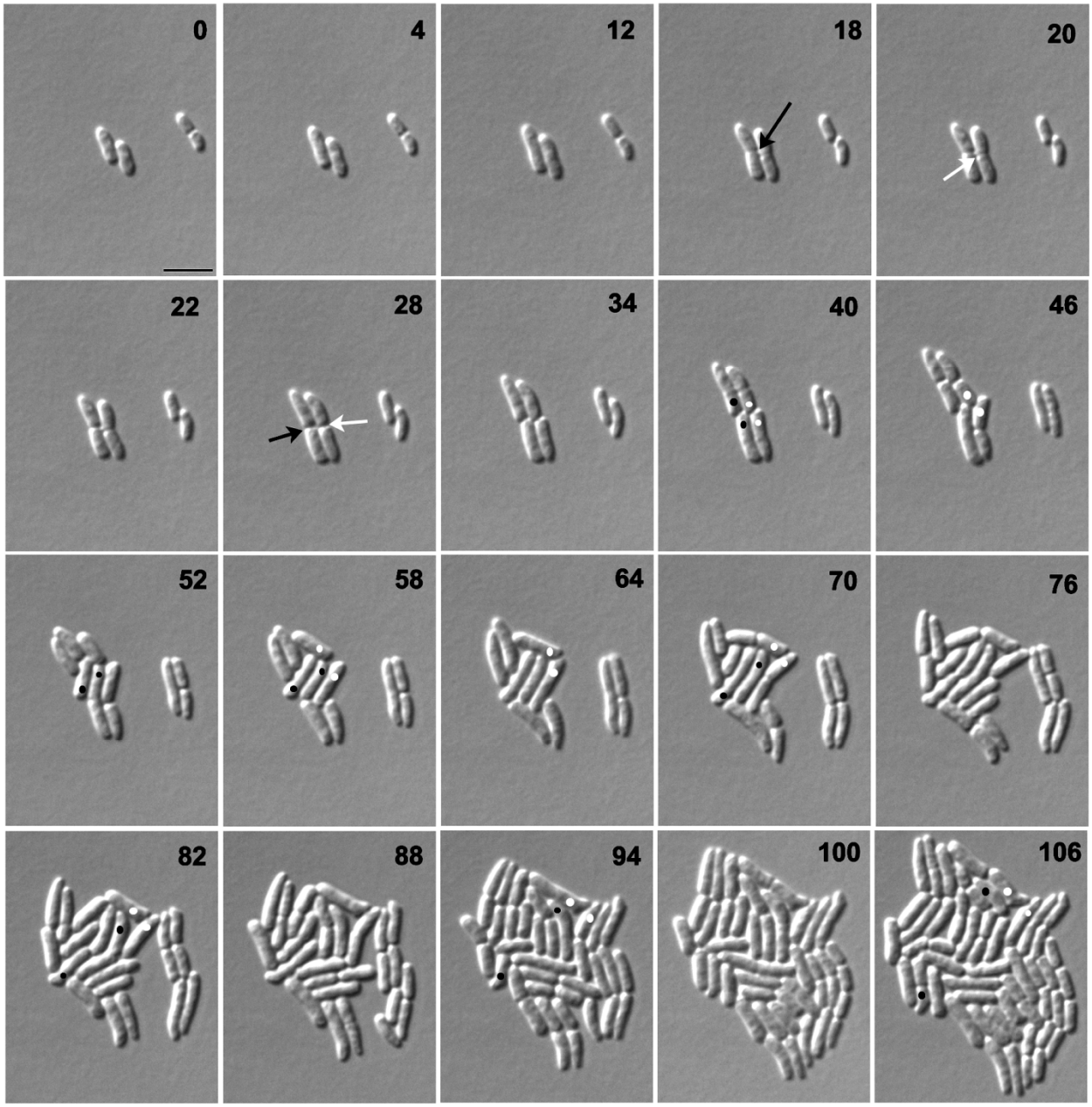

Figure S3

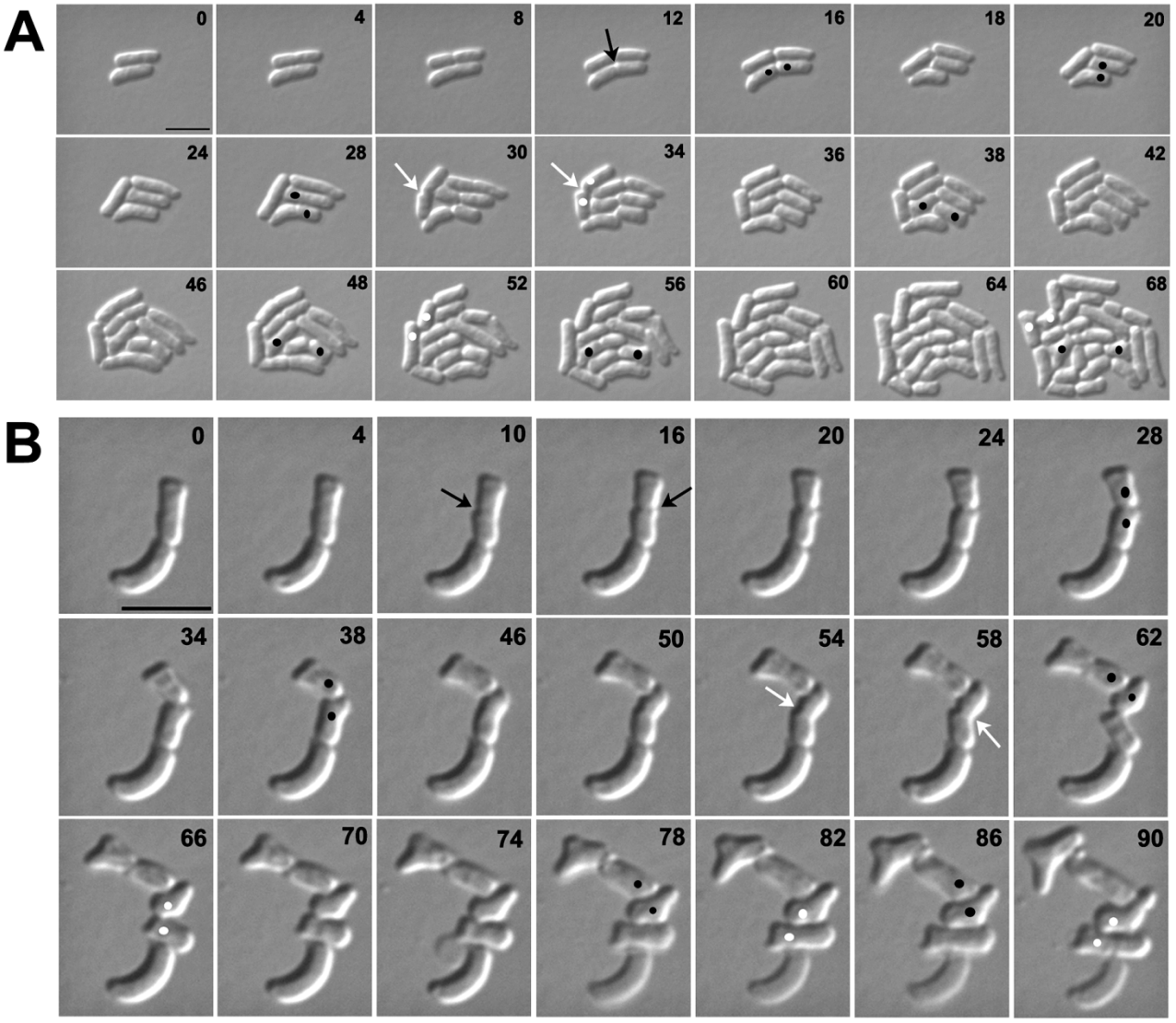
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Figure S4

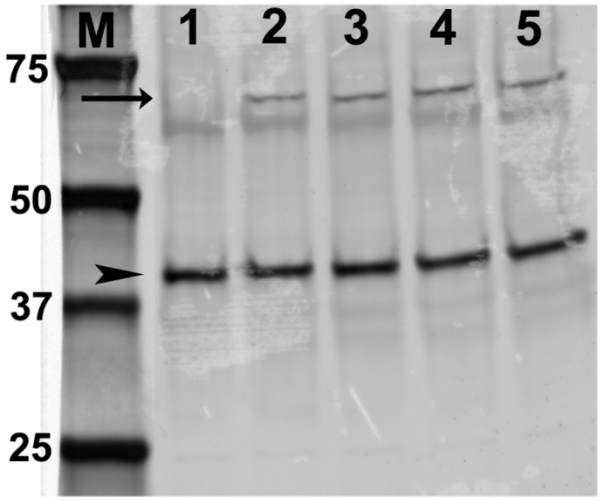
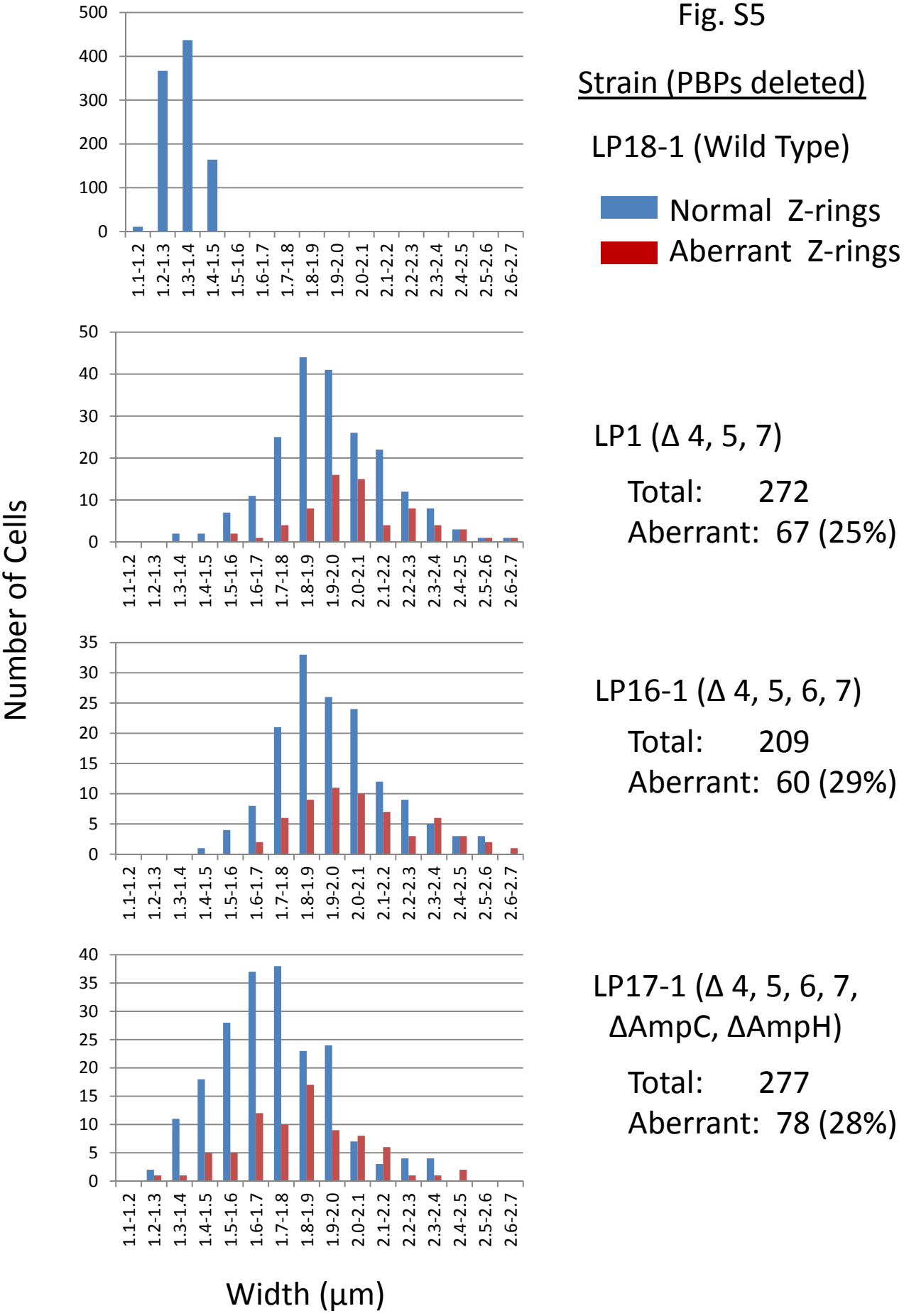


Fig. S5



Strain (PBPs deleted)

LP18-1 (Wild Type)

Normal Z-rings

Aberrant Z-rings

LP1 (Δ 4, 5, 7)

Total: 272

Aberrant: 67 (25%)

LP16-1 (Δ 4, 5, 6, 7)

Total: 209

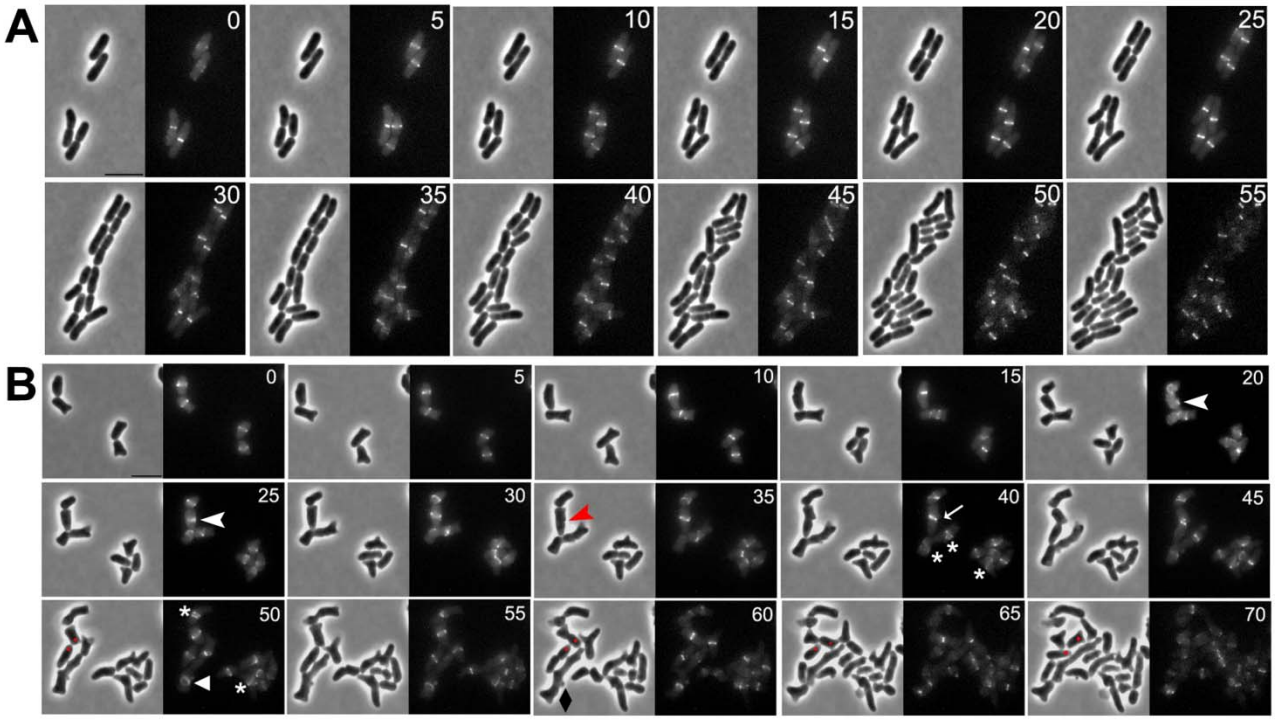
Aberrant: 60 (29%)

LP17-1 (Δ 4, 5, 6, 7, ΔAmpC, ΔAmpH)

Total: 277

Aberrant: 78 (28%)

Figure S6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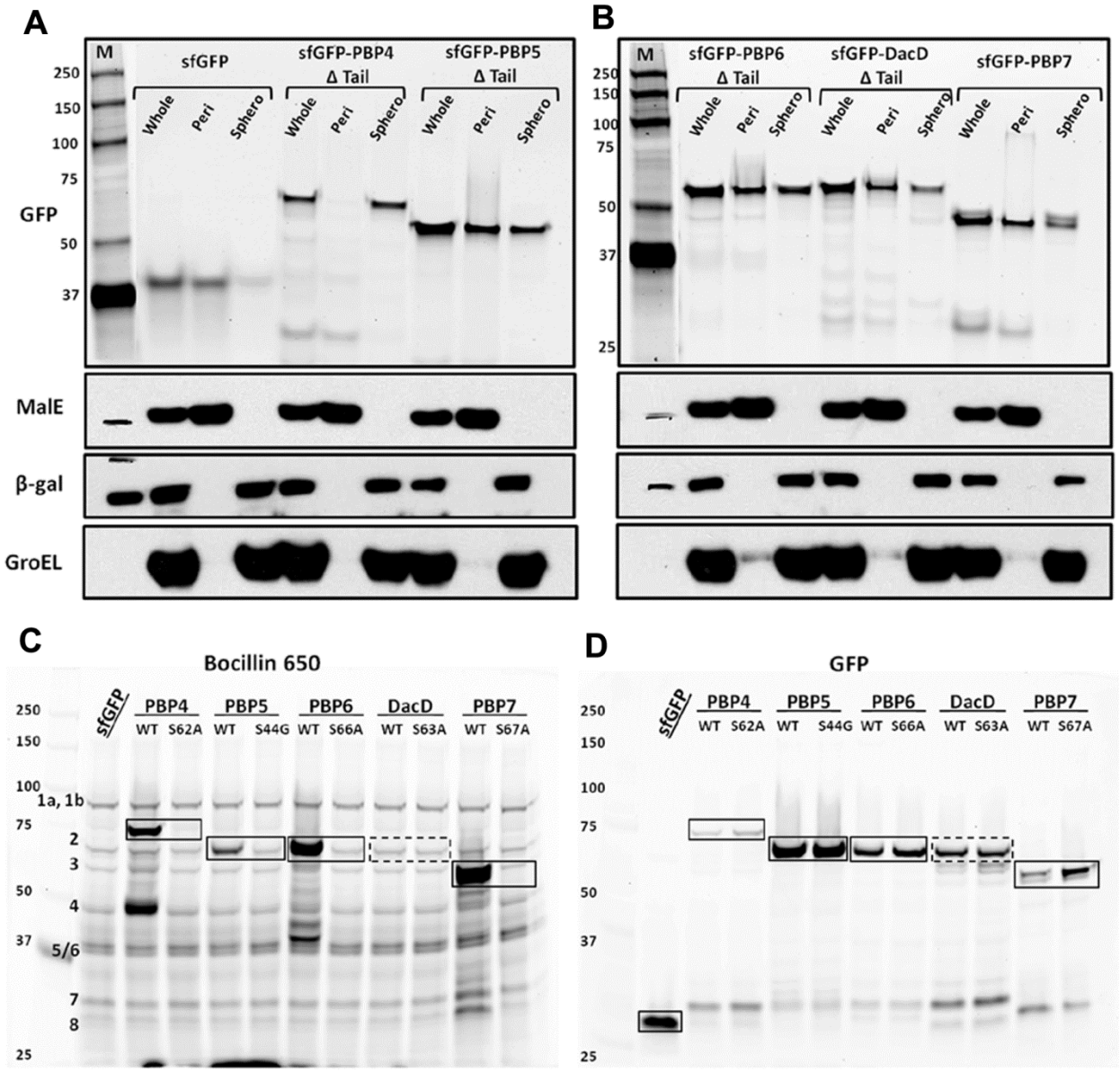
45
50
55
60
65
70

Figure S7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sfGFP-PBP5 Δ Tail
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Peri
Sphero
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150
100
75
50
37
GFP
MalE
β-gal
GroEL
B
M
sfGFP-PBP6 Δ Tail
sfGFP-DacD Δ Tail
sfGFP-PBP7
Whole
Peri
Sphero
250
150
100
75
50
37
25
C
Bocillin 650
sfGFP
PBP4 WT S62A
PBP5 WT S44G
PBP6 WT S66A
DacD WT S63A
PBP7 WT S67A
250
150
100
75
50
37
25
1a, 1b
2
3
4
5/6
7
8
D
GFP
sfGFP
PBP4 WT S62A
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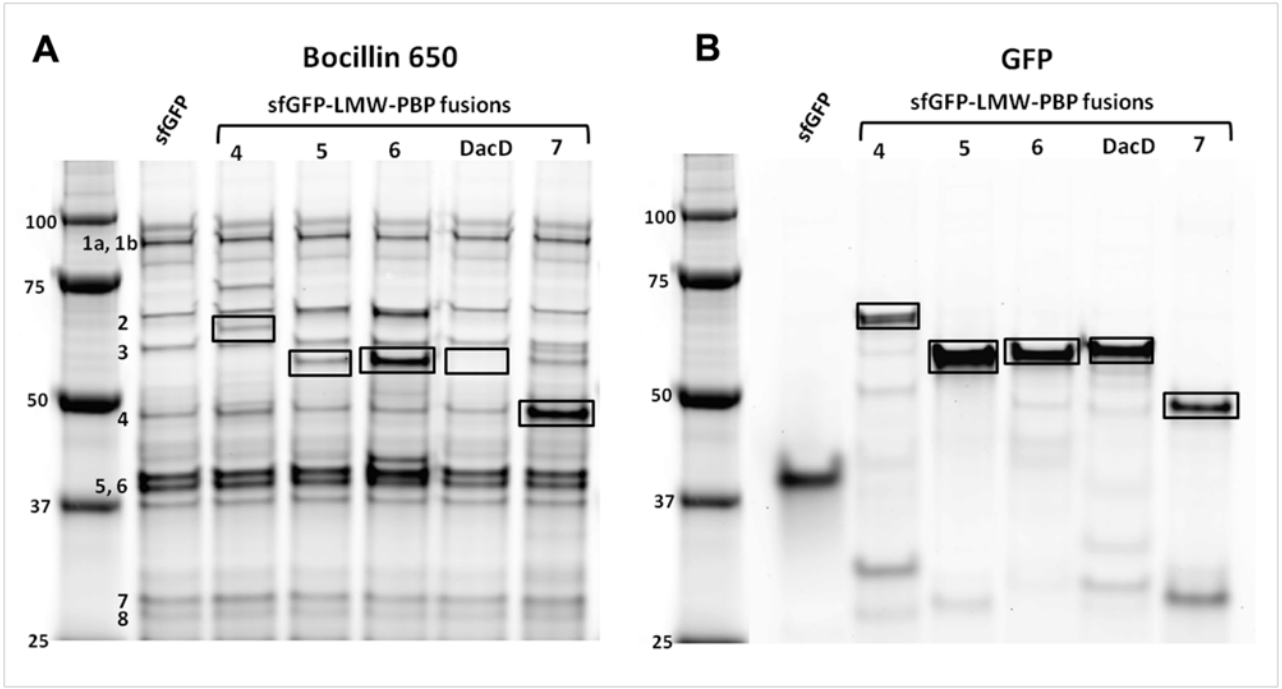
100
75
50
37
25

Figure S8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Bocillin 650
sfGFP
sfGFP-LMW-PBP fusions
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5
6
DacD
7
100
75
50
37
25
1a, 1b
2
3
4
5, 6
7
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DacD
7
100
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50
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25

Figure S9

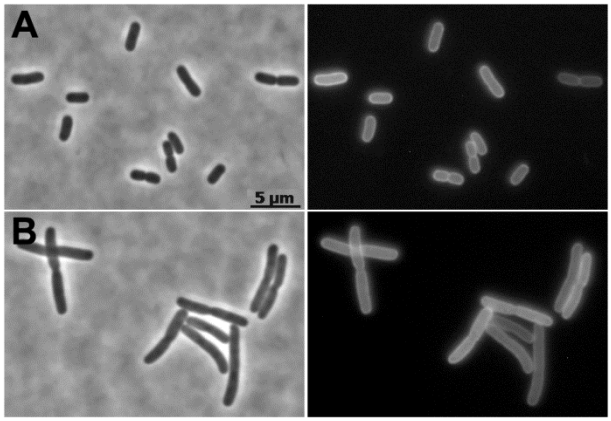


Figure S10

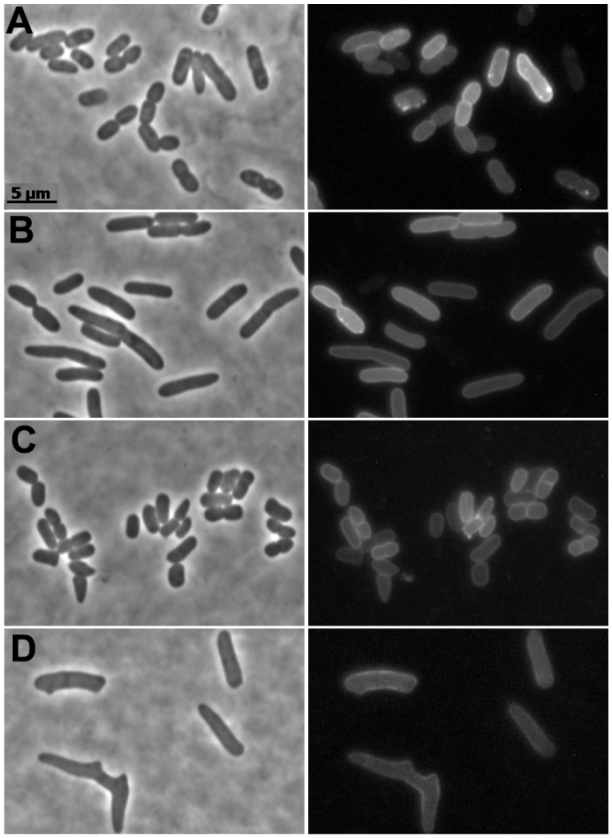
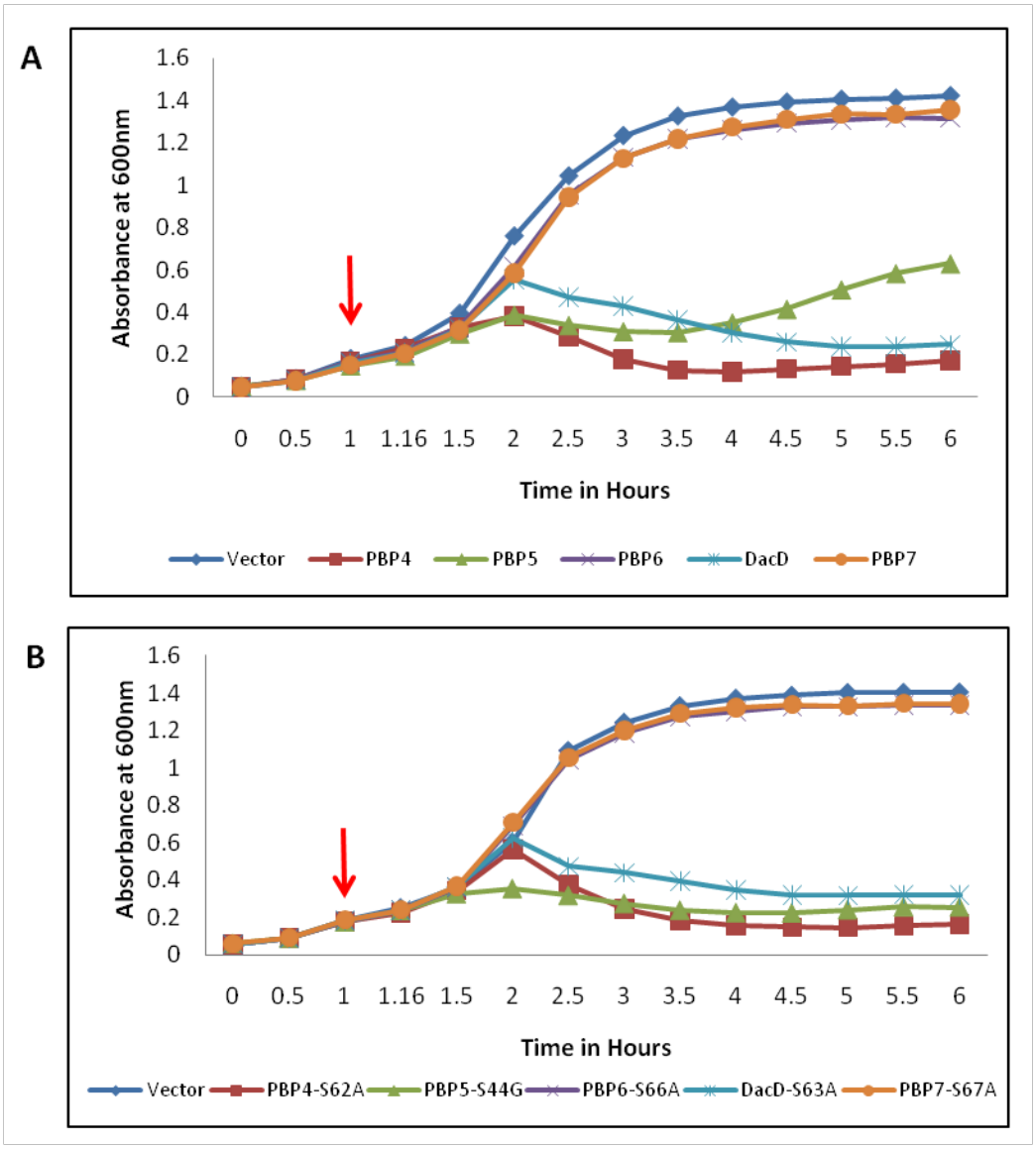


Figure S11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1.2
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0.6
0.4
0.2
0
0 0.5 1 1.16 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
Time in Hours
Vector
PBP4
PBP5
PBP6
DacD
PBP7
B
Absorbance at 600nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 0.5 1 1.16 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
Time in Hours
Vector
PBP4-S62A
PBP5-S44G
PBP6-S66A
DacD-S63A
PBP7-S67A

Figure S12