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SUPPLEMENTARY INFORMATION

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CozE is a member of the MreCD complex that directs cell elongation in *Streptococcus pneumoniae*

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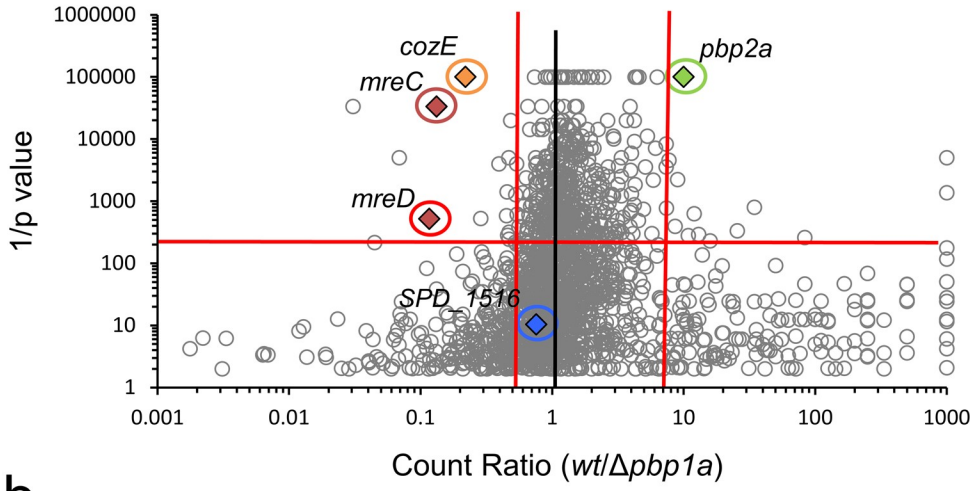
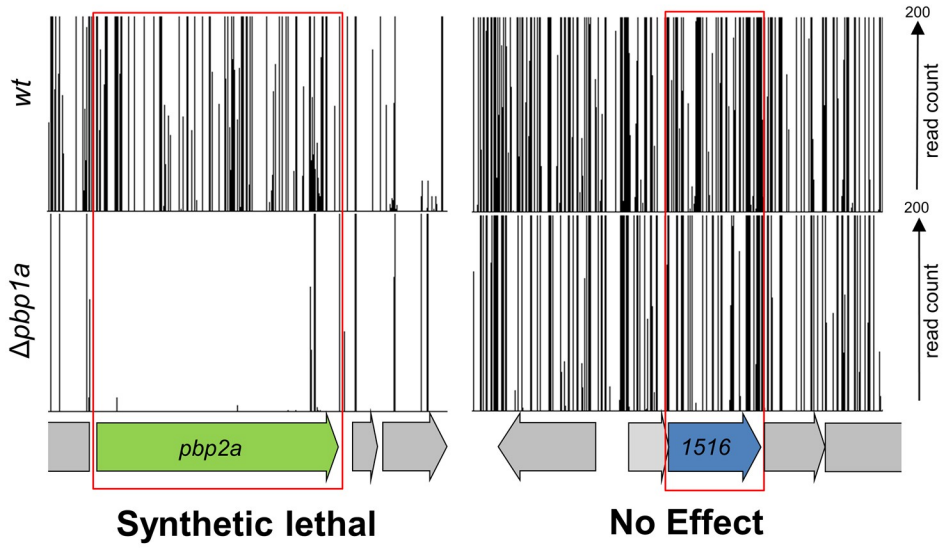


b

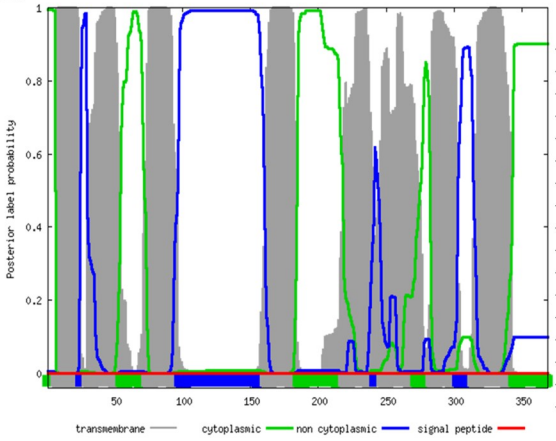
Locus Name	Number of TA sites in ORF	Count Ratio (*wt*/Δ*pbp1a*)	U stat	p-value
cozE	112	0.22	4507	0.00
mreC	63	0.12	1592	0.0019
mreD	49	0.13	745	0.00003
pbp2a	145	10	3814	0.00
SPD_1516	69	0.75	2081	10.34

c



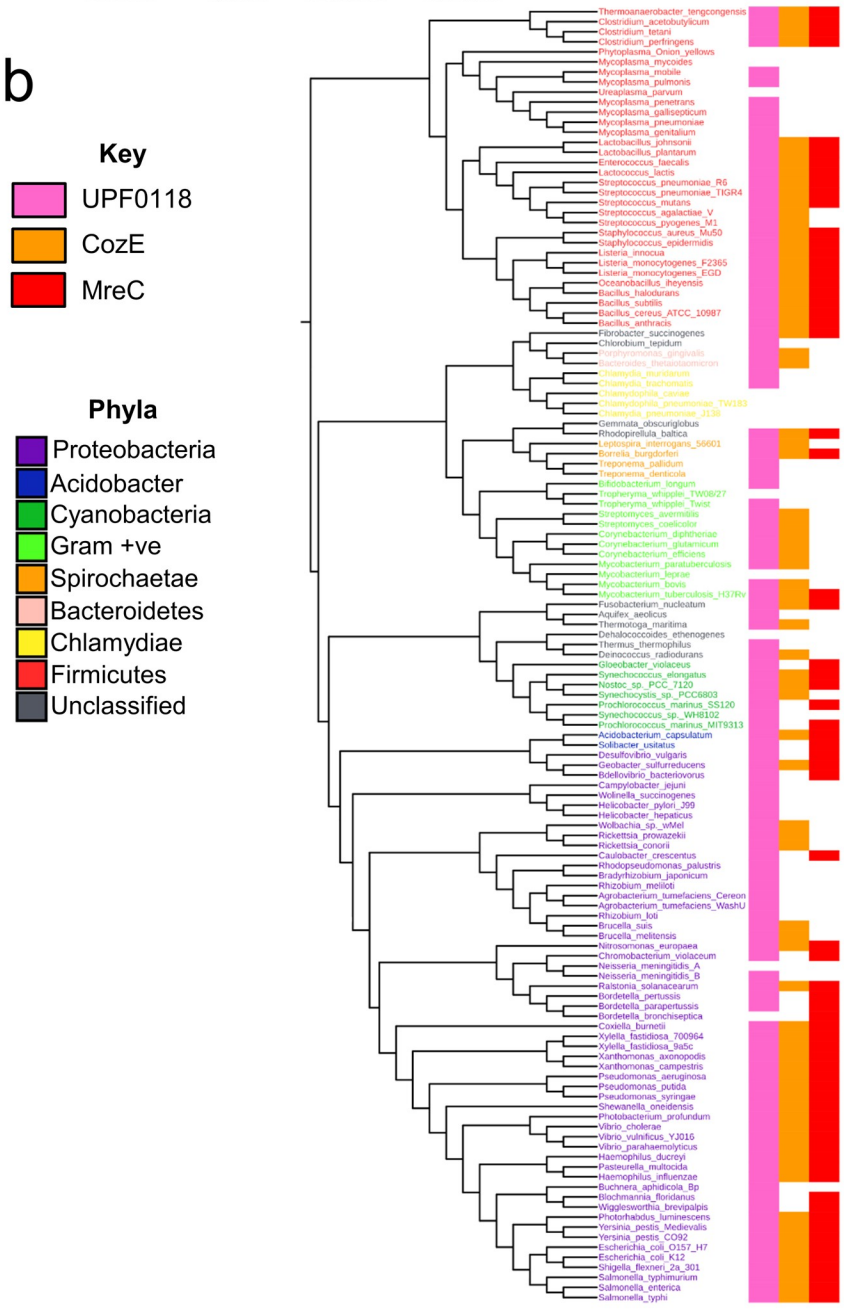
Supplementary Figure 1 | Δ*pbp1a* Tn-Seq data reveals novel genetic relationships.
Transposon sequencing of a Δ*pbp1a* strain compared to a *wt* control. Libraries were generated by direct transformation of *in vitro* transposed gDNA into both strains, resulting libraries had transposon positions sequenced using next generation sequencing. For both libraries, whole genome transposon insertion data was divided into TA site insertion profiles for each ORF. The raw number of sequence reads at each insertion site was calculated for each condition (strain) and the ratio calculated (the more skewed the ratio, the more likely the hit will verify).TA insertion profiles were compared by a Mann-Whitney U test to evaluate differential insertion profiles (the more significant, the more likely the hit will verify).
a, An inversed volcano plot showing *wt* vs Δ*pbp1a* Tn-Seq data. Red lines indicate cut off values used by this study to identify hits in the screen, p value < 0.0005 and count ratio > 7 fold. The *cozE* ORF is highlighted in orange. Examples of a known synthetic lethal gene (*pbp2a*), synthetic viable (*mreCD*) genes and an ORF not essential in either library (SPD_1516) are also highlighted.
b, Table showing *wt* vs Δ*pbp1a* Tn-Seq output statistics of the ORFs highlighted in **a**. A Mann-Whitney U test is used find significant differences in transposon insertion profiles across each ORF.
c, Transposon insertion profiles for the known synthetically lethal gene: *pbp2a* and a gene non-essential in either library: SPD_1516. The synthetic lethal gene has a dramatic reduction in apparent transposon insertions in the Δ*pbp1a* strain, whereas SPD_1516 has multiple insertions in each strain. Profiles contrast those of *cozE* and *mreCD* shown in **Fig. 1a**.

a

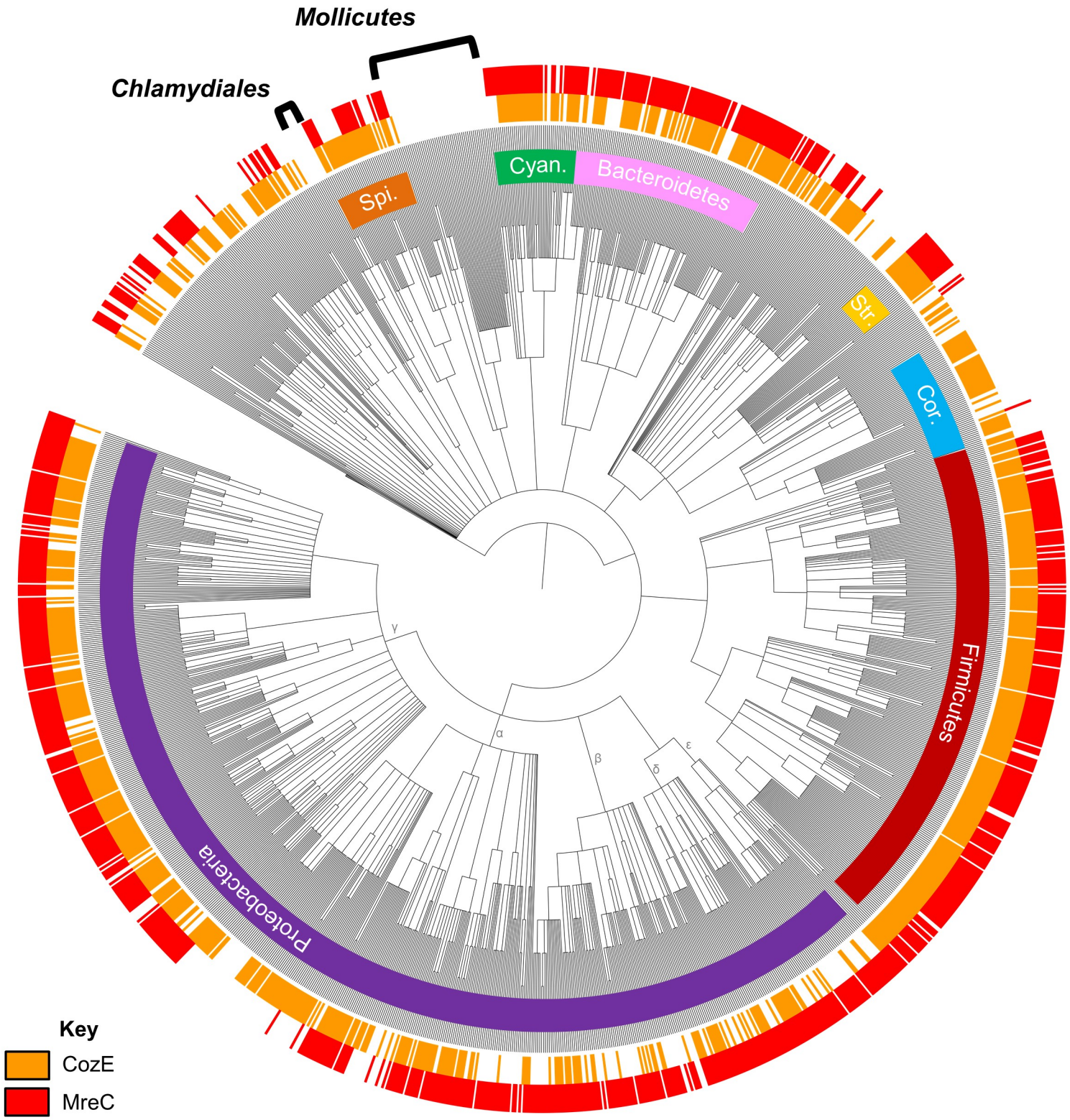


Amino Acid Start	Amino Acid End	Prediction
1	6	CYTOPLASMIC
7	24	TRANSMEMBRANE
25	29	NON CYTOPLASMIC
30	54	TRANSMEMBRANE
55	73	CYTOPLASMIC
74	97	TRANSMEMBRANE
98	160	NON CYTOPLASMIC
161	184	TRANSMEMBRANE
185	218	CYTOPLASMIC
219	241	TRANSMEMBRANE
242	246	NON CYTOPLASMIC
247	271	TRANSMEMBRANE
272	282	CYTOPLASMIC
283	302	TRANSMEMBRANE
303	313	NON CYTOPLASMIC
314	343	TRANSMEMBRANE
344	369	CYTOPLASMIC

b

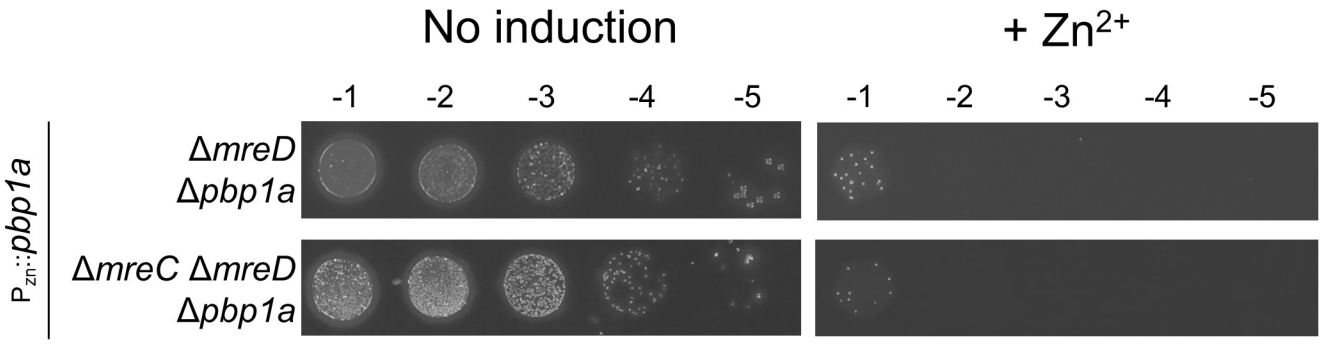


Supplementary Figure 2 | CozE is a conserved polytopic membrane protein.
a, Output from the Phobius web server for CozE (SPD_0768) topology. Phobius uses a hidden Markov model which assesses sub-regions of a protein in a series of interconnected submodels to generate a topological prediction. Graph indicates the probability that sequences are cytoplasmic or non-cytoplasmic and the presence of transmembrane regions long the length of the protein sequence. The corresponding amino acid positions for predicted non-cytoplasmic/membrane/cytoplasmic transitions are shown in the table on the right. Phobius can be found at: (http://phobius.sbc.su.se/). Data was used to draw the schematic of the predicted membrane topology of CozE shown in **Fig. 1b**.
b, Annotated linearised version of the phylogenetic tree shown in **Fig. 1c**, showing the species names of all 129 diverse bacterial species. The phylum each species belongs to is indicated by the colour of the text. The presence of UPF0118 family members are indicated in pink on each leaf. Organisms with a *S. pneumoniae* CozE/MreC homologue are indicated in orange/red (e-value cut off = $1x10^{-4}$). CozE and MreC show strong co-occurrence particularly in the Firmicutes and Proteobacteria. The tree was constructed using the Interactive Tree Of Life (v2) web-based tool, currently running version 3 (http://itol.embl.de/).

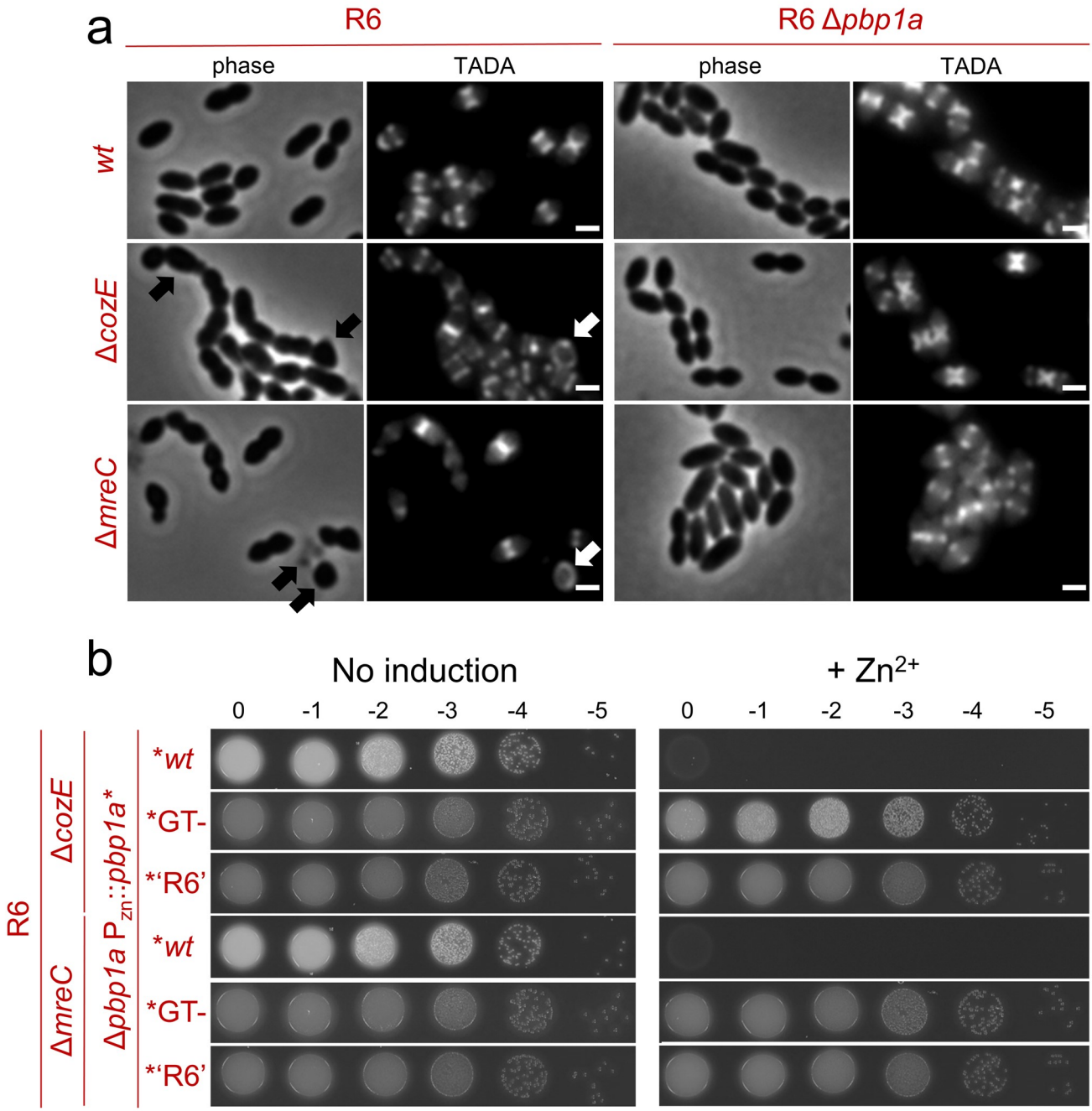


Supplementary Figure 3 | *S. pneumoniae* CozE and MreC co-occur across bacterial species and are absent in bacteria lacking a cell wall.

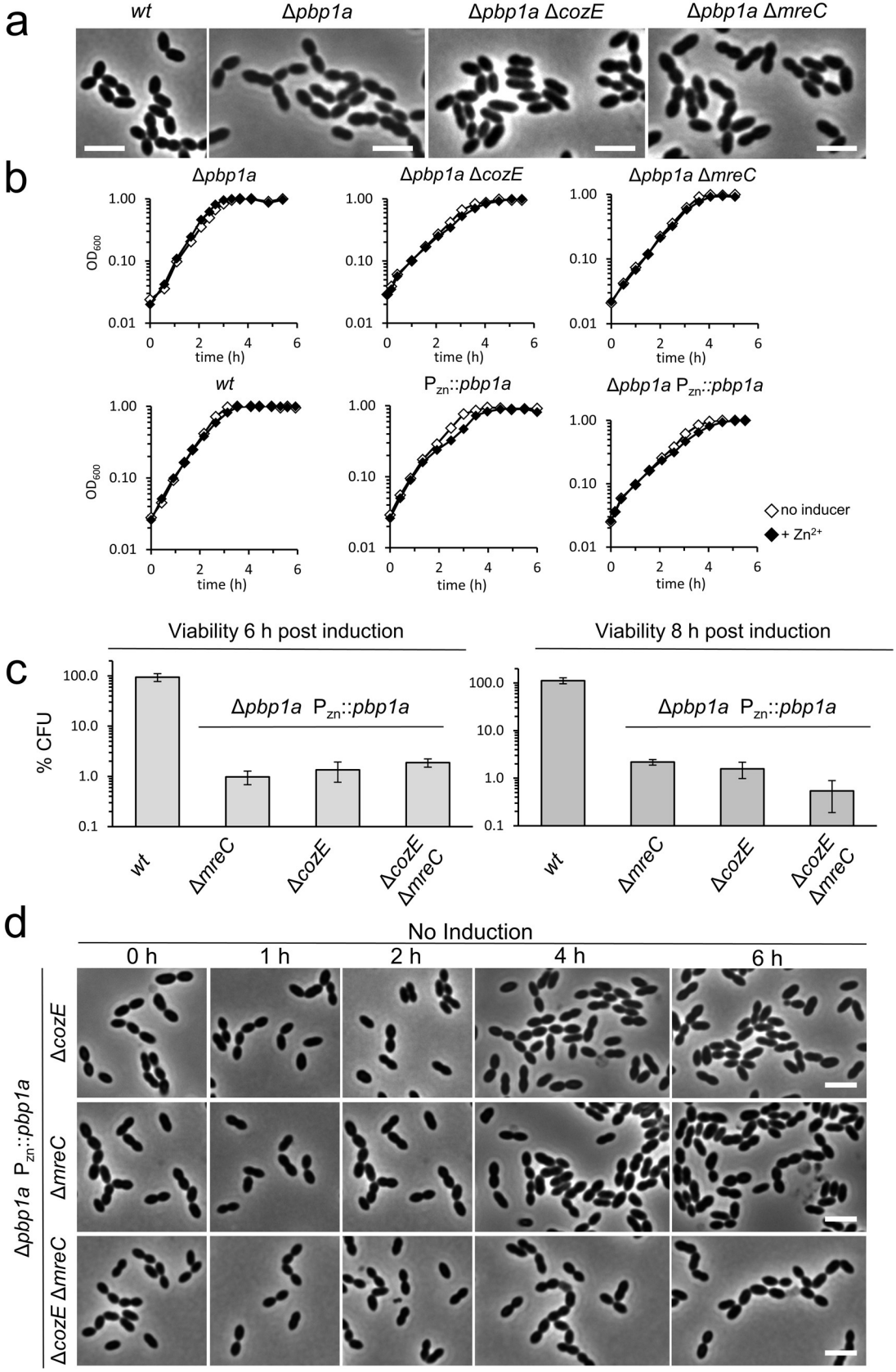
Phylogenetic tree showing CozE *S. pneumoniae* homologues across 1,576 bacterial species. Strains containing a homologous sequence are indicated by an orange (CozE) or red (MreC) bar on each leaf respectively. CozE and MreC show strong co-occurrence, particularly in the Firmicutes and Proteobacteria phyla. Notable groups without CozE and MreC homologues are the Mollicutes Class, which are known to lack a cell wall suggesting these proteins are required for cell wall biogenesis. In addition both homologues are absent from the Chlamydiales which carry out cryptic cell wall biosynthesis with a reduced set enzymes [1]. Cor. = Corynebacteriales, Cyan. = Cyanobacteria, Spi. = Spirochaetae and Str. = Streptomycetes. The tree was constructed using NCBI BLASTp, using the *S. pneumoniae* CozE protein sequence as the query to search against a database of bacterial genomes with an e-value cut off of $1x10^{-4}$. To identify the diverse MreC homologues across species a mixed query was used consisting of: *S. pneumoniae*, *E. coli*, *C. crescentus*, *C. jejuni*, *S. coelicolor* and *B. fragilis* sequences; again with an e-value cut off of $1X10^{-4}$. This analysis was carried out using the Harvard Medical School research computing cluster Orchestra (https://rc.hms.harvard.edu/#orchestra). The tree was drawn using the Interactive Tree Of Life (v3) web-based tool (http://itol.embl.de/) [2].



Supplementary Figure 4 | Both *mreD* and the *mreCD* operon are essential in *wt* (*pbp1a*$^{+}$) D39 *S. pneumoniae* cells.
The indicated S. pneumoniae strains were grown to exponential phase and normalised to an OD_{600} of 0.2. Resulting cultures were serially diluted and 5 µl of each dilution spotted onto TSAII 5%SB plates in the presence or absence of 600 µM $ZnCl_2$. Plates were incubated at 37^{o}C in 5% CO_2 cabinet and imaged. Representative image from three replicates is shown.



Supplementary Figure 5 | The *pbp1a*^{D39} allele is lethal in *S. pneumoniae* R6 strains lacking CozE and MreC.
a, Microscopic analysis of R6 strains mutant strains. Strains were grown to mid-exponential phase, back diluted to an OD_{600} of 0.025 in THY and incubated at 37°C in a 5% CO_2 cabinet for 3 h 45 min. Where necessary cultures were further back diluted at 2 h to avoid high cell densities. Cells were labelled with TADA for 15 min prior to imaging on 2% agarose pads. Δ*cozE* and Δ*mreC* deletions are viable in the R6 strain background. However, strains show mild morphological defects and evidence of lysis (black arrows). Rarely cells show delocalised TADA incorporation (highlighted by white arrows). This mild phenotype is suppressed entirely upon Δ*pbp1a* deletion Representative image from two replicates is shown,
scale bars = 1 μm.
b, Expression of *wt* D39 *pbp1a* is lethal in R6 lacking CozE or MreC. The indicated S. pneumoniae strains were grown to exponential phase and normalised to an OD_{600} of 0.2. Resulting cultures were serially diluted and 5 μl of each dilution spotted onto TSAII 5%SB plates in the presence or absence of 600 μM $ZnCl_2$. Plates were incubated at 37°C in 5% CO_2 cabinet and imaged. Spot dilutions show P_{Zn}::*pbp1a*^{D39} '*wt*' was lethal in the R6 background on induction, whereas P_{Zn}::*pbp1a*^{R6} and a glycosyltransferase P_{Zn}::*pbp1a*(GT-) mutant was not lethal under the same conditions. Images are representative of two replicates.



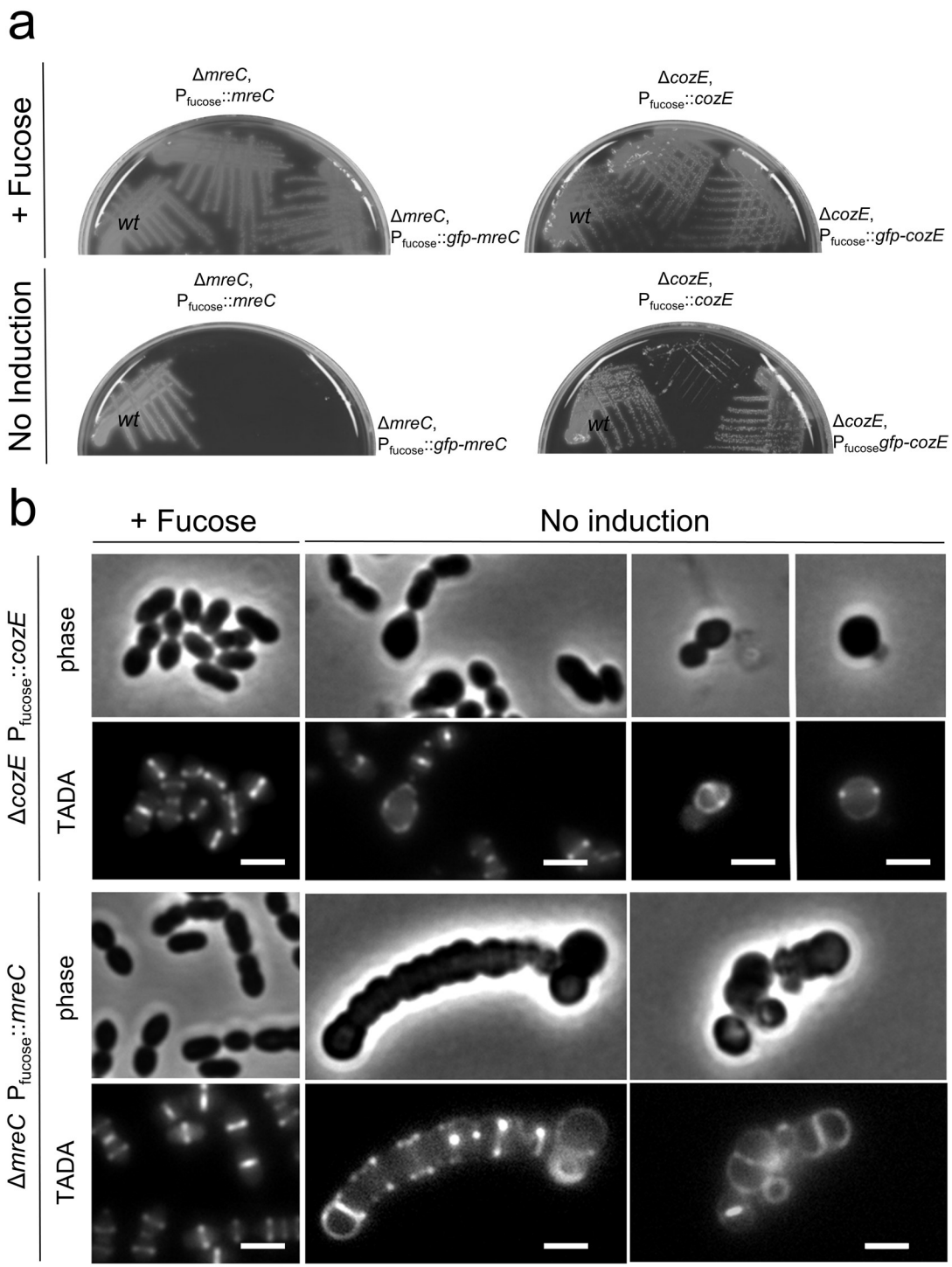
Supplementary Figure 6 | Cells lacking both PBP1a and CozE or PBP1a and MreC have no growth or morphological defects.

a, Representative images of *wt*, Δ*pbp1a*, Δ*pbp1a* Δ*cozE* and Δ*pbp1a* Δ*mreC* deletion strains. In all cases strains were grown in THY at 37°C in a 5% CO_2 cabinet to mid-exponential phase. Cells were imaged on a THY 2% agarose pad, $n = 4$, scale bars = 3 μm.

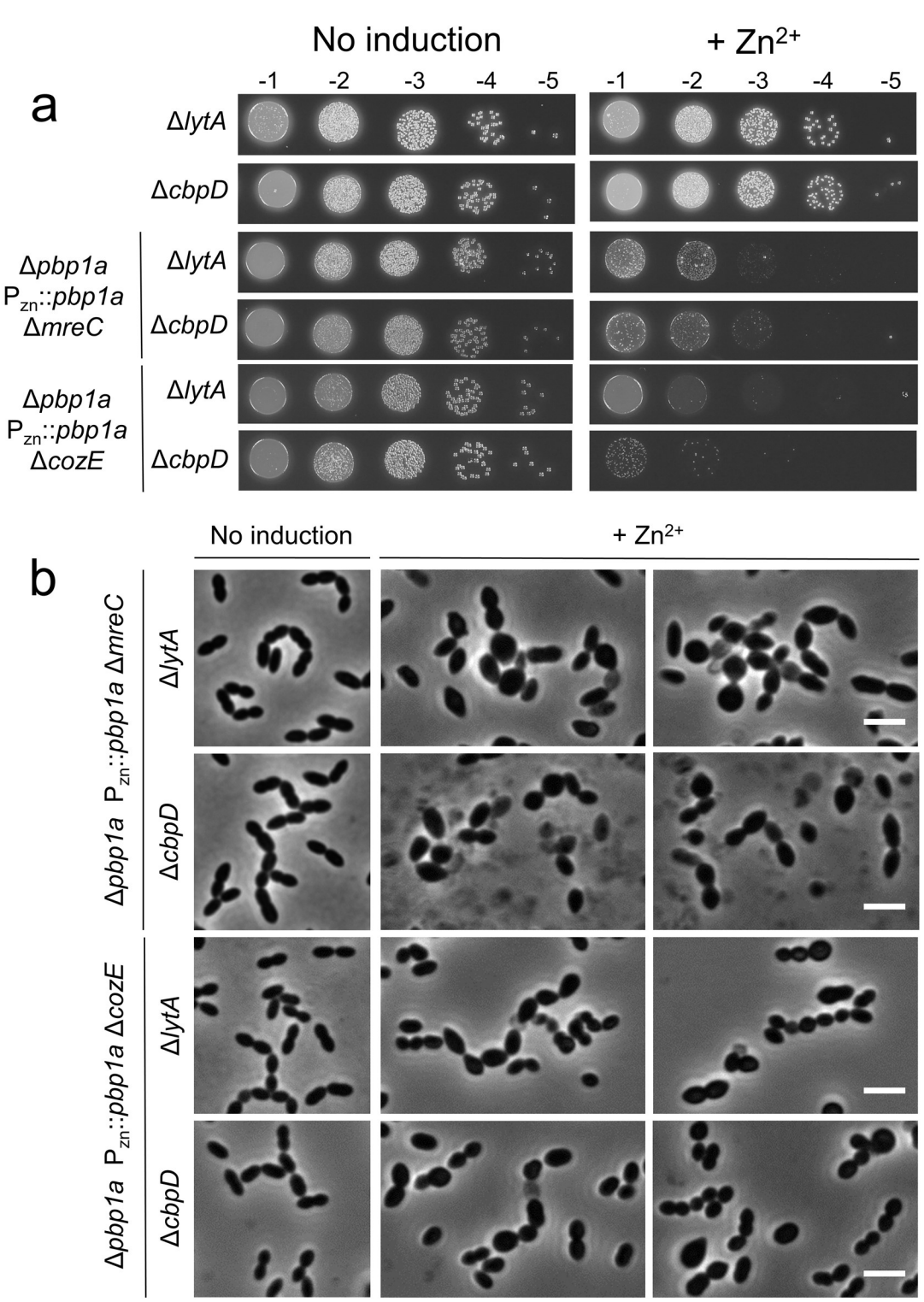
b, Control growth curves for all single/double deletion strains and strains containing the P_{zn}::*pbp1a* construct. All strains were grown in THY to mid-exponential phase, diluted in fresh THY to an OD_{600} of 0.025 in the presence or absence of 600 μM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet. OD_{600} was recorded approximately every 30 min for 6 h. Growth curves are representative of four replicates.

c, CFU counts of Δ*cozE* and Δ*mreC* strains show no difference in strain viability on P_{zn}::*pbp1a* induction after 8 h. Cultures were diluted in fresh THY to an OD_{600} of 0.025 in the presence or absence of 600 μM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet. Viable counts are shown for the 6 h and 8 h post-induction time points and show no statistically significant differences. $n = 3$, error bars = standard deviation.

d, Representative images of Δ*cozE*, Δ*mreC* and Δ*cozE* Δ*mreC* strains containing the P_{zn}::*pbp1A* construct but without induction. Strains were grown in the same manner as the growth curves above (b). At indicated time points cells were removed and placed on a THY 2% agarose pad and immediately imaged. $n = 2$, scale bars = 3 μm. Images serve as the no induction control of strains shown in **Fig 2b**.



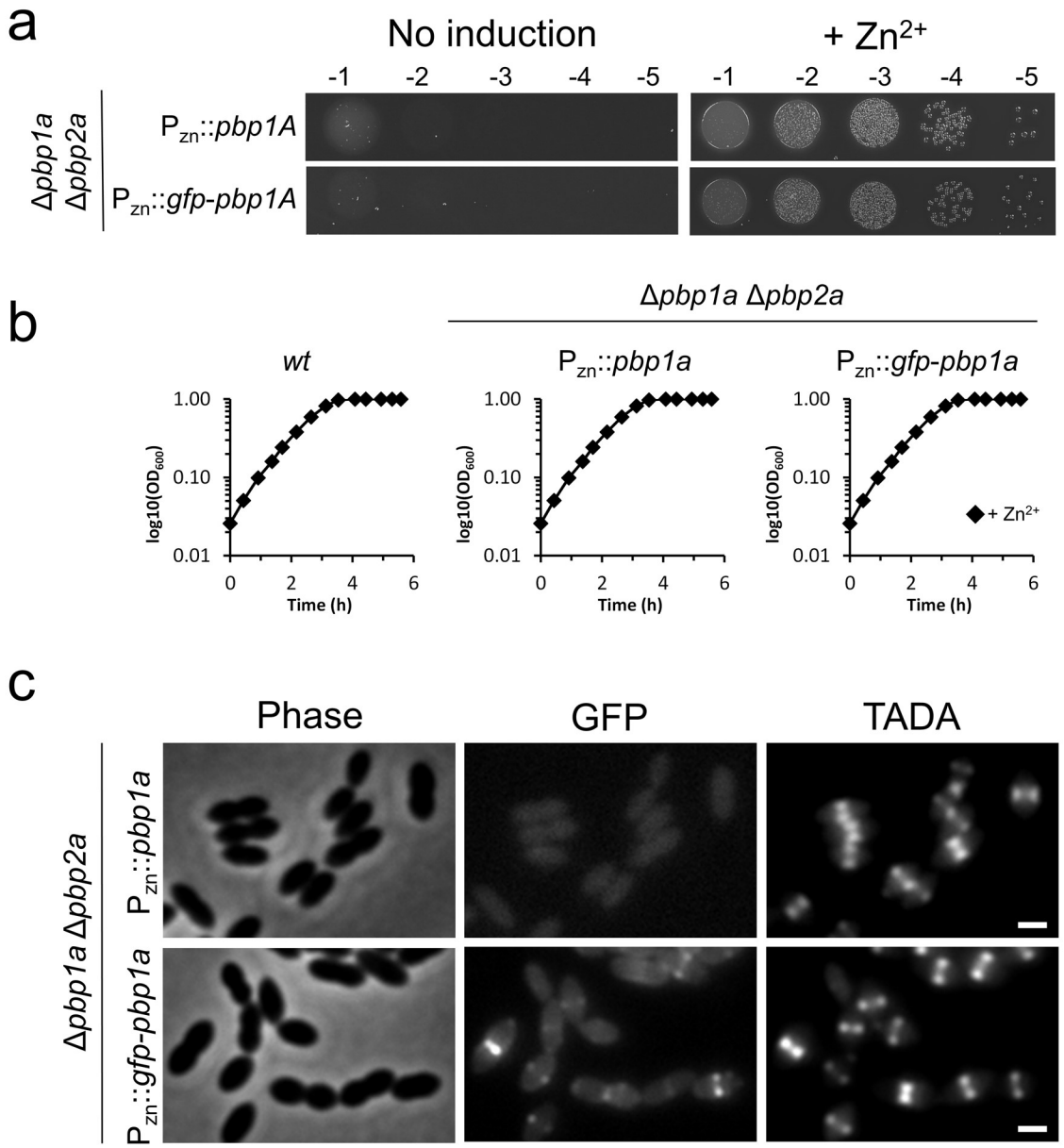
Supplementary Figure 7 | CozE and MreC are essential and show a lytic phenotype upon depletion.
a, Depletion of MreC or CozE in *pbp1a*$^{+}$ strains. Deletion strains of the essential genes *cozE* or *mreC* were constructed in strain backgrounds supplying a second copy of the ORF eptopically from a fucose inducible promoter. Strains were streaked on TSAII 5%SB overlay plates containing either 0.4% fucose (+ Fucose) or 0.4% sucrose to repress the promoter (No induction). Complementation with both native- and GFP-tagged CozE and MreC are shown. Plates were incubated at 37^{o}C in a 5% CO_2 cabinet and imaged. Representative images of two replicates are shown.
b, Aberrant fluorescent D-amino acid (TADA) incorporation in CozE and MreC depletion strains. Strains were grown to mid-exponential phase in THY containing 0.4% fucose, washed in THY and back diluted to an OD_{600} of 0.025 in fresh THY without fucose. Cultures were incubated at 37^{o}C in a 5% CO_2 cabinet for 3 h 45 min. TADA labelling was carried out for 15 min prior to imaging on 2% agarose pad. Scale bars = 1 µm. Phenotypes are similar to those resulting from P_{zn}::*pbp1A* or P_{zn}::*gfp-pbp1A* expression in Δ*cozE* or Δ*mreC* strains shown in **Fig. 2b** and **Fig. 3b**. Representative images of two replicates are shown.



Supplementary Figure 8 | PBP1a-induced lethality in the absence of CozE or MreC does not require the activity of the major autolysins LytA or CbpD.

a, Indicated *S. pneumoniae* strains were grown to mid-exponential phase and normalised to an OD_{600} of 0.2. Resulting cultures were serially diluted and 5 µl of each dilution spotted onto TSAII 5%SB plates in the presence or absence of 600 µM $ZnCl_2$. Plates were incubated at 37°C in a 5% CO_2 cabinet and imaged. Plate images are representative of four replicates.

b, Δ*cozE*, Δ*mreC* strains lacking major autolysins lyse upon induction of *pbp1a* from a zinc inducible promoter. Strains containing a zinc inducible *pbp1a* construct were grown in THY to mid-exponential phase. Cultures were diluted in fresh THY to an OD_{600} of 0.025 in the presence or absence of 600 µM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet. Where necessary cultures were further back diluted at 2 h to avoid high cell densities. At 4 h post-induction cells were removed and placed on THY 2% agarose pad and immediately imaged. Two representative images for the induced condition are shown, $n = 3$, scale bars = 3 µm. Uninduced cells have a morphology similar to *wt*, however cells expressing *pbp1a* show aberrant morphologies similar to those reported in **Fig. 2b**.

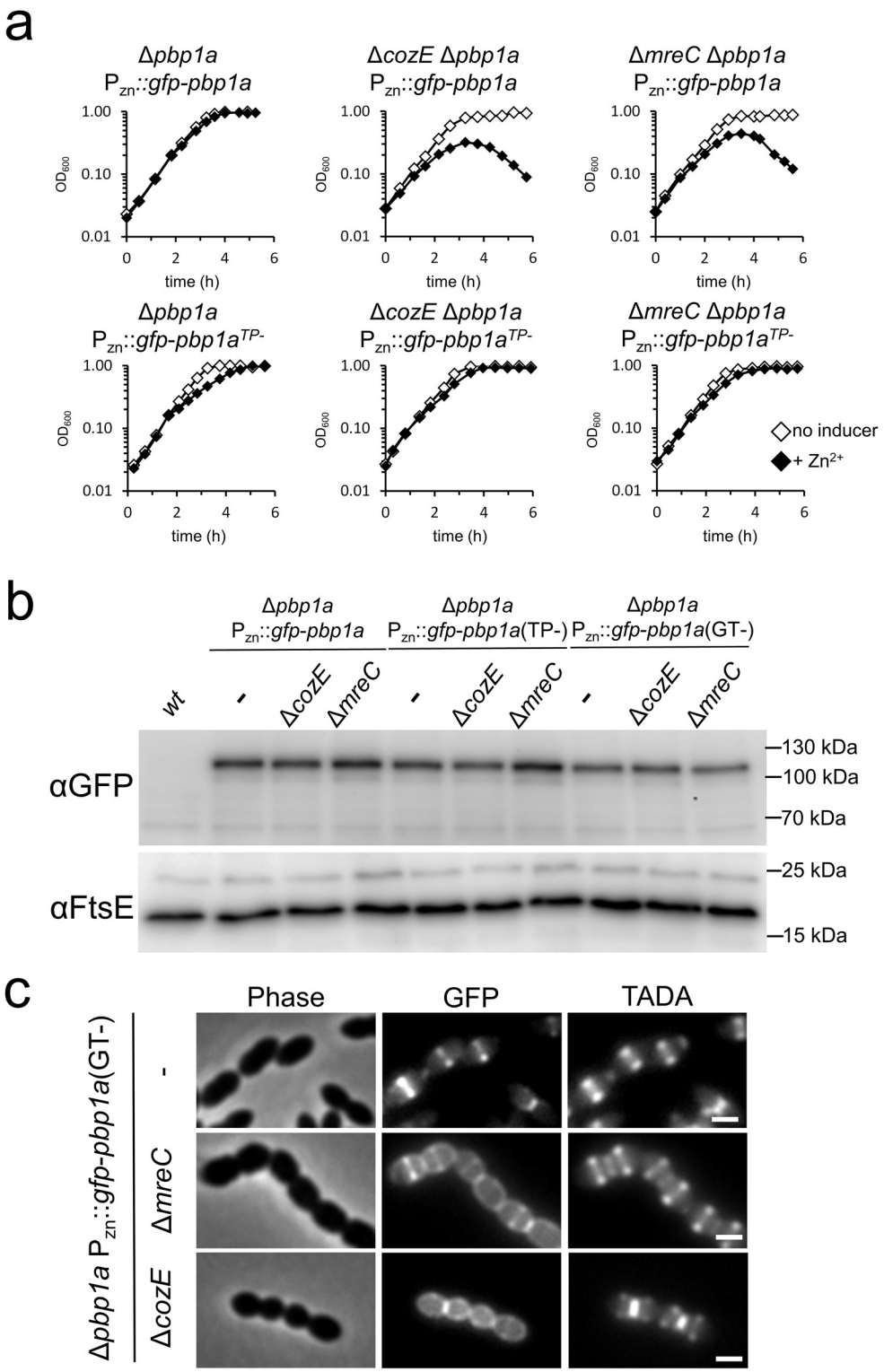


Supplementary Figure 9 | GFP-PBP1a fusion protein functionality assays.

a, Inducing the P_{zn}::*gfp-pbp1a* fusion can complement *pbp1a*/*pbp2a* synthetic lethality. The indicated S. pneumoniae strains were grown to exponential phase and normalised to an OD_{600} of 0.2. Resulting cultures were serially diluted and 5 µl of each dilution spotted onto TSAII 5%SB plates in the presence or absence of 200 µM $ZnCl_2$. Plates were incubated at 37°C in 5% CO_2 cabinet and imaged. Plate images are representative of two replicates.

b, The growth rate of cells expressing the GFP-PBP1a fusion as the only essential class A PBP enzyme are is indistinguishable from *wt*. Strains containing a zinc inducible pbp1A or *gfp-pbp1a* allele were grown in THY with 200 µM $ZnCl_2$ to mid-exponential phase. Cultures were diluted in fresh THY to an OD_{600} of 0.025 in the presence or absence of 200 µM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet. OD_{600} were recorded approximately every 30 min for 6 h. A wt growth curve in the presence of 200 µM $ZnCl_2$ is shown for reference. Growth curves are representative of at least six replicates.

c, GFP-PBP1a is localised to mid-cell. Strains were grown to mid-exponential phase in THY 200 µM $ZnCl_2$ at 37°C in a 5% CO_2 cabinet to mid-exponential phase. Cells were labelled with TADA for 15 min prior to imaging on 2% agarose pads. Representative images of two replicates are shown, scale bars = 1 µm.

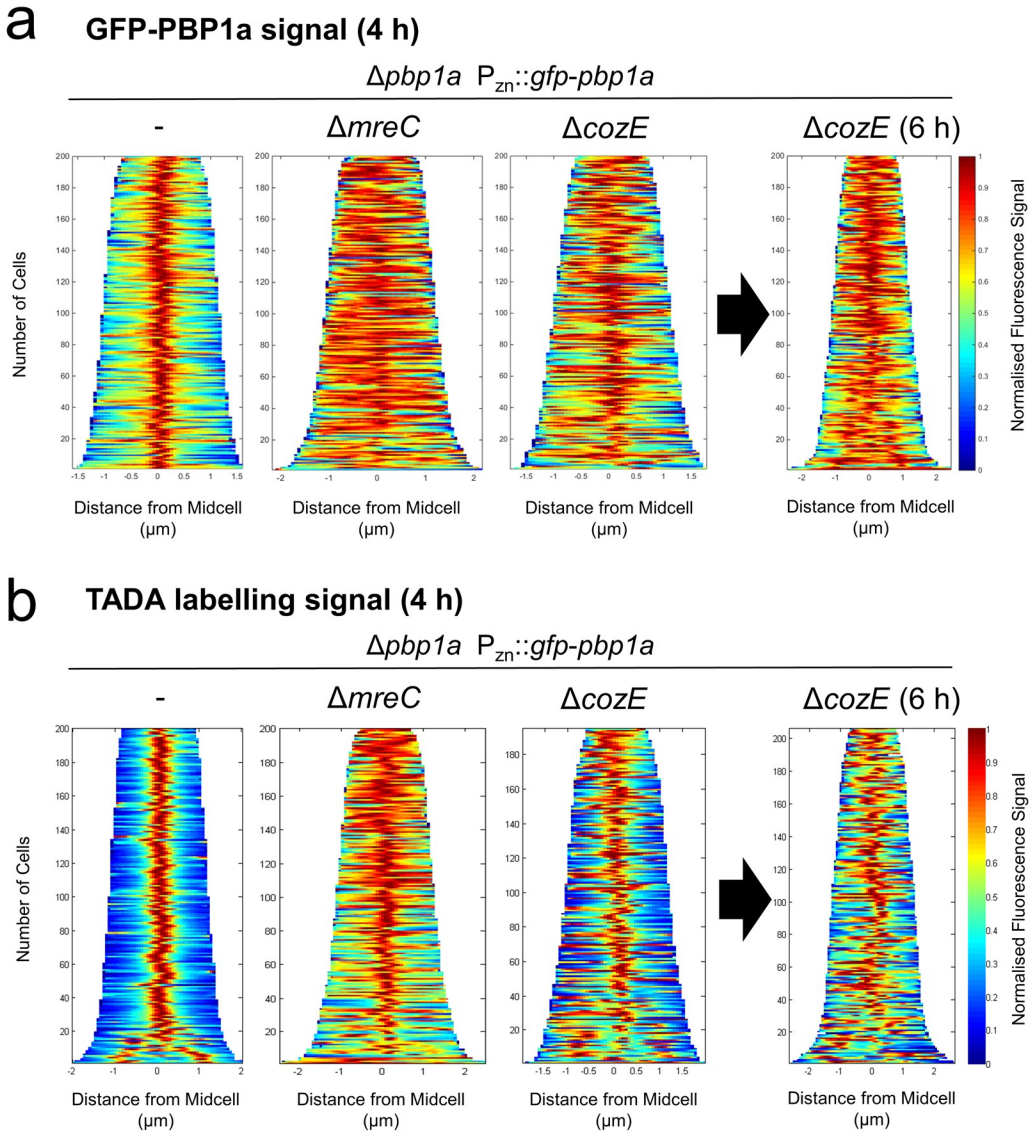


Supplementary Figure 10 | GFP-PBP1a activity in the absence of *cozE* and *mreC* results in growth defects and delocalised PG synthesis.

a, Growth curves for strains containing *gfp-pbp1a* constructs. Both *gfp* fused *pbp1a* wild type and catalytically inactive transpeptidase mutant constructs (TP-) were placed under the control of a zinc inducible promoter in Δ*cozE* or Δ*mreC* strain backgrounds. Strains were grown in THY to mid-exponential phase, diluted in fresh THY to an OD_{600} of 0.025 in the presence or absence of 600 μM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet. OD_{600} was recorded approximately every 30 min for 6 h. Growth curves are representative of two replicates.

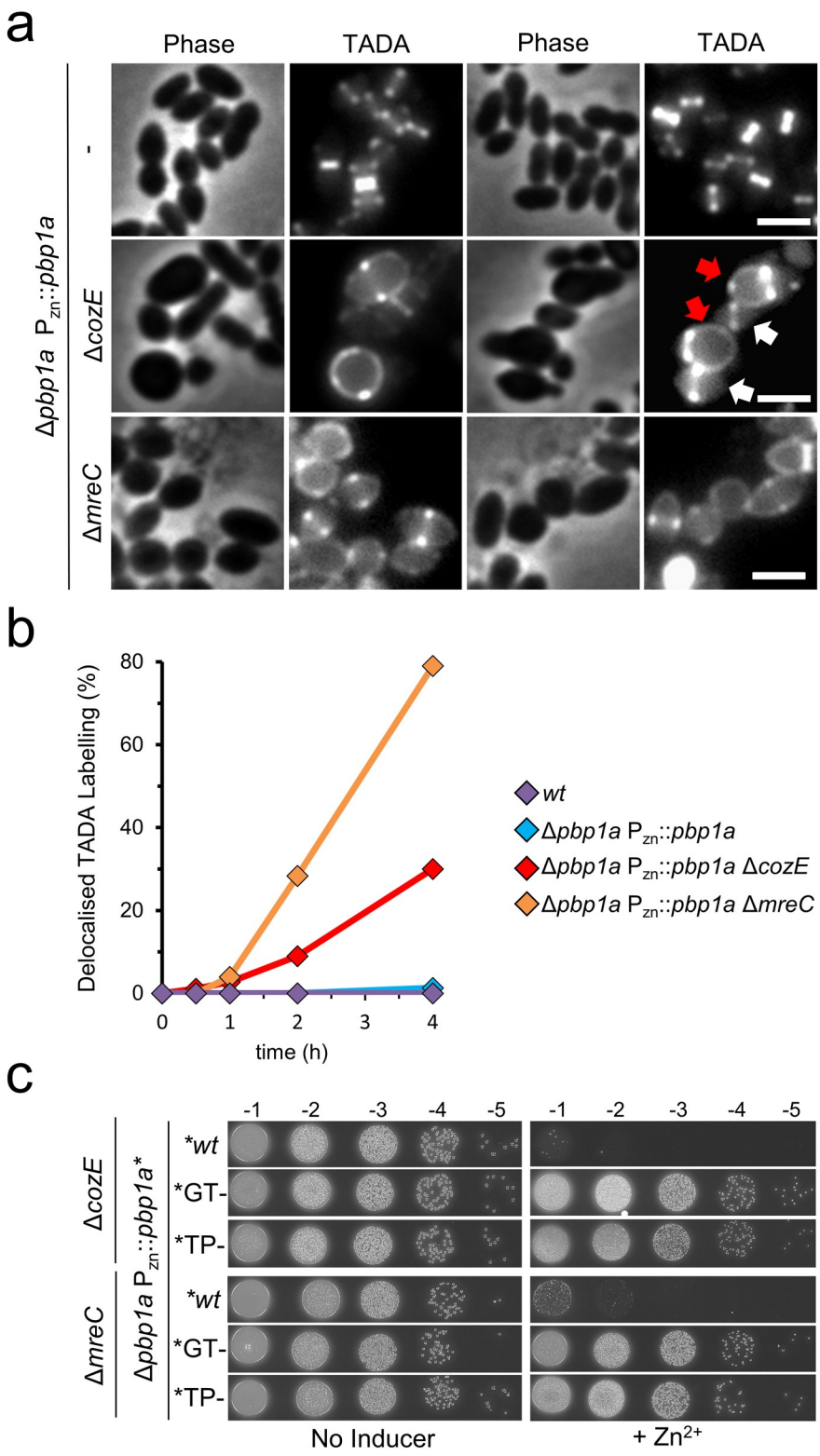
b, Immunoblot analysis of strains expressing *gfp-pbp1a* after 4 h of induction. Strains were grown to mid-exponential phase, back diluted to an OD_{600} of 0.025 in THY + 600 μM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet for 4 h. Where necessary cultures were further back diluted at 2 h to avoid high cell densities and autolysis. Cells were sampled 4 h after induction, making these blots consistent with microscopy images shown in **Fig. 3b**. Cultures were normalised to an OD_{600} of 0.3 immediately before lysis. Cell lysates were used for immunoblots using either affinity-purified anti-GFP antibodies or an anti-FtsE antibody used as a loading control. The anti-GFP blot image is representative two replicates, the anti-FtsE loading control was carried only on the preparations shown. Expected sizes of GFP-PBP1a = 107 kDa and FtsE = 26 kDa.

c, Glycosyltransferase defective GFP-PBP1a variants (GT-) are not retained at mid-cell in Δ*cozE* or Δ*mreC* strains but do not cause aberrant TADA incorporation similar to the transpeptidase mutant shown in **Fig. 3b**. Strains were grown to mid-exponential phase, back diluted to an OD_{600} of 0.025 in THY +600 μM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet for 3 h 45 min. Where necessary cultures were further back diluted at 2 h to avoid high cell densities and autolysis. Cells were labelled with TADA for 15 min prior to imaging on 2% agarose pads. Images are representative of two replicates, scale bars = 1 μm.

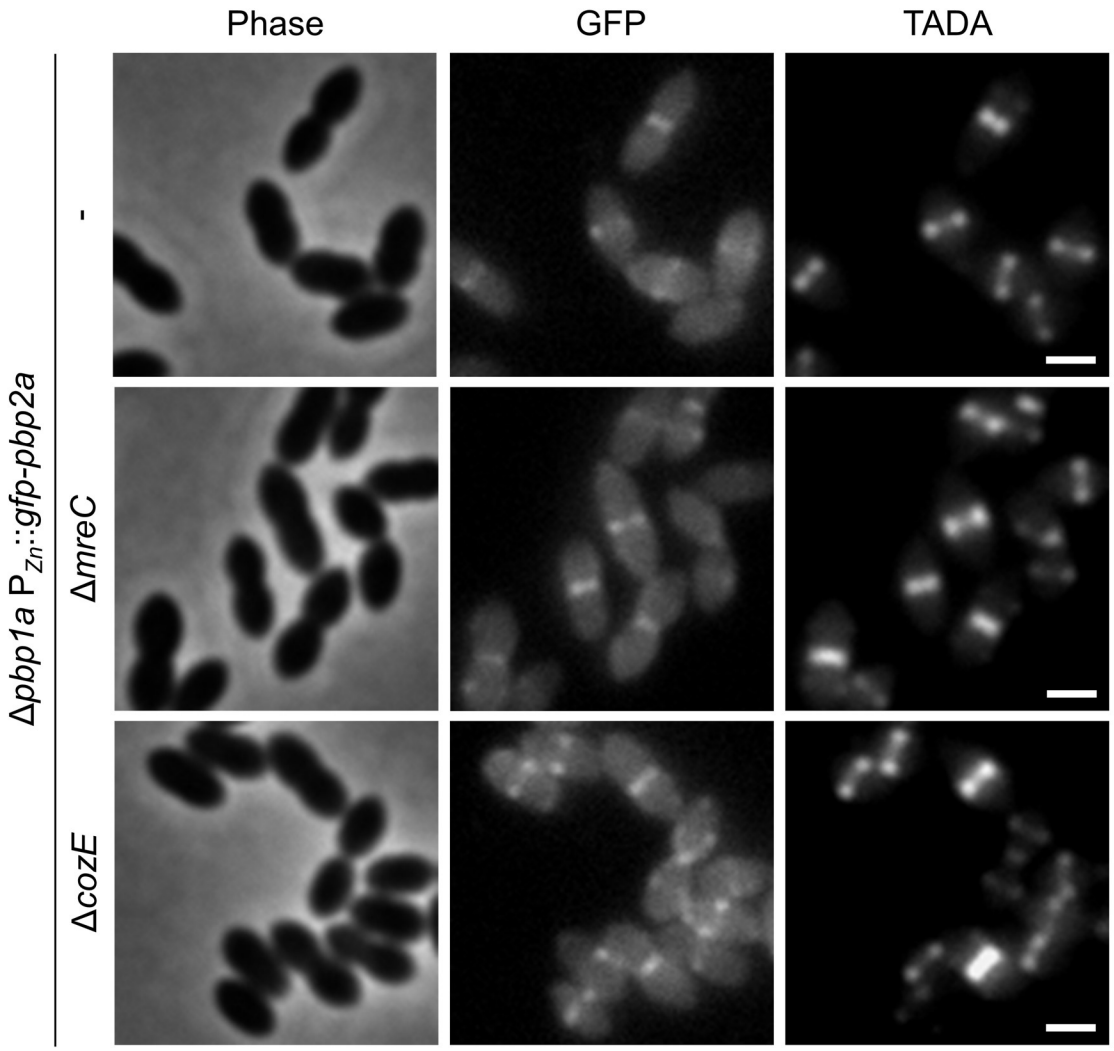


Supplementary Figure 11 | Demographs showing delocalised GFP-PBP1a and TADA labelling signals at 4 h post induction.

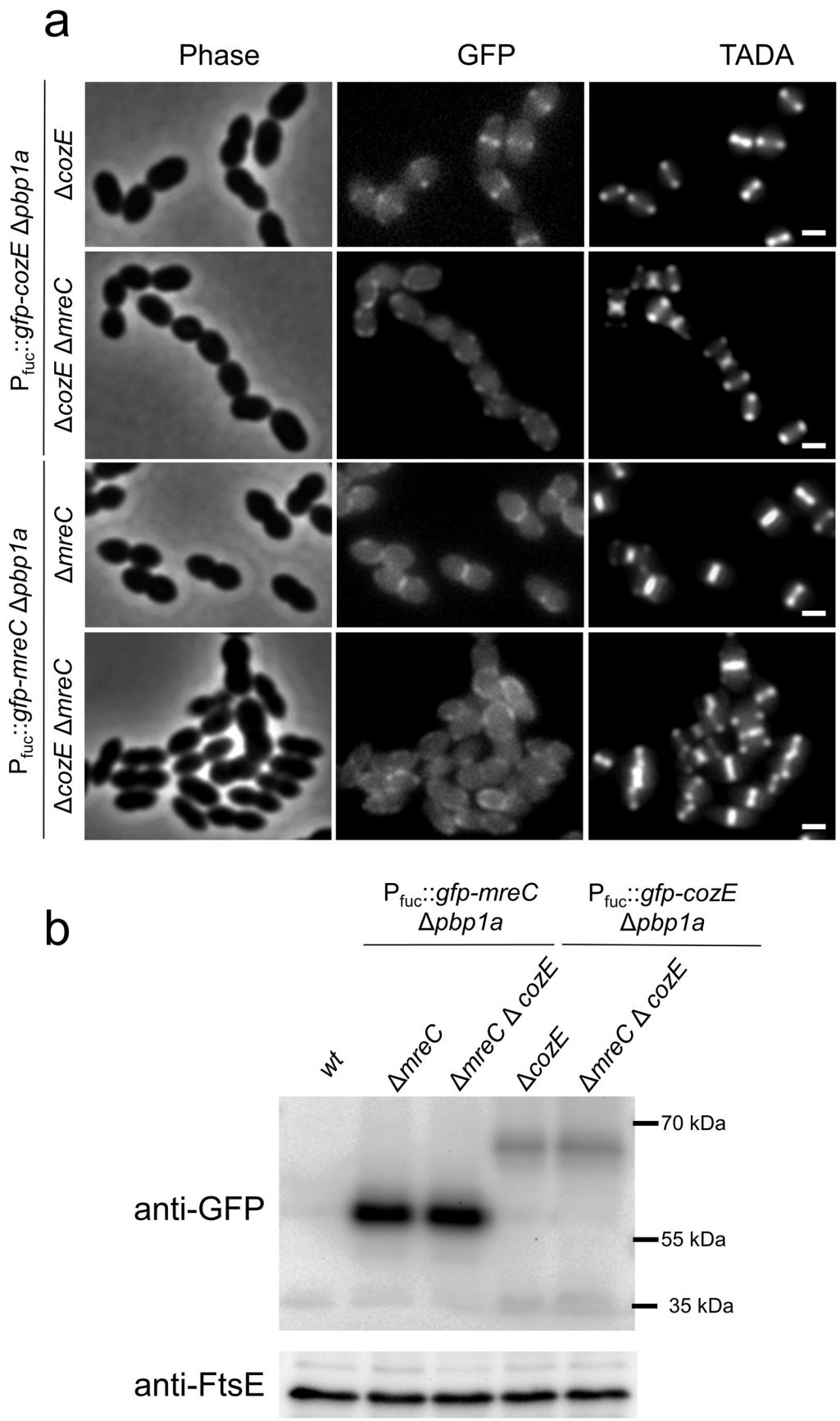
Demographs show aberrant GFP-PBP1a localisation and TADA incorporation patterns in Δ*cozE* and Δ*mreC* strains upon induction of P_{zn}::*gfp-pbp1a*. Strains were grown to mid-exponential phase, back diluted to an OD_{600} of 0.025 in THY + 600 μM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet for 3 h 45 min (and 5 h 45 min). Cells were labelled with TADA for 15 min prior to imaging on 2% agarose pads. Demographs shows normalised fluorescence signal profiles for single cells which have been integrated and collapsed to 1 pixel in width. Profiles are sorted by cell size and plotted. Demographs show delocalised GFP-PBP1a and TADA labelling in Δ*cozE* and Δ*mreC* cells compared to a control strain. Delocalisation is more pronounsed in Δ*mreC* backgrounds compared to Δ*cozE* at the 4 h timepoint verifying signal localisation quantifications shown in **Fig.3c**. However, signals continue to delocalise in the Δ*cozE* backgrounds showing more severe delocalisation pattern at 6 h. Fluorescence profiles for 200 cells are shown in each demograph. Demographs were constructed using the open-source software package Oufti [3].



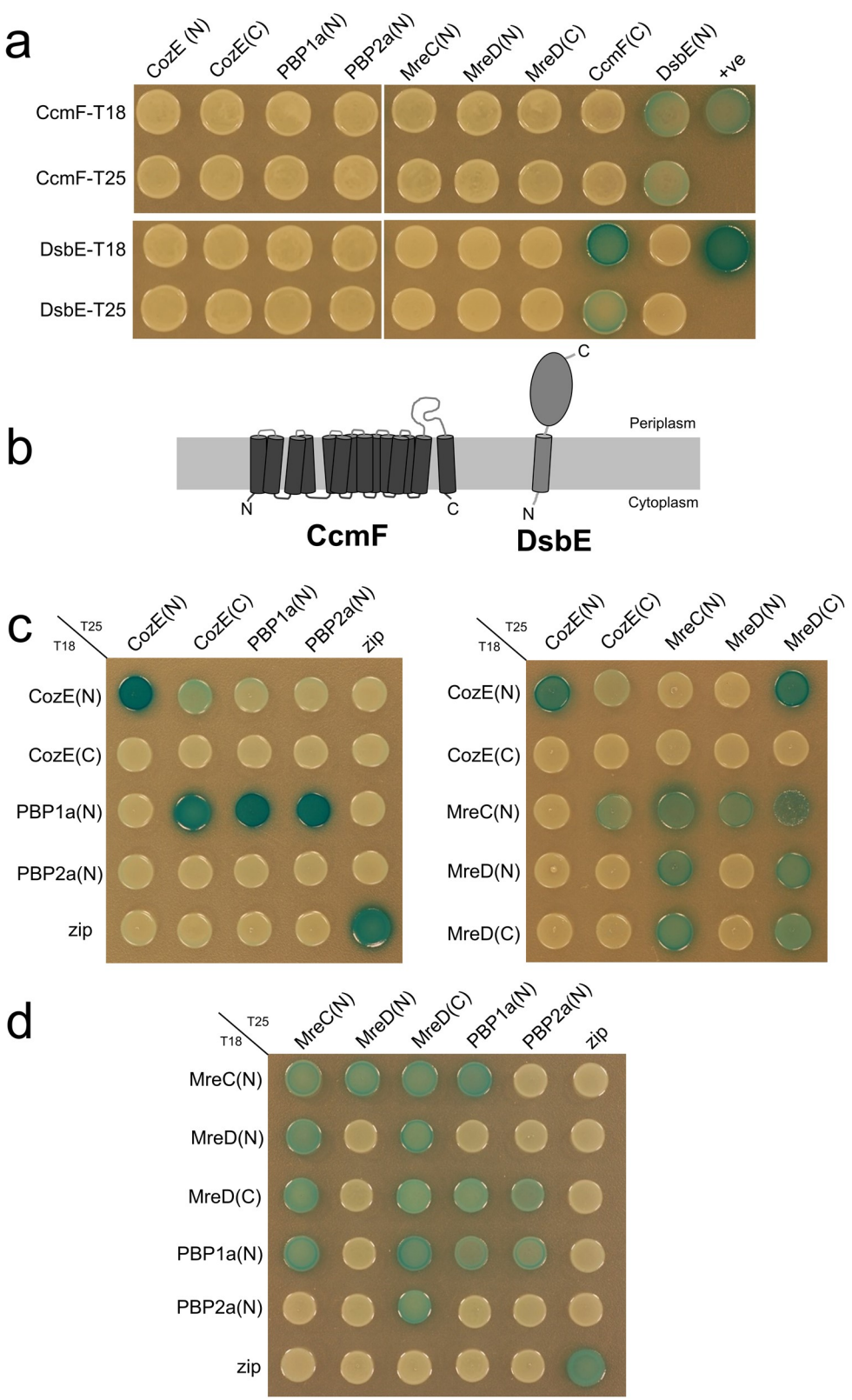
Supplementary Figure 12 | Untagged-PBP1a in the absence of CozE or MreC leads to delocalised TADA labelling.
a, Aberrant TADA incorporation in Δ*cozE* and Δ*mreC* cells expressing untagged *pbp1a*. Strains were grown to mid-exponential phase, back diluted to an OD_{600} of 0.025 in THY + 600 µM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet for 3 h 45 min. Where necessary cultures were further back diluted at 2 h to avoid high cell densities and autolysis. Cells were labelled with TADA for 15 min prior to imaging on 2% agarose pads. Two representative images for each strain are shown, *n* = 3, scale bars = 1 µm. Delocalised TADA labelling patterns shown here are very similar to those reported for the Δ*cozE* and Δ*mreC* cells producing GFP-PBP1a variants in **Fig. 3b**. Examples of mid-cell and delocalised TADA signals in a Δ*cozE* strain are highlighted with white and red arrows respectively.
b, Quantification for aberrant TADA incorporation patterns in Δ*cozE* and Δ*mreC* strains upon induction of P_{zn}::*pbp1a*. Cultures were grown and treated exactly as described in (**a**).
Cells were imaged and scored for localization/incorporation at the indicated time. Each cell in the joined diplococcus was treated as a single cell unit and scored as wt mid-cell localized signal or delocalized signal (examples of which are indicated by arrows in **a**). The percentage of cells with delocalized signal for TADA or GFP-PBP1a localisation in the indicated strains are shown. >700 cell units were scored per time point, n = 2. Quantification of TADA incorporation patterns shown here are very similar to those reported in **Fig. 3c**.
c, The indicated S. pneumoniae strains were grown to exponential phase and normalised to an OD_{600} of 0.2. Resulting cultures were serially diluted and 5 µl of each dilution spotted onto TSAII 5%SB plates in the presence or absence of 600 µM $ZnCl_2$. Plates were incubated at 37°C in 5% CO_2 cabinet and imaged. Plates show PBP1a GT- and TP- variants are not lethal in Δ*cozE* and Δ*mreC* strains. Representative images of two replicates are shown. These results are identical to those shown in **Fig. 3c** when GFP tagged PBP1a variants are expressed in Δ*cozE* and Δ*mreC* strains.



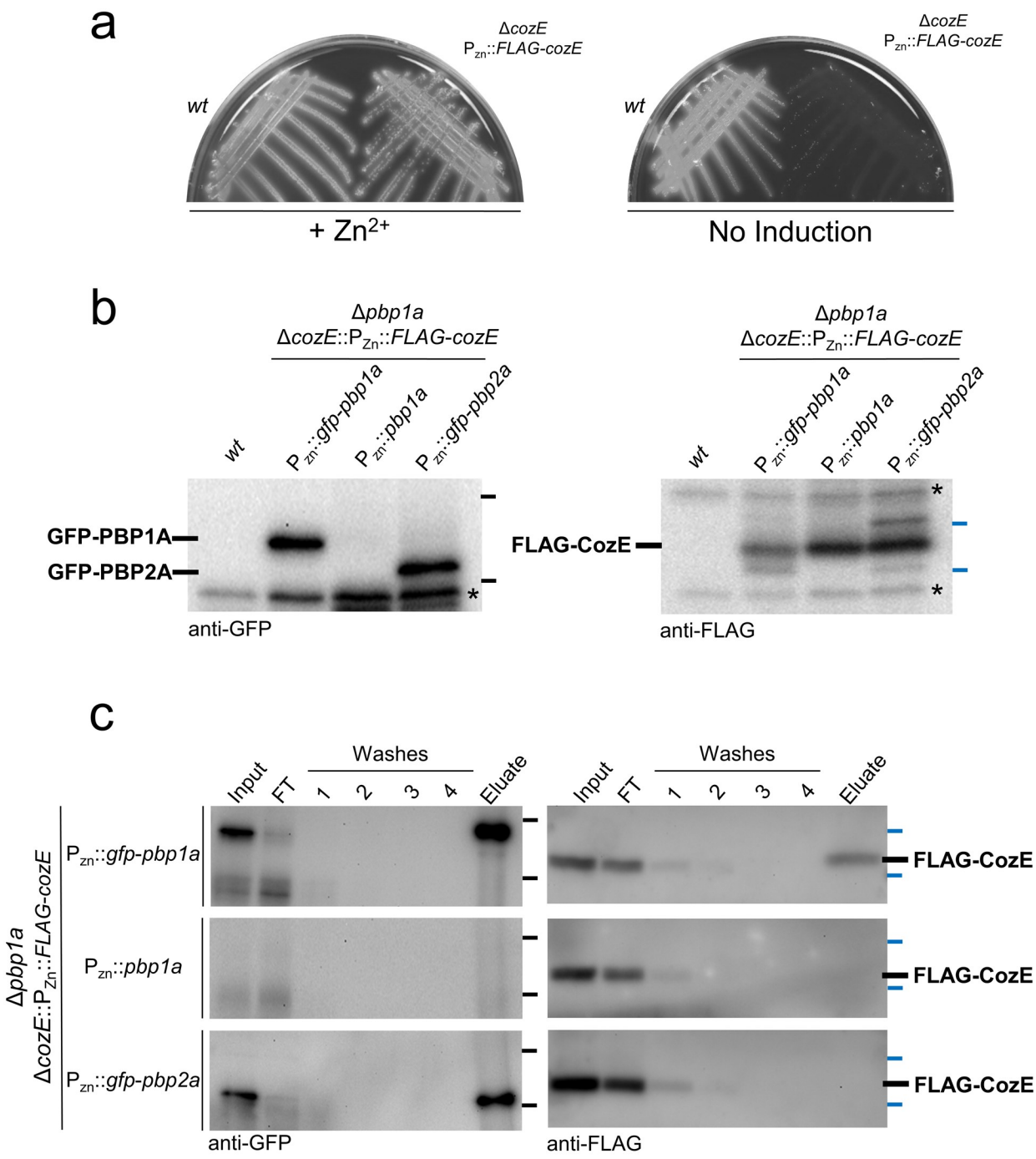
Supplementary Figure 13 | Mid-cell localisation of GFP-PBP2a in the absence of CozE and MreC.
Strains were grown to mid-exponential phase, back diluted to an OD_{600} of 0.025 in THY + 600 µM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet for 3 h 45 min. Where necessary cultures were further back diluted at 2 h to avoid high cell densities and autolysis. Cells were labelled with TADA for 15 min prior to imaging on 2% agarose pads. Representative images are shown, n = 2, scale bars = 1 µm.



Supplementary Figure 14 | Mid-cell localisation of GFP-CozE requires *mreC*.
a, Midcell localisation of GFP-CozE and GFP-MreC is lost in double Δ*cozE* Δ*mreC* mutants. Strains were grown to mid-exponential phase in THY 0.4% fucose at 37°C in a 5% CO_2 cabinet. Cells were labelled with TADA for 15 min prior to imaging on 2% agarose pads. GFP-CozE and GFP-MreC localise at midcell to sites of active PG synthesis (suggested by the TADA labelling). In both cases localisation is lost in the double mutant: GFP-CozE forms delocalised spots at the cell periphery and GFP-MreC adopts a dispersed pattern which is typically more intense at one cell pole. GFP-CozE and GFP-MreC fusion proteins are functional as they suppress Δ*cozE* and Δ*mreC* essentiality (**Supplementary Fig. 7a**). Representative images for each strain are shown, *n* = 2, scale bars = 1 μm.
b, Immunoblot analysis of strains expressing *gfp-cozE* and *gfp-mreC*. Strains were grown to mid-exponential phase in THY + 0.4% fucose and incubated at 37°C in a 5% CO_2 cabinet for 4-6 h. Cultures were normalised to an OD_{600} of 0.3 immediately before lysis. Cell lysates were used for immunoblots using either affinity-purified anti-GFP antibodies or an anti-FtsE antibody used as a loading control. Blot image is representative two biological replicates. Expected molecular weight of GFP-CozE = 68 kDa and GFP-MreC = 56 kDa. Protein fusions are not degraded and expressed to the same level regardless of strain background, suggesting the aberrant localisation patterns observed in (**a**) are not due to partial degradation of the protein fusion.



Supplementary Figure 15 | Bacterial two hybrid controls. BTH101 *E. coli* cells containing plasmids expressing T18- and T25-protein fusions were grown to stationary phase in LB containing: Amp 50 μg ml^{-1}, Kan 25 μg ml^{-1} and IPTG 500 μg ml^{-1}. 5 μl of stationary phase culture was spotted onto LB agar containing: Amp 50 μg ml^{-1}, Kan 25 μg ml^{-1} and X-gal 40 μg ml^{-1}. Plates were incubated at 30^{o}C and imaged. The terminus used for 'T25' or 'T18' protein fusion to *S. pneumoniae* proteins are shown in parenthesis, (N) = N-terminal or (C) = C-terminal. **a,** Both DsbE and CcmF *E. coli* fusion proteins were labelled at the C-terminus. All fusion-proteins tested in this study are putative membrane proteins, therefore false positive signals could arise through artificial concentration of partner proteins constrained in space by the membrane. This control plate tests all protein fusions against two *E. coli* membrane protein fusions: DsbE, a transmembrane thiol:disulphide oxidoredutase enzyme and CcmF, a multi-pass inner membrane protein predicted to interact with DsbE (see diagram in **Fig. 15b**). Representative image are shown of two biological replicates. No interaction is expected between these protein fusions and therefore they serve as a negative control for the bacterial two-hybrid results shown in **Fig. 4b**. However these proteins interact with each other showing both DsbE and CcmF fusion proteins are expressed in these cells. **b,** Schematic of the DsbE and CcmF bacterial two hybrid negative controls. The output files of the Phobius web server were used to draw the schematic of the membrane topology of the control proteins used in (**a**). (http://phobius.sbc.su.se/). **c,** Plates show bacterial two-hybrid interactions of CozE with known members of the PG biosynthetic complex. Plates show CozE fusions labelled at both termini with the class A PBP enzymes and MreCD proteins. Representative image are shown of at least three biological replicates. Plates show every combination and serves as a control and additional verification of the two-hybrid data shown in **Fig. 4b**. **d,** Plates show bacterial two hybrid interactions of MreCD with the synthetic lethal Class A PBP enzymes. Representative image are shown of at least three biological replicates. Plates show every combination and serves as a control and additional verification of the two-hybrid data shown in **Fig. 4b**.



Supplementary Figure 16 | GFP-PBP1a, FLAG-CozE co-Immunoprecipitation controls.
a, The P_{zn}::*FLAG-cozE* fusion can complement a *cozE* deletion in the presence of zinc. A *cozE* deletion strain was constructed in strain backgrounds expressing the *FLAG-cozE* fusion ectopically from a zinc inducible promoter. Strains were streaked on TSAII 5%SB overlay plates with either 200 µM $ZnCl_2$ or without (No Induction). Plates were incubated at 37°C in a 5% CO_2 cabinet and imaged. Plate image is representative of three replicates. Plates indicate the FLAG-CozE fusion protein is functional.
b, Antibody specificity tests on whole cell lysates used for co-immunoprecipitation experiments. Strains were grown to mid-exponential phase in THY + 400 µM $ZnCl_2$ and incubated at 37°C in a 5% CO_2 cabinet for 4-6 h. Cultures were normalised to an OD_{600} of 0.3 immediately before lysis. Cell lysates were used for immunoblots using monoclonal anti-GFP antibody or anti-FLAG antibody. Expected molecular weight of GFP-PBP1a = 125 kDa and GFP-PBP2a = 108 kDa. The FLAG-CozE fusion runs at ≈ 30 kDa. Blot image is representative of a single experiment. The position of protein markers are indicated and non-specific bands are highlighted with an asterisk (*).The position of protein markers are indicated by short marks to the right of the blot: 130 kDa and 100 kDa are shown in in black and 35 kDa and 25 kDa are indicated in blue.
c, GFP-PBP1a interacts with FLAG-CozE in a co-immunoprecipitation assay. Indicated strains were grown and harvested in the presence of 400 µM $ZnCl_2$ and their membranes solubilised in 0.5% Digitonin. Solubilised membranes were passed over an anti-GFP sepharose resin and incubated for 4 h allowing the binding of the target GFP-fusion protein. The protein-bound resin was washed four times and bound proteins eluted in SDS-PAGE sample buffer. Proteins in complex with the GFP-fusion will be retained by the resin and therefore be detectable in the eluate fraction. Fractions were analysed by immunoblot using monoclonal anti-GFP antibodies or monoclonal anti-FLAG antibodies. Analysis reveals PBP1a specifically pulls down CozE, as a FLAG-CozE fusion can be detected in the GFP-PBP1a co-IP eluate but is not present in the GFP-PBP2a or PBP1a controls. Representative blots are shown, *n* = 4. The position of protein markers are indicated by short marks to the right of the blot: 130 kDa and 100 kDa are shown in in black and 35 kDa and 25 kDa are indicated in blue.

Figure 4c

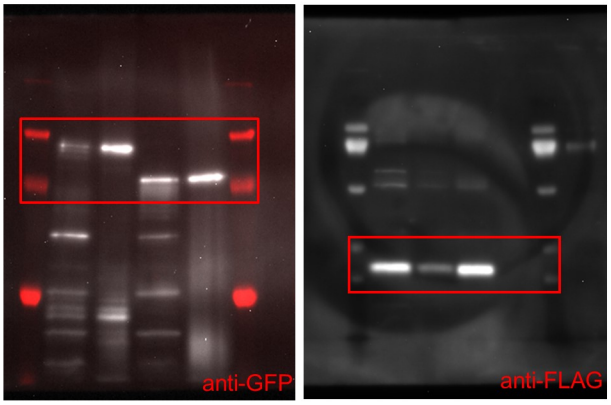


Figure S14b

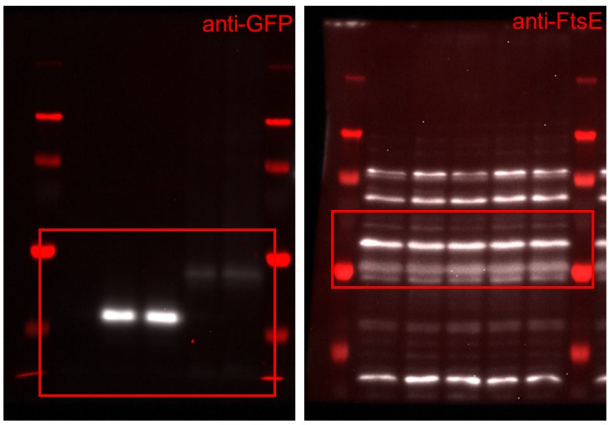


Figure S16b

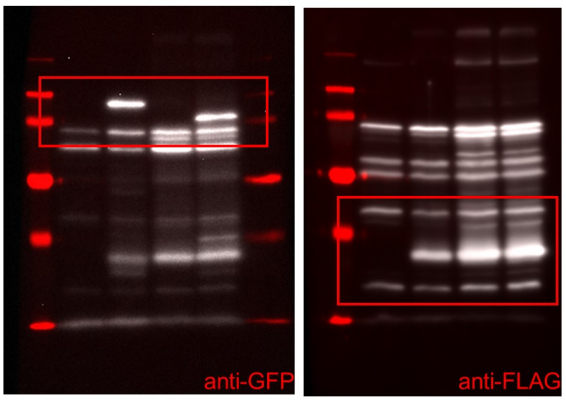


Figure S16c

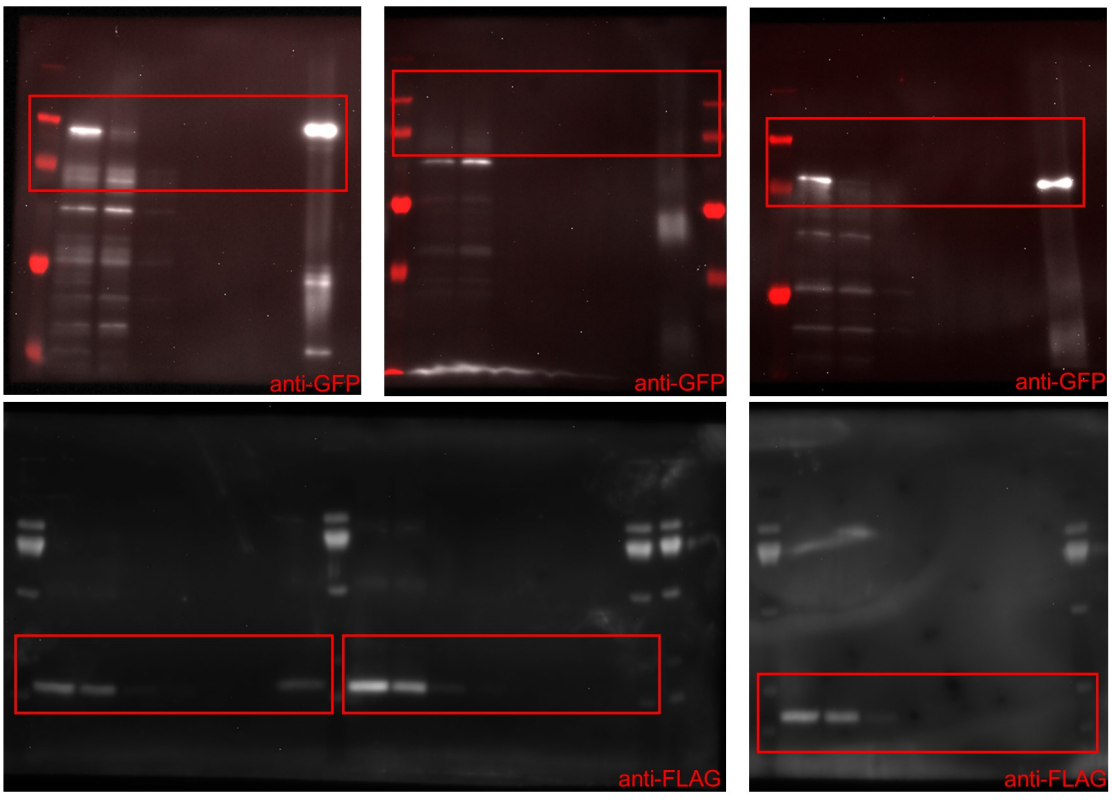
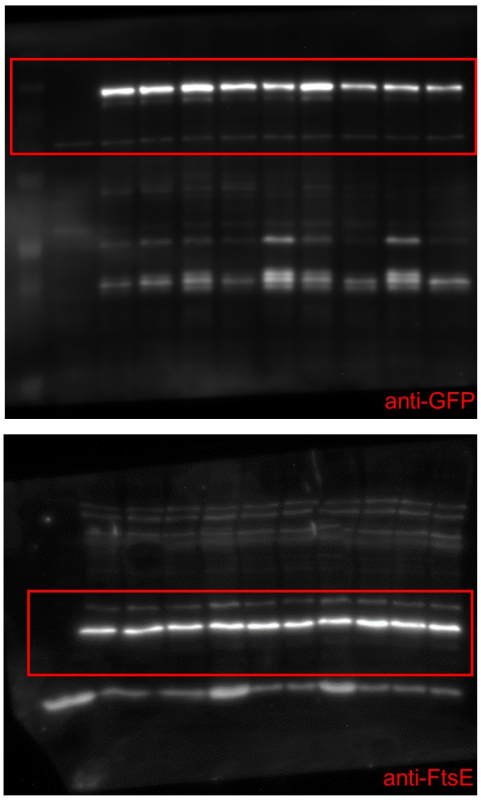


Figure S10b



Supplementary Figure 17 | Full Images of Immunoblots blots used in this study
Regions used to generate figures are indicated by a red box

Supplementary Table 1 | *S. pneumoniae* strains used in this study

Strain Name	Genotype	Resistance Marker(s)	Source or Reference
D39	[Type Strain]	-	Lanie *et al* 2007 ([17])
D39 Δ*cps*	Δ*cps2A'*-Δ*cps2H'* [Wild Type]	-	Lanie *et al* 2007 ([17])
R6	non-pathogenic domesticated laboratory strain, derivative of D39	-	([17,31,32]) Vernet Lab
AKF_Spn001	Δ*bgaA::kan*	Kan	This Study
AKF_Spn002	Δ*bgaA::add9*(*spec*)	Spec	This Study
AKF_Spn003	Δ*bgaA::tetM*(*tet*)	Tet	This Study
AKF_Spn004	Δ*bgaA::cat*	Cam	This Study
AKF_Spn005	Δ*bgaA::erm*	Erm	This Study
AKF_Spn009	Δ*pbp1a*::*kan*	Kan	This Study
AKF_Spn011	Δ*pbp2a*::*erm*	Erm	This Study
AKF_Spn042	Δ*pbp1a*::*kan* Δ*cozE*::*spec*	Kan, Spec	This Study
AKF_Spn277	Δ*pbp1a*::*kan* Δ*cozE*::*erm*	Kan,	This Study
AKF_Spn040	Δ*pbp1a*::*kan* Δ*mreC*::*spec*	Kan, Spec	This Study
AKF_Spn075	Δ*pbp1a*::*kan* Δ*mreD*::*spec*	Kan, Spec	This Study
AKF_Spn388	Δ*pbp1a*::*kan* Δ*mreCD*::*spec*	Kan, Spec	This Study
AKF_Spn279	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*cozE*::*erm*	Kan, Spec, Erm	This Study
AKF_Spn024	Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Tet	This Study
AKF_Spn036	Δ*pbp1a*::*kan* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Tet	This Study
AKF_Spn343	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*:: (P_{zn}::*pbp1a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn346	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*:: (P_{zn}::*pbp1a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn412	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*cozE*::*erm* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Spec, Erm, Tet	This Study
AKF_Spn082	Δ*pbp1a*::*kan* Δ*mreD*::*spec* Δ*bgaA*:: (P_{zn}::*pbp1a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn396	Δ*pbp1a*::*kan* Δ*mreCD*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Spec, Tet	This Study

Supplementary Table 1 | *S. pneumoniae* strains used in this study (*continued*)

Strain Name	Genotype	Resistance Marker(s)	Source or Reference
AKF_Spn538	R6: Δ*cozE*::*spec*	Spec,	This Study
AKF_Spn540	R6: Δ*mreC*::*spec*	Spec,	This Study
AKF_Spn534	R6: Δ*pbp1a*::*kan* Δ*cozE*::*spec*	Kan, Spec,	This Study
AKF_Spn542	R6: Δ*pbp1a*::*kan* Δ*mreC*::*spec*	Kan, Spec,	This Study
AKF_Spn550	R6: Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn548	R6: Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(E91A, E150A)[GT-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn596	R6: Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a*^{R6}, *tet*)	Kan, Spec, Tet	This Study
AKF_Spn552	R6: Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn554	R6: Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(E91A, E150A)[GT-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn556	R6: Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a*^{R6}, *tet*)	Kan, Spec, Tet	This Study
AKF_Spn391	Δ*bgaA*::(P_{zn}::*gfp-pbp1a, tet*)	Tet	This Study
AKF_Spn452	Δ*pbp1a*::*kan* Δ*bgaA*::(P_{zn}::*gfp-pbp1a, tet*)	Kan, Tet	This Study
AKF_Spn400	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn393	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn482A	Δ*pbp1a*::*kan* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(E91A, E150A)[GT-], *tet*)	Kan, Tet	This Study

Supplementary Table 1 | *S. pneumoniae* strains used in this study (*continued*)

Strain Name	Genotype	Resistance Marker(s)	Source or Reference
AKF_Spn486	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(E91A, E150A)[GT-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn484	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(E91A, E150A)[GT-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn480A	Δ*pbp1a*::*kan* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(S370A) [TP-], *tet*)	Kan, Tet	This Study
AKF_Spn477	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(S370A) [TP-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn479	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*gfp-pbp1a*(S370A) [TP-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn367	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a*(E91A, E150A)[GT-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn359	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a*(E91A, E150A)[GT-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn365	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a*(S370A) [TP-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn357	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{zn}::*pbp1a*(S370A) [TP-], *tet*)	Kan, Spec, Tet	This Study
AKF_Spn641	Δ*pbp1a*::*kan* Δ*bgaA*::(P_{Zn}::*gfp-pbp2a*, *tet*)	Kan, Spec, Tet	This Study
AKF_Spn643	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*bgaA*::(P_{Zn}::*gfp-pbp2a*, *tet*)	Kan, Spec, Tet	This Study
AKF_Spn645	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*bgaA*::(P_{Zn}::*gfp-pbp2a*, *tet*)	Kan, Spec, Tet	This Study
AKF_Spn402	Δ*bgaA*::(P_{fucose}::*cozE*, *tet*)	Tet	This Study
AKF_Spn408	Δ*bgaA*::(P_{fucose}::*gfp-cozE*, *tet*)	Tet	This Study

Supplementary Table 1 | *S. pneumoniae* strains used in this study (*continued*)

Strain Name	Genotype	Resistance Marker(s)	Source or Reference
AKF_Spn283	Δ*bgaA*::(P_{fucose}::*mreC*, *tet*)	Tet	This Study
AKF_Spn406	Δ*bgaA*::(P_{fucose}::*gfp-mreC*, *tet*)	Tet	This Study
AKF_Spn296	Δ*mreC*::*spec* Δ*bgaA*::(P_{fucose}::*mreC*, *tet*)	Spec,Tet	This Study
AKF_Spn328	Δ*mreC*::*spec* Δ*bgaA*::(P_{fucose}::*gfp-mreC*, *tet*)	Spec,Tet	This Study
AKF_Spn422	Δ*cozE*::*spec* Δ*bgaA*::(P_{fucose}:*cozE*, *tet*)	Spec,Tet	This Study
AKF_Spn442	Δ*cozE*::*spec* Δ*bgaA*::(P_{fucose}:*gfp-cozE*, *tet*)	Spec,Tet	This Study
AKF_Spn351	Δ*lytA*::*cat*	Tet	This Study
AKF_Spn385	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*lytA*::*cat* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Erm, Tet, Spec, Cam	This Study
AKF_Spn446	Δ*pbp1a*::*kan* Δ*cozE*::*spec* Δ*lytA*::*cat* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Erm, Tet, Spec, Cam	This Study
AKF_Spn382	Δ*cbpD*::*cat*	Tet	This Study
AKF_Spn410	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*cbpD*::*cat* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Erm, Tet, Spec, Cam	This Study
AKF_Spn448	Δ*pbp1a*::*kan* Δ*mreC*::*spec* Δ*cbpD*::*cat* Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Erm, Tet, Spec, Cam	This Study
AKF_Spn638	Δ*pbp1a*::*kan* Δ*cozE*::(P_{zn}::*FLAG*(3X)-*cozE, spec*)	Kan, Spec	This Study
AKF_Spn_653	Δ*pbp1a*::*kan* Δ*cozE*::(P_{zn}::*FLAG*(3X)-*cozE, spec*) Δ*bgaA*::(P_{zn}::*gfp-pbp1a, tet*)	Kan, Spec, Tet	This Study

Supplementary Table 1 | *S. pneumoniae* strains used in this study (*continued*)

Strain Name	Genotype	Resistance Marker(s)	Source or Reference
AKF_Spn_655	Δ*pbp1a*::*kan* Δ*cozE*::(P_{zn}::*FLAG*(3X)-*cozE, spec*) Δ*bgaA*::(P_{zn}::*gfp-pbp2a, tet*)	Kan, Spec, Tet	This Study
AKF_Spn_664	Δ*pbp1a*::*kan* Δ*cozE*::(P_{zn}::*FLAG(*3X)-*cozE, spec*) Δ*bgaA*::(P_{zn}::*pbp1a, tet*)	Kan, Spec, Tet	This Study

The D39 Δ*cps* genotype (Δ*cps2A'*-Δ*cps2H'*) was excluded from derivative strains for clarity. Strains are arranged as first introduced into the manuscript. Cam = chloramphenicol, Erm = erythromycin, Kan = kanamycin, Spec = spectinomycin, Tet = tetracycline. [GT-] and [TP-] denote putative *pbp1a* constructs lacking either glycosyltransferase or transpeptidase activity respectively.

Supplementary Table 2 | *E. coli* strains used in this study

Strain Name	Genotype	Resistance Marker(s)	Source or Reference
BTH101	F- *cya*-99 *ara*D139 *gal*E15 *gal*K16 *rps*L1 *hsd*R2 *mcr*A1 *mcr*B1	-	Euromedex
DH5α	F- hsdR17, Δ*(argF-lacZ)U169 phoA glnV44 Φ80dlacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 supE44 deoR*	-	Gibco BRL

Supplementary Table 3 | Plasmids used in this study

Name	Genotype	Replicon	Resistance Marker(s)	Source or Reference
pAC1000	5' malP::rlrA::cat::malM	pBR322	Cat	Hava *et al* 2003 ([35])
pAKF200	*bgaA*'::P_{zn}::*pbp2a*::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF201	*bgaA*'::P_{zn}::*pbp1a*::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF205	*bgaA*'::P_{fucose}::*tetM*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF207	*bgaA*'::P_{fucose}::*mreCD*::*tetM*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF208	*bgaA*'::P_{fucose}::*cozE*::*tetM*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF212	*bgaA*'::P_{zn}::*pbp1a*(S370A)::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF213	*bgaA*'::P_{zn}::*pbp1a*(E91A, E150A)::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF214	*bgaA*'::P_{zn}::*gfp-pbp1a*::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF215	*bgaA*'::P_{fucose}::'TTG'-*cozE*::*tetM*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF217	*bgaA*'::P_{fucose}::*gfp-mreCD* ::*tetM*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF218	*bgaA*'::P_{fucose}::*gfp-cozE* ::*tetM*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF221	*bgaA*'::P_{fucose}::'TTG'-*gfp-cozE* ::*tetM*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF222	*bgaA*'::P_{zn}::*gfp-pbp1a*(S370A) ::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF223	*bgaA*'::P_{zn}::*gfp-pbp1a*(E91A, E150A)::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF227	*bgaA*'::P_{zn}::*FLAG-cozE*::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pAKF228	*bgaA*'::P_{zn}::*gfp-pbp2a*::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
pDR240	*cwlH*::P_{amiA}::*kan*::*yqeD, bla* (Janus cassette[36] is the original source of *kan* marker)	pACYC	Kan, Amp	Cloned by Harvey Kimsey

Supplementary Table 3 | Plasmids used in this study (*continued*)

Name	Genotype	Replicon	Resistance Marker(s)	Source or Reference
pDR242	*cwlH*::P_{pe}::*erm*::*yqeD bla*	pACYC	Erm, Amp	Cloned by Harvey Kimsey
pJWV025	*bgaA*'::P_{czc}::*gfp*, *tetM*::*spr0564' bla*	pBR322	Tet, Amp	Eberhardt *et al* 2009 ([37])
pKNT25	P_{lac}::-*T25, kan*	pACYC	Kan	Karimova *et al* 1998 ([22])
pKNT25-*mreD*	P_{lac}::*mreD*(*S.pn*)-*T25 kan*	pACYC	Kan	This study
pKNT25-*ccmF*	P_{lac}::*ccmF-T25 kan*	pACYC	Kan	This study
pKNT25-*cozE*	P_{lac}::*cozE*(*spd0768*)-*T25 kan*	pACYC	Kan	This study
pKT25	P_{lac}::*T25- kan*	pACYC	Kan	Karimova *et al* 1998 ([22])
pKT25-*mreC*	P_{lac}::*T25-mreC*(*S.pn*) *kan*	pACYC	Kan	This study
pKT25-*mreD*	P_{lac}::*T25-mreD*(*S.pn*) *kan*	pACYC	Kan	This study
pKT25-*pbp1a*	P_{lac}::*T25-pbp1a*(*S.pn*) *kan*	pACYC	Kan	This study
pKT25-*pbp2a*	P_{lac}::*T25-pbp2a*(*S.pn*) *kan*	pACYC	Kan	This study
pKT25-*cozE*	P_{lac}::*T25-cozE*(*spd0768*), *kan*	pACYC	Kan	This study
pKT25-*zip*	P_{lac}::*T25-leucine zipper region from yeast GCN4. kan*	pACYC	Kan	Karimova *et al* 1998 ([22])
pKT25-*zip*	P_{lac}::*T25-leucine zipper region from yeast GCN4. kan*	pACYC	Kan	Karimova *et al* 1998 ([22])
pLEM019	*bgaA'*::MCS::*tetM*::*bgaA' bla*	pACYC	Tet, Amp	This study
pLEM023	*bgaA'*::P_{zn}::MCS::*tetM*::*bgaA' bla*	pACYC	Tet, Amp	This study
pLEM025	*bgaA*'::P_{zn}::*pbp1a*(R6)::*tet*::*bgaA*' *bla*	pACYC	Tet, Amp	This study
p*Magellan6*	IRL(MmeI)::*add9::*IRR(MmeI) *bla*	ColE1	Spec, Amp	van Opijnen *et al* 2009 ([5])
'pMalC9'	*MBP*::*Himar1 bla*	pMB1 (*rop*-)	Amp	Lampe *et al* 1999 ([42])
pRY102	Plac::*dspE(ccmG)-T18 bla*	pUC	Amp	Cloned by Rachel Yunk
pRY103	Plac::*dspE(ccmG)-T25 kan*	pACYC	Kan	Cloned by Rachel Yunk
pUC57-*gfp*	*gfp, lacZ, bla*	pUC	Amp	Gift from the Campo lab

Supplementary Table 3 | Plasmids used in this study (*continued*)

Name	Genotype	Replicon	Resistance Marker(s)	Source or Reference
pUT18	P_{lac}::-*T18 bla*	pUC	Amp	Karimova *et al* 1998 ([22])
pUT18C	P_{lac}::*T18- bla*	pUC	Amp	Karimova *et al* 1998 ([22])
pCH363	P_{lac}::-*T18 lacI^{q}, bla*	pUC	Amp	Bendezú *et al* 2009 ([41])
pCH363-*ccmF*	P_{lac}::-*T18-ccmF lacI^{q} bla*	pUC	Amp	This study
pUT18C-*mreC*	P_{lac}::*T18-mreC*(*S.pn*) *bla*	pUC	Amp	This study
pUT18C-*mreD*	P_{lac}::*T18-mreD*(*S.pn*) *bla*	pUC	Amp	This study
pUT18C-*pbp1a*	P_{lac}::*T18-pbp1a*(*S.pn*) *bla*	pUC	Amp	This study
pUT18C-*pbp2a*	P_{lac}::*T18-pbp2a*(*S.pn*) *bla*	pUC	Amp	This study
pUT18C-*cozE*	P_{lac}::*T18-cozE*(*spd0768*) *bla*	pUC	Amp	This study
pUT18-*cozE*	P_{lac}::*cozE-T18*(*spd0768*) *bla*	pUC	Amp	This study
pUT18-*mreD*	P_{lac}::*mreD-T18*(*S.pn*) *bla*	pUC	Amp	This study
pUT18-zip	P_{lac}::*T18-leucine zipper region from yeast GCN4. bla*	pUC	Amp	Karimova *et al* 1998 ([22])

Cam = chloramphenicol, Erm = erythromycin, Kan = kanamycin, Spec = spectinomycin, Tet = tetracycline, MCS = multiple cloning site, S.pn = ORF amplified from *S. pneumoniae* D39 Δ*cps*.

Supplementary Table 4 | Oligonucleotides used in this study

Name	Sequence (5'-3')
AntibioticMarker_F	GAGGGAGGAAAGGCAGGA
AntibioticMarker_R	CGCCGTATCTGTGCTCTC
BgaA_3ORF_F	GAGAGCACAGATACGGCGGCTTCAGTCGGTGTTCTGTTTGG
BgaA_3ORF_R	TGCAAAGAAGTGAAGTTTGGCTTGAGC
BgaA_5FLANK_F	CCTACATTTGATGACCTTCTTAACGCC
BgaA_5FLANK_R	AACTTCGTCAGTGTCGCCTTGC
bgaA_FLANK_Pfucose_F	CCTGTATCTGTTCTTGAAGTTTGGCG
bgaA_FLANK_Pfucose_R	CGTCCTTGAAGACGTTTAGTAGCAAC
bgaA_FLANK_F	GTTGCTACTAAACGTCTTCAAGGACG
bgaA_FLANK_R	CGCCAAACTTCAAGAACAGATACAGG
cbpD_5FLANK_F	GGCAATTGACAGATGAGGAGTGG
cbpD _5FLANK_R	TCCTGCCTTTCCTCCCTCGCTATAAACGGTAAAATTTTCATTCTTCCTCC
cbpD _3FLANK_F	GAGAGCACAGATACGGCGCAGTGATGGAGAACGAGTATAGAAAATTGG
cbpD _3FLANK_R	CCGTCCAAAATACTAACGATGATGGG
cbpD _SEQ_F	GTGCGGAAAGCTTGGTAGACCG
ccmF_BTH_C_F	CGTCTAGATATGATGCCAGAAATTGGTAACGGACTGCTGTGC
ccmF_BTH_C_R	GCGAATTCTACGGCCTCCGGCGCAGTTTTTTGC
Chlor_isoT_F	GAGGGAGGAAAGGCAGGACCGGTATCGATAAGCTTGATG
Chlor_isoT _R	CGCCGTATCTGTGCTCTCAAACCTTCTTCAACTAACGGGG
GFP/YFP _N_F	ATGGTTTCTAAAGGTGAAGAATTGTTTACAGGTGTTGTTCCAATTTTGG
GFP/YFP _N_R	GAGACCTGCTGGCCCTTCCAATTTATACAATTCATCCATACCATGTGTAATACCAGC
GFP_TTG_F	GAGGGAGGTAACTCTTGGTTTCTAAAGGTGAAGAATTGTTTACAGGTGTTGTTCC
GFP_TTG_R	CCTTTAGAAACCAAGAGTTACCTCCCTCACTTTATTTTACCATATTTTCAAAAAGCTTAACAGATGAATTATTAAAGC
lytA_5FLANK_F	GATGAGTTCAATTGTATCTATCGGCAGTG

Supplementary Table 4 | Oligonucleotides used in this study (*continued*)

Name	Sequence (5'-3')
lytA _5FLANK_R	TCCTGCCTTTCCTCCCTCCTACTCCTTATCAATTAAAACAACTCATTTTTTACAATCC
lytA _3FLANK_F	GAGAGCACAGATACGGCGCCAGATGGCTTGATTACAGTAAAATAATAATGG
lytA _3FLANK_R	CTCAATCTATATAACATAGCTTTATGACTGATACC
lytA_SEQ_F	GGACTTGCTACCATTATTTCGCAAGG
MreC_BTH_N_F	CGTCTAGATATGAACCGTTTTAAAAAATCAAAATATGTCATTATTGTTTTTGTCACTG
MreC _BTH_N_R	ATCCCGGGTTATGAATTCCCCACTAATTCTATCACATCTACATTATGAG
MreC_GFP/YFP_N_3F	TTGGAAGGGCCAGCAGGTCTCATGAACCGTTTTAAAAAATCAAAATATGTCATTATTGTTTTTGTCACTGTTC
MreC_GFP/YFP_N_5R	GTAAACAATTCTTCACCTTTAGAAACCATATCCCTACCTTTATATCAAAAACTGTTACAGTAACTTTTTA
MreC_5FLANK_F	CTGATAGAGGCGTGTCATTTAAACG
MreC_5FLANK_R	TCCTGCCTTTCCTCCCTCAAACGGTTCATATCCCTACC
MreC_3FLANK_F	GAGAGCACAGATACGGCGGTGGGGAATTCATAATGAGAC
MreC_3FLANK_R	GTTTTTCCAACGATTGGTTACGAGAAAC
MreC_SEQ_F	CAAGTGATGTCTTTCAAGACGTTG
MreC_nativeRBS_F	TATCTCGAGGCTTTCAGGAATTGATAAAAAGTTACTGTAACAG
MreD_R	ATAGGATCCGACAATACTAGTATACCAAAAAAAGGCC
MreD _BTH_C_F	CAGAAGCTTATGAGACAGTTGAAGCGAGTTGGAG
MreD _BTH_C_R	AGTGGATCCTCTAGATAATATTTTTCAAAAATAAATTGAAAAACAGTAATCC
MreD_BTH_N_F	CGTCTAGATATGAGACAGTTGAAGCGAGTTGGAG
MreD _BTH_N_R	GCGAATTCTTATAGATAATATTTTTCAAAAATAAATTGAAAAACAGTAATCC
MreD_5FLANK_F	CTGACCATTCTTGTTATTCTCGTACTTGG
MreD_5FLANK_R	TCCTGCCTTTCCTCCCTCCGCTTCAACTGTCTCATTATGAATTCC
MreD_3FLANK_F	GAGAGCACAGATACGGCGTAAGAACGACATATAAATGTAACAAAGGCG

Supplementary Table 4 | Oligonucleotides used in this study (*continued*)

Name	Sequence (5'-3')
MreD_3FLANK_R	CATTCTCCAATTGGATAACTTGAAGCG
MreD_SEQ_F	GGCTATCAGGGTGGAAAAGGC
oSp104	CGGGATCCCGGTCAATGTTAGTCATATGG
oSp105	ATTTGCCTCCTTAAGATCCGC
oSp106	GCGGATCTTAAGGAGGCAAATATGAACAAACCAACGATTCTGCGC
oSp107	CGGCTCGAGTTATGGTTGTGCTGGTTGAGG
oSp108	GCGGATCTTAAGGAGGCAAATATGAAATTAGATAAATTATTTGAGAAATTTC
oSp109	CGGCTCGAGTTAGCGAAATAGATTGACTATC
oSp00X	TTTGAATTCGAATTCTAGATGGCTTTTTTGG
oSp00Y	TATCTCGAGTTATAATAGATTTATGAACACC
P_fucose_F4	CGAGAATTCGGAGGAATTTGAATTATTTTTATGAATATTGGG
P_fucose_R4	TAACTCGAGCGTCCTTGATTAACTTTATTATAATCCCC
pbp1a_BTH_N_F	CGTCTAGATATGAACAAACCAACGATTCTGCGCC
pbp1a _BTH_N_R	ATCCCGGGTTATGGTTGTGCTGGTTGAGGATTCTG
PBP1A_GFP/YFP_N_3F2	TTGGAAGGGCCAGCAGGTCTCAACAAACCAACGATTCTGCGCCTAATC
PBP1A_GFP/YFP_N_5R	GTAAACAATTCTTCACCTTTAGAAACCATATTTGCCTCCTTAAGATCCGCAGACTC
pbp1a_5FLANK_F	GTAAACACAAGCCAAGACACCCC
pbp1a_5FLANK_R	TCCTGCCTTTCCTCCCTCTTTGTTCATCTTGTTTTACCACC
pbp1a_3FLANK_F	GAGAGCACAGATACGGCGGCACAACCATAACATTTATCATCC
pbp1a_3FLANK_R	CACGTGGATCAGGTTCAAATGG
pbp1a_E150A _F	GCTCAGGCTGCTTGGTTAGCGATTCAGTTAGAACAAAAAGCAACC
pbp1a_E150A _R	GCTAACCAAGCAGCCTGAGCCTTACGAGAAATAGTCTGGTCG
pbp1a_E91A _F	CGTTTCTATCGCTGACCATCGCTTCTTCGACCACAGGGGGATTG

Supplementary Table 4 | Oligonucleotides used in this study (*continued*)

Name	Sequence (5'-3')
pbp1a_E91A _R	GCGATGGTCAGCGATAGAAACGATTGCCTTAACCAAATCTGTGGGAATATCATTAG
pbp1a_S370A _F	GACTGGGGAGCTACTATGAAACCGATCACAGACTATGCTCCTGC
pbp1a_S370A _R	GGTTTCATAGTAGCTCCCCAGTCGCGGTTTGTTTCTACTGCTTGG
pbp2a_5FLANK_F	GCCTCTCTAAAGTAAGTGGG
pbp2a_5FLANK_R	TCCTGCCTTTCCTCCCTCCATCTTCATCATAGGAAGAC
pbp2a_3FLANK_F	GAGAGCACAGATACGGCGGATGCTTGTCAAAGCCTAGC
pbp2a_3FLANK_R	CGTACAGTTTGACCAATCTC
pbp2a_BTH_N_F	CGTCTAGAATATGAAATTAGATAAATTATTTGAGAAATTTCTTTCTCTTTTTAAAAAAGAAACAAG
pbp2a_BTH_N_R	ATCCCGGGTTAGCGAAATAGATTGACTATCGAATCCC
PBP2a_GFP/YFP_N_F	TTGGAAGGGCCAGCAGGTCTCAAATTAGATAAATTATTTGAGAAATTTCTTTCTC
PBP2a_GFP/YFP_N_R	GTAAACAATTCTTCACCTTTAGAAACCATATTTGCCTCCTTAAGATCCGCAGAC
SPD_0768_nativeRBS_F	TAACTCGAGGCTTTAATAATTCATCTGTTAAGCTTTTTGAAAATATGG
SPD_0768_R	ATAGGATCCGACTTTTACTTAGCTAATTCTCTTTCTCG
SPD_0768_5FLANK_F	CTGCATTTAAAACAACTGTGATGACTC
SPD_0768_5FLANK_R	TCCTGCCTTTCCTCCCTCTTCTACGAAACATGAGTTACCTCC
SPD_0768_3FLANK_F	GAGAGCACAGATACGGCGGAAAGAGAATTAGCTAAGTAAAAGTCAGG
SPD_0768_3FLANK_R	GGTTATGTATTATTTTAACAGCCCCTCG
SPD_0768_Seq_F	CGATTTTGCGAAGTGTAAATGTAGAAG
SPD0768_FLAG_N_R	TTTATCGTCGTCATCCTTGTAGTCAATGTCATGGTCTTTGTAGTCTCCGTCATGGTCCTTATAGTCCATGAGTTACCTCCCTCACTTTATTTTACC
SPD0768_FLAG_N_F	CATTGACTACAAGGATGACGACGATAAATTGGAAGGGCCAGCAGGTCTCTTTCGTAGAAATAAATTATTTTTTTGGACCACAGAAATTTTACTCTTAACC

Supplementary Table 4 | Oligonucleotides used in this study (*continued*).

Name	Sequence (5'-3')
SPD0768_FLAG_Pzn_F	ATAACTCGAGAGGAGGTAACTCATGGACTATAAGGACCATGACGG
SPD0768_FLAG_Pzn_R	TATGGATCCTTACTTAGCTAATTCTCTTTCTCGTTCTTTCATTATTTTATG
FLAG_Seq_F	CCATGACGGAGACTACAAAGACC
SPD0768_5FLANK_insert_R	GGAGATCCCCAAGTAATCGTGTTCTACGAAACATGAGTTACCTCC
pLEM023_F	CACGATTACTTGGGGATCTCCCCGCGAAAGCGGG
pLEM023_R	TCCTGCCTTTCCTCCCTCGTCATACCATGTATACCACTTGG
SPD_0768_TTG_F	GGGAGGTAACTCTTGTTTCGTAGAAATAAATTATTTTTTTGGACCACAGAAATTTTACTCTTAACC
SPD_0768_TTG_R	CTACGAAACAAGAGTTACCTCCCTCACTTTATTTTACCATATTTTCAAAAAGCTTAACAGATG
SPD0768_GFP/YFP_N_5R	GTAAACAATTCTTCACCTTTAGAAACCATGAGTTACCTCCCTCACTTTATTTTACCATATTTTCAAAAAGC
SPD0768_GFP/YFP_N_3F	TTGGAAGGGCCAGCAGGTCTCATGTTTCGTAGAAATAAATTATTTTTTTGGACCACAGAAATTTTACTCTTAACC
SPD0768_BTH_C_F	CAGAAGCTTATGTTTCGTAGAAATAAATTATTTTTTTGGACCACAG
SPD0768_BTH_C_R	AGTGGATCCTCCTTAGCTAATTCTCTTTCTCGTTCTTTC
SPD0768_BTH_N_F	CGTCTAGATATGTTTCGTAGAAATAAATTATTTTTTTGGACCACAG
SPD0768_BTH_N_R	GCGAATTCTTACTTAGCTAATTCTCTTTCTCGTTCTTTC
Spec_isoT_F	GAGGGAGGAAAGGCAGGACCCGTTTGATTTTTAATGGTAATG
Spec_isoT _R	CGCCGTATCTGTGCTCTCAATTTTTTTATAATTTTTTTAATCTG
Tet_isoT_F	GAGGGAGGAAAGGCAGGACCAAGTAATCGTGAATGTCGCTG
Tet_isoT_R	CGCCGTATCTGTGCTCTCTGCGCTCCGCTAGCTTTACAGAC

Where restriction enzyme sites have been introduced into the primers, these are underlined. Any primer containing relevant sequence from an ORF are shown in red. Overlapping sequences used for isothermal assembly when generating gene knockout constructs are shown in blue. Regions where codons have been altered are highlighted in green. Linker regions used for GFP- fusions are shown in purple. Sequences used to generate the FLAG tag fusion proteins are shown in orange.

Supplementary References

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