

1 **SUPPLEMENTAL INFORMATION (SI)**

2 **Suppression of Deletion Mutation in the Gene Encoding Essential PBP2b Reveals**
3 **a New Lytic Transglycosylase Involved in Peripheral Peptidoglycan Synthesis in**
4 ***Streptococcus pneumoniae* D39**

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15 **SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

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20
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23 observed hydrolase activities in zymogram assays and phenotypes of deletion mutants
24 in a $\Delta pbp1a \Delta mltG$ background.

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reconstruction of $\Delta pbp2b$ strains with original *mltG* suppressor alleles (Fig. S4)

Genotypes of strains in these constructions are listed in Table S1 and primers used in constructions are compiled in Table S2. Two approaches were used to reconstruct $\Delta pbp2b$ strains with *mltG* suppressor alleles (see Fig. S4). The first approach used intermediate strains with insertions of a P_c -[*kan-rpsL*⁺] cassette either upstream or downstream *mltG* in a *pbp1a*⁺ genetic background. This approach was only successful in reconstructing $\Delta pbp2b$ *sup3* (IU9783, $\Delta pbp2b$ *mltG*(Y488D)). To obtain IU9783, an intermediate strain IU8986 (D39 Δcps *rpsL1* *mltG*⁺- P_c -[*kan-rpsL*⁺]) with an insertion of P_c -[*kan-rpsL*⁺] cassette immediately after *mltG* was constructed. An *mltG*(Y488D) amplicon was obtained using IU7567 as a template and primers P1348 and P1349 and transformed into IU8986 to obtain IU9760, which was sequenced to confirm the Y488D change, and no other mutation in *mltG* or *pbp1a*. IU9760 was transformed with $\Delta pbp2b$ <*aad9* from IU7397 to obtain IU9783 (D39 Δcps *rpsL1* *mltG*(Y488D) $\Delta pbp2b$ <*aad9*) (Fig. S4).

We attempted a similar strategy to reconstruct the other suppressor strains without success. The transformation of a *mltG*($\Delta 5bp$) amplicon into IU8986 (*mltG*⁺- P_c -[*kan-rpsL*⁺]) and the transformation of *mltG*($\Delta 488bp$) or *mltG*($\Omega 45bp$)² amplicons into IU8980 (P_c -[*kan-rpsL*⁺]-*mltG*⁺) yielded only strains with the wild-type *mltG* sequence. One isolate was found to contain the *mltG*($\Delta 488bp$) mutation, but it was found to also contain a frameshift mutation in *pbp1a*.

The second approach (Fig. S4) was to transfer the *mltG* [$\Delta 5bp$, $\Delta 488bp$ or ($\Omega 45bp$)²] mutations into a stabilizing $\Delta pbp1a$ genetic background as the first step (see *Results*).

52 Amplicons were synthesized from the original suppressor strains as templates using
53 primers P1348 and P1349 and transformed into strain IU7325 ($\Delta cps rpsL1 \Delta pbp1a$
54 $\Delta mltG::P_c-[kan-rpsL^+]$). The resulting strains were transformed with the $\Delta pbp2b \langle aad9$
55 amplicon. The $\Delta pbp1a$ mutations in strains IU8565, IU8567 and IU8569 were repaired
56 by transformation first with a $\Delta pbp1a::P_c-[kan-rpsL^+]$ amplicon, followed by
57 transformation with a $pbp1a^+$ amplicon. Strains IU9777, IU9905 and IU9907 were
58 sequenced to confirm the correct *mltG* mutation and no initial mutation in *pbp1a*.

59

60 **Reconstruction of *pbp1a* alleles in a clean genetic background (Fig. 3)**

61 PCR amplicons containing *pbp1a* alleles from original $\Delta mltG$ suppressor strains
62 IU7258 ($\Delta mltG pbp1a$ (G494E)), IU7260 ($\Delta mltG pbp1a$ (G deletion at Gly451)), IU7286
63 ($\Delta mltG pbp1a$ (S89F)), IU7287 ($\Delta mltG pbp1a$ (A deletion at Lys160)), IU7288 ($\Delta mltG$
64 $pbp1a$ (T insertion at Phe33)) were prepared from strain lysates and primers P234 and
65 P235. Amplicons were transformed into intermediate strain IU6726 (D39 $\Delta cps rpsL1$
66 $\Delta pbp1a::P_c-[kan-rpsL^+]$) as described above (see Fig. S4). The resulting strains were
67 designated IU7837 (*pbp1a* (G494E)), IU7839 (*pbp1a* (G deletion at Gly451)), IU7840
68 (*pbp1a* (S89F)), IU7843 (*pbp1a* (A deletion at Lys160)), and IU7845 (*pbp1a* (T insertion
69 at Phe33)). *pbp1a* alleles in reconstructed strains were confirmed by DNA sequencing.
70 A markerless $\Delta pbp1a$ allele in strain IU6741 was constructed by transforming a $\Delta pbp1a$
71 fusion amplicon containing 60 bp (20 codons) from the N-terminus and 60 bp (20
72 codons) from the C-terminus of *pbp1a* into strain IU6726 (see top, Fig. S4). The short
73 regions at the beginning and end of *pbp1a* were included in the $\Delta pbp1a$ allele to
74 maintain possible translational coupling of closely spaced pneumococcal genes.

75 **Construction of zinc-dependent *MltG* merodiploid strain IU9102 (Fig. 3 and 6)**

76 The $\Delta bgaA::tet\text{-}P_{Zn}\text{-RBS}_{m\text{lt}G}\text{-}m\text{lt}G$ fusion amplicon was obtained by fusion PCR of a
77 5' PCR fragment containing $bgaA'::tet\text{-}P_{Zn}$ ($bgaA'::tet\text{-}P_{czcD}$ DNA template from pJWV25
78 (Eberhardt *et al.*, 2009)), a middle fragment containing $\text{RBS}_{m\text{lt}G}\text{-}m\text{lt}G$ (24bp upstream
79 and ORF sequences of *m\text{lt}G*), and 3' fragment of flanking *bgaA'* sequence. The fusion
80 amplicon was transformed into IU1945 to obtain strain IU8872 (D39 $\Delta cps \Delta bgaA::tet\text{-}$
81 $P_{Zn}\text{-RBS}_{m\text{lt}G}\text{-}m\text{lt}G$). A $\Delta m\text{lt}G::P_c\text{-}aad9$ fusion amplicon was transformed into IU8872 to
82 obtain zinc-dependent, merodiploid strain IU9102 (D39 $\Delta cps \Delta m\text{lt}G::P_c\text{-}$
83 $aad9//\Delta bgaA::tet\text{-}P_{Zn}\text{-RBS}_{m\text{lt}G}\text{-}m\text{lt}G$). IU9102 was maintained in media containing 0.2
84 mM ZnCl_2 and 0.02 mM MnSO_4 during all steps in the transformation procedure, single
85 colony isolation, and growth for storage. MnSO_4 was added at a concentration of 0.1X
86 of ZnCl_2 in all media to minimize Zn^{+2} toxicity (Jacobsen *et al.*, 2011). Growth and cell
87 morphology of wild type strain IU1945 in BHI media containing 0.2 mM ZnCl_2 and 0.02
88 mM MnSO_4 is similar to growth in BHI media without additional ZnCl_2 and MnSO_4 .

89
90 **Construction of markerless *ftsZ-L2-mKate2*, *gfp-L1-m\text{lt}G*, and double *ftsZ-L2-*
91 *mKate2 gfp-L1-m\text{lt}G* strains (Fig. 9)**

92 *mKate2* is a far-red monomeric fluorescent protein, whose codons have been
93 optimized for expression in *S. pneumoniae* (Beilharz *et al.*, 2015). Because of its
94 relative stability, *mKate2* is preferred over *mCherry* for localization studies. To construct
95 a markerless carboxyl fusion of *ftsZ* to *mKate2*, intermediate strain IU7614 ($ftsZ^+\text{-}P_c\text{-}$
96 [*kan-rpsL*⁺]) was constructed by insertion of the $P_c\text{-}[kan\text{-}rpsL^+]$ cassette after the *ftsZ*
97 stop codon. The $P_c\text{-}[kan\text{-}rpsL^+]$ cassette was followed by a duplicated 60 bp of the 3'-

98 end of *ftsZ* and the downstream gene *ylmE*. An *ftsZ*-L₂-*mKate2* fusion amplicon with
99 *ftsZ* replaced by *ftsZ*-L₂-*mKate2*, followed by 17 bp of the 3'-end of *ftsZ* was constructed
100 and transformed into strain IU7614 to obtain IU9148 (*ftsZ*-L₂-*mKate2*). The L₂ linker
101 sequence KLDIEFLQ was used for the C-terminal fusion as described in (Fleurie *et al.*,
102 2014).

103 To construct a markerless N-terminal fusion of *gfp* to *mltG*, intermediate strain
104 IU8980 (P_c[*kan-rpsL*⁺]-*mltG*⁺) was constructed with the P_c[*kan-rpsL*⁺] cassette placed
105 80 bp upstream of *mltG*. A *gfp*-L₁-*mltG* fusion amplicon with *mltG* replaced by *gfp*-L₁-
106 *mltG* was constructed and transformed into IU8980 to obtain IU10228 (*gfp*-L₁-*mltG*).
107 The original *mltG* start codon TTG is replaced with ATG of *gfp*. The L₁ linker sequence
108 LEGSG was used for the N-terminal fusion as described in (Fleurie *et al.*, 2014). The
109 DNA template for *gfp* is pUC57-*gfp*(*Sp*) (Martin *et al.*, 2010), which was codon
110 optimized for *S. pneumoniae* and has an aa substitution (A206K) that prevents GFP
111 dimerization.

112 To construct double *ftsZ*-L₂-*mKate2* *gfp*-L₁-*mltG* strain IU10353, IU10228 (*gfp*-L₁-
113 *mltG*) was transformed with the *ftsZ*⁺-P_c-[*kan-rpsL*⁺] amplicon from IU7614 to obtain
114 IU10318 (*gfp*-L₁-*mltG* *ftsZ*⁺-P_c-[*kan-rpsL*⁺]). IU10318 was transformed with a *ftsZ*-L₂-
115 *mKate2* amplicon from IU9148 to obtain final strain IU10353.

116 117 **Construction of *mltG*(Δ DUF_1346) strains**

118 Initially we did not know whether the DUF_1346 domain was essential for MltG
119 function. Since Δ *mltG* Δ *pbp1a* strains are viable, whereas Δ *mltG* *pbp1a*⁺ strains are not,
120 a *mltG*(Δ DUF_1346) mutation was first constructed in strain IU6741 (D39 Δ *cps* *rpsL1*

121 $\Delta pbp1a$). IU6741 was transformed with $\Delta mltG::P_c-[kan-rpsL^+]$ from strain K637,
122 producing strain IU7325 (D39 Δcps $rpsL1$ $\Delta pbp1a$ $\Delta mltG::P_c-[kan-rpsL^+]$). A
123 $mltG(\Delta DUF_1346)$ fusion amplicon was then constructed by fusion PCR and
124 transformed into IU7325. The resulting strain was designated as IU8910 (D39 Δcps
125 $rpsL1$ $\Delta pbp1a$ $mltG(\Delta DUF_1346)$). To evaluate whether the $mltG(\Delta DUF_1346)$ allele is
126 functional in IU8910, we attempted to transform a $\Delta pbp2b$ amplicon (Table 2) into
127 IU8910. No colonies were recovered, indicating that $MltG(\Delta DUF_1346)$ is functional in
128 IU8910.

129 We synthesized an $mltG(\Delta DUF_1346)$ amplicon from strain IU8910 and transformed
130 the amplicon into strain IU8980 ($P_c-[kan-rpsL^+]-mltG^+$). Hundreds of well-formed
131 streptomycin resistant colonies appeared after 20 hours of incubation. Two isolates
132 (strain IU9025 and IU9026) were stored from independent transformations, and DNA
133 sequencing confirmed that both strains contain the $mltG(\Delta DUF_1346)$ allele. DNA
134 sequencing also confirmed that $pbp1a^+$ of strain IU9025 had not accumulated any
135 mutations.

136

137 **Conditions comparing growth of $mltG^+$ and $mltG(\Delta DUF_1346)$ strains (Fig. S9)**

138 Growth of $mltG^+$ parent strain IU1824 and $mltG(\Delta DUF_1346)$ mutant IU9025 was
139 compared in media containing high salt, β -lactam antibiotic penicillin G, and at low pH.
140 To test growth in high salt conditions, IU1824 and IU9025 were grown in BHI broth
141 without additional NaCl (0.08 M NaCl in BHI formula), or in BHI broth with additional
142 NaCl to a final concentration of 0.3 M. Strains were grown overnight in BHI broth, and
143 diluted to $OD_{620} \approx 0.003$ in BHI broth with or without additional NaCl. To test the growth

144 of IU1824 and IU9025 in the presence penicillin G, strains were grown overnight in BHI
145 broth, and diluted to $OD_{620} \approx 0.003$ in BHI broth for culture growth. At $OD_{620} \approx 0.1$,
146 penicillin G sodium salt (Sigma PEN-NA) was added to the cultures to final
147 concentrations of 0, 0.004 (0.5x MIC, Kocaoglu *et al.*, 2015), and 0.006 $\mu\text{g/ml}$. This
148 experiment was repeated once with addition of penicillin G concentrations of 0, 0.002,
149 0.004 and 0.008 $\mu\text{g/ml}$. To test the growth of IU1824 and IU9025 at low pH condition,
150 strains were grown overnight in BHI broth (pH 7.2), and diluted to $OD_{620} \approx 0.003$ in BHI
151 broth for culture growth. At $OD_{620} \approx 0.1$, 100 or 150 μL of 1 M HCl were added to the 5
152 mL cultures to decrease the pH to 5.8 or 5.0. This growth experiment was repeated
153 once. To test the effect of temperature on growth of *mltG*⁺ and *mltG*(ΔDUF_1346)
154 strains, ice from frozen cultures of both strains was streaked onto two TSAII-BA plates.
155 One plate was incubated at 37°C in an atmosphere of 5% CO₂ overnight, and the other
156 was incubated at 42°C in an atmosphere of 5% CO₂ overnight. The size and
157 morphologies of the colonies of the two strains were compared.

158

159 **Western blotting (Fig. S2)**

160 Strains were grown exponentially in BHI broth to $OD_{620} \approx 0.15$. Lysates were
161 prepared as described previously (Wayne *et al.*, 2012) and separated using 10 % or 4-
162 15% mini-protean TGX pre-cast gels (Bio-Rad). FLAG-, HA-, and Myc-tagged proteins
163 were detected by Western blotting as described previously (Tsui *et al.*, 2014).
164 Chemiluminescent signal in protein bands was quantitated by using an IVIS imaging
165 system as described in (Wayne *et al.*, 2010).

167 ***DNA library construction, Illumina MiSeq DNA sequencing, and bioinformatic***
168 ***analyses (Table 1)***

169 One μg of genomic DNA of each sample was diluted to 130 μL with TE and sheared
170 using the following settings on the S220 focused-ultrasonicator (Covaris): 105W Peak
171 Incident Power; 5 % Duty Factor, 200 Cycles Per Burst; 40 seconds Treatment Time.
172 Sheared samples were purified using AmpureXP beads (Agencourt/Seradyn/Beckman
173 Coulter, A63881) to sample ratio of 0.5X and eluted with 54 μL of EB (Qiagen). Sheared
174 samples were visualized using a D1K High Sensitivity tape (Agilent, 5067-5363) on an
175 Agilent 2200 TapeStation and consisted mostly of ≈ 650 to 700 bp fragments. Library
176 construction was carried out on the Biomek FX^P (Beckman Coulter) using a modified
177 SWHT (SPRIworks High Throughput for Illumina, Beckman Coulter) method to
178 accommodate a 700 bp distribution. Bioo Scientific NextFlex DNaseq Library Kit for
179 Biomek FX^P (5140-42) was used in conjunction with Bioo Scientific NextFlex DNA
180 barcodes-96 adaptors (514105). Following library construction, 15 μL of the 25 μL pre-
181 enrichment library was used as template in a 10-cycle PCR amplification. Library sizes
182 and concentrations were determined using TapeStation and Quant-iT Picogreen
183 (Molecular Probes, P7589), respectively. Dilutions were made to 20 nM and pooled in
184 preparation for a MiSeq run. 30 μL of 20 nM library was diluted to 500 μL using Illumina
185 buffer HT1 and loaded onto a 500 cycle MiSeq (version 2) flowcell (Illumina, MS-102-
186 2003). Paired-end run cycle parameters were 260 (Read1) + 8 (Index read) + 260
187 (Read2). DNA sequences were assembled based on the published encapsulated D39
188 genome sequence (Lanie *et al.*, 2007). Bioinformatic analyses was performed using
189 cutadapt (<https://code.google.com/p/cutadapt/>) with a min length of 100 and quality

190 cutoff of 30, and assembled using newbler (Margulies *et al.*, 2005), mapped using
191 bowtie (Langmead *et al.*, 2009) and called SNPs using mpileup (Li *et al.*, 2009). Full
192 coverage was obtained for the genomes of each mutant with >175 reads of most base
193 pairs. Gaps were only present at the expected *cps2A* to *cps2H* and *pbp2b* regions.

194

195 ***MltG* sequence alignment and secondary structure information (Fig. 5 and S6)**

196 The MltG_{Eco} sequence (gi accession 687676267) was aligned with the MltG_{Spn} (gi:
197 116076234) and YceG_{Lmo} (gi: 16803539) using Clustal Omega (Sievers *et al.*, 2011)
198 with default parameters. Secondary structure information of MltG_{Eco} was obtained from
199 the PDB database (PDB ID: 2R1F).

200

201 ***RNA-Seq analysis (Table 4)***

202 cDNA libraries were prepared from total RNA by the Center for Genomics and
203 Bioinformatics at Indiana University in Bloomington, Indiana. The mRNA was enriched
204 from 1 µg of total RNA using RiboZero™ rRNA Removal Kit (Bacteria) (EpiCentre, Inc.).
205 rRNA-depleted mRNA samples were purified using RNeasy Minelute Cleanup Kit
206 (Qiagen). Double-stranded cDNA synthesis was performed following ScriptSeq™
207 Complete Kit (Bacteria) Preparation guide (EpiCentre, Inc.) in accordance with the
208 manufacturer's standard protocol. Entire resultant mRNA sample was fragmented using
209 divalent cations via incubation for 5 min at 85°C. The first strand of cDNA was
210 synthesized by reverse transcription using random-sequence primers containing a
211 tagging sequence at their 5' ends. Di-tagged cDNA was synthesized by random
212 annealing of a terminal-Tagging Oligo to the 3' end of the cDNA for extension of the

213 cDNA by DNA polymerase. Di-tagged cDNA was purified using Agencourt AMPure® XP
214 beads (Beckman Coulter) followed by PCR amplification for 15 cycles using Failsafe™
215 PCR enzyme and ScriptSeq™ Index PCR Primer set (EpiCentre, Inc.). This step
216 generated the second strand of cDNA and completed the addition of Illumina adapter
217 sequences incorporating a user-defined barcode. The amplified libraries were purified
218 using Agencourt AMPure® XP beads. Quality and quantity were assessed using D1000
219 TapeStation (Agilent Technologies) and Quant-iT™ PicoGreen® dsDNA Assay Kit (Life
220 Technologies), respectively. Sequencing was performed at the Greehey Children's
221 Cancer Research Institute Genome Sequencing Facility at the University of Texas
222 Health Science Center at San Antonio, Texas. Cluster generation was performed using
223 TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Single-end, 50 bp sequencing was
224 performed using TruSeq SBS Kit v3-HS (Illumina) on a HiSeq 2000 sequencer
225 (Illumina). Image analysis, base calling, sequencing reads de-multiplexing were
226 followed by default Illumina procedure.

227 The raw sequencing reads were quality and adapter trimmed using Trimmomatic
228 version 33 (Lohse *et al.*, 2012) with a minimum final read length of 35. The trimmed
229 reads were mapped onto the *S. pneumoniae* D39 (RefSeq NC_008533) genome and
230 D39 plasmid pDP1 sequence (RefSeq NC_005022) using bowtie2 version 2.1
231 (Langmead & Salzberg, 2012). Custom PERL scripts were used to generate read
232 counts for the genes and 100 bp non-overlapping intergenic regions of the genome.
233 Differential gene expression was identified using DESeq2 (version 1.8.1) (Love *et al.*,
234 2014) using default parameters (Robinson *et al.*, 2010). The false-discovery rate (FDR)
235 was calculated using Benjamini and Hochberg's algorithm (Benjamini & Hochberg,

236 1995) and a gene or region was defined as differentially expressed if it had an up- or
237 down-fold change of 1.8 and their FDR was less than 0.05.

238 239 **QRT-PCR analysis (Fig. S15)**

240 RNA from each strain was prepared as described in *Experimental procedures* for
241 RNA-Seq analysis. Five µg of purified RNA were further treated with DNA-free DNA
242 Removal Kit (Ambion). 125 ng of treated RNA were used to synthesize cDNA by qScript
243 Felex cDNA synthesis kit (Quanta Biosciences). RT-PCR was performed using the
244 Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies). Each
245 reaction contained 10 µl of 2xBrilliant III Ultra-Fast SYBR Green QPCR Master Mix
246 (Agilent), 2 µl of each 2 µM primers (Table S2), 0.3 µl of a 1:500 dilution of ROX
247 reference dye and 6 µl of diluted cDNA. Samples were run in an MX3000P thermocycler
248 (Stratagene) with Program MxPro v. 3.0. QRT-PCR reactions were performed 3 times
249 each for three independent biological samples. The transcript amounts were normalized
250 to *gyrA* RNA amount (Table S2). Data were analyzed with the SYBR Green (with
251 dissociation curve) program on the thermocycler. Four dilutions of cDNA from a wild-
252 type *S. pneumoniae* strain were used to generate standard curves for each primer set.
253 For statistical analysis, normalized transcript amounts of *spd_1874*, *spd_0104*, and
254 *pcsB* were tested with a one sample t test (GraphPad Prism) to determine if the mean
255 was significantly different from a hypothetical value of 1.

259 **Membrane preparation from *S. pneumoniae* cells for zymogram analysis (Fig. S8)**

260 Membrane preparations from strains IU1945 (D39 Δcps parent), K5 ($\Delta lytC$), K27
261 ($\Delta lytB$) and K43 ($\Delta lytA$) were made as previously described with minor modifications
262 (Wayne *et al.*, 2010). Briefly, 20 mL cultures of each strain were grown in BHI broth to
263 $OD_{620} \approx 0.3$. Cells were collected by centrifuging at 16,500 xg for 5 min at 4°C. Pellets
264 were washed with 2 mL 1 x SMM buffer (0.5 M sucrose, 20 mM $MgCl_2$, 20 mM MES pH
265 6.5) at room temperature and resuspended in 2 mL 1 x SMM buffer. 100 μL of 10
266 mg/mL lysozyme (Sigma), 8 μL of 1mg/mL of mutanolysin (Sigma), and 20 μL of
267 protease inhibitor cocktail set III (Calbiochem) were added to each resuspended pellet,
268 and mixtures were incubated at 37°C for 1 hour to digest cell wall. Protoplasts were
269 collected as pellets following centrifugation at 8,000 xg for 10 min at room temperature.
270 Protoplast pellets were frozen on dry ice for 10 min and resuspended in 2 mL of cold
271 Buffer H (20 mM HEPES, pH 8.0, 200 mM NaCl, 1 mM DTT, and protease inhibitor
272 cocktail set III). 20 μL of 0.1 M $MgCl_2$, 20 μL of 0.1 M $CaCl_2$, 4 μL of 5 mg/mL DNase
273 (Sigma), and 4 μL of 10 mg/mL RNase (Sigma) were added to each resuspended pellet,
274 and the mixtures were incubated on ice for 1 h. Following centrifugation at 16,000 xg for
275 30 min at 4°C, the pellets were collected as membrane fractions, and were resuspended
276 in 60 μL solubilization buffer (1% (wt/vol) SDS, 0.1% vol/vol Triton-X-100). 5 μL was
277 taken for protein concentration determination with the DCTm protein assay kit (Bio-Rad).
278 Equal volumes of 2X Laemmli sample buffer (Bio-Rad, 161-0737) without added β -
279 mercaptoethanol, were added to the remaining membrane solutions. Mixtures were
280 incubated at 100°C for 10 min before loading into gel lanes for zymogram analysis.

281

Zymogram analysis (Fig. S8)

Zymogram analysis was performed with a protocol modified from Bartual *et al.*, (2014). Lysed cell preparation from strain IU1945 was used as the PG substrate. 300 mL of strain IU1945 was grown up to $OD_{620} \approx 0.3$ in BHI broth and collected by centrifugation at 10,000 xg for 10 min at 4 °C. Pellets were washed once with 5 mL of ice-cold buffer containing 100 mM NaCl, 20 mM Tris-HCl pH 7.4 buffer, and resuspended in 1.5 mL of the same buffer. Resuspended cells were incubated at 100 °C for 10 min, followed by centrifugation at 16,000 xg for 10 min. The pellet was resuspended in 1.5 mL of 1.5 M Tris-HCl, pH 8.8 and stored at -20 °C. The 1.5 mL suspension of lysed IU1945 cells was added to a 12% SDS-PAGE resolving gel solution (total volume 7.5 mL) containing 0.33 mL of 1.5 M Tris-HCl (pH 8.8), 75 μ L of 10% SDS, 2.25 mL of 40% acrylamide (19:1 acrylamide/bis-acrylamide, A9926 Sigma), 3.3 mL water, 37.5 μ L 10% ammonium persulfate (APS) and 3.75 μ L TEMED (Bio-Rad, 1610800). 4% SDS-PAGE stacking gel solution was made with a mixture of 470 μ L of 0.5M Tris-HCl, pH 6.8, 19 μ L of 10 %SDS, 190 μ L of 40 % acrylamide, 1.2 mL water, 11 μ L 10 % APS, and 1.1 μ L TEMED. 35 μ g of membrane proteins from strains IU1945, K7, K27 or K43, or 10 μ L of PageRuler prestained protein ladder (Thermo Scientific) was loaded into each lane. Gel electrophoresis was performed in Tris-glycine SDS buffer for 3h at 150V. The gel was washed with distilled water twice for 30 min each and incubated in \approx 300 mL of refolding buffer (50 mM NaCl, 20 mM $MgCl_2$, 0.5% (vol/vol) Triton X-100, and 20 mM Tris-HCl, pH 7.4) with gentle shaking at 37 °C for 12 to 14 h. Gel images were obtained by photography against a black background.

TABLE S1. Bacterial strains used in this study^a

<i>S. pneumoniae</i> strains			
Strain number	Genotype (description) ^b	Antibiotic resistance ^c	Reference or source
IU1690	D39 <i>cps</i> ⁺	None	Lanie <i>et al.</i> , 2007
IU1751	R6 $\Delta mreCD \langle \rangle aad9$	Sp ^c ^R	Land and Winkler, 2011
IU1824	D39 <i>rpsL1</i> $\Delta cps2A'-cps2H'$ = D39 <i>rpsL1</i> Δcps	Str ^R	Lanie <i>et al.</i> , 2007
IU1945	D39 $\Delta cps2A'-cps2H'$ = D39 Δcps	None	Lanie <i>et al.</i> , 2007
IU2519	D39 $\Delta bgaA'::kant1t2-P_{fcsK}-pcsB^+$	Kan ^R	Barendt <i>et al.</i> , 2009
IU3286	D39 <i>rpsL1</i> $\Delta cps2E::P_c-[kan-rpsL^+]$	Kan ^R Str ^S	Ramos-Montanez <i>et al.</i> , 2010
IU3877	D39 $\Delta cps \Delta lytB \langle \rangle P_c-[kan-rpsL^+]$	Kan ^R	Barendt <i>et al.</i> , 2011
IU3878	D39 $\Delta cps \Delta spd_{0873} \langle \rangle P_c-[kan-rpsL^+]$	Kan ^R	Barendt <i>et al.</i> , 2011
IU3897	D39 $\Delta cps \Delta mreCD \langle \rangle P_c-erm$	Erm ^R	Land & Winkler, 2011
IU4970	D39 $\Delta cps mreC-L-FLAG^3-P_c-erm$	Erm ^R	Land & Winkler, 2011
IU6647	D39 $\Delta cps \Delta pbp1a::P_c-erm$ (IU1945 X $\Delta pbp1a::P_c-erm$ from E177)	Erm ^R	This study
IU6649	D39 $\Delta cps \Delta pbp1b::P_c-erm$ (IU1945 X $\Delta pbp1b::P_c-erm$ from E193)	Erm ^R	This study
IU6662	D39 $\Delta cps \Delta pbp1a::P_c-[kan-rpsL^+]$ (IU1945 X $\Delta pbp1a::P_c-[kan-rpsL^+]$ from K164)	Kan ^R	This study
IU6680	D39 $\Delta cps \Delta pbp2a::P_c-[kan-rpsL^+]$ (IU1945 X $\Delta pbp2a::P_c-[kan-rpsL^+]$ from K166)	Kan ^R	This study
IU6726	D39 $\Delta cps rpsL1 \Delta pbp1a::P_c-[kan-rpsL^+]$ (IU1824 X $\Delta pbp1a::P_c-[kan-rpsL^+]$ from K164)	Kan ^R Str ^S	This study
IU6741	D39 $\Delta cps rpsL1 \Delta pbp1a$ (IU6726 X fusion $\Delta pbp1a$)	Str ^R Kan ^S	This study
IU7258	D39 $\Delta cps \Delta mltG::P_c-[kan-rpsL^+] sup1$ (<i>pbp1a</i> (G494E)(GGA→GAA) (single colony isolate of K637)	Kan ^R	This study
IU7260	D39 $\Delta cps \Delta mltG::P_c-erm sup1$ (<i>pbp1a</i> (ΔG at G451)(GGT ₁₇ →G-T)(single	Erm ^R	This study

	colony isolate of E681)		
IU7286	D39 $\Delta cps \Delta mltG::P_c-[kan-rpsL^+] sup2$ (<i>pbp1a</i> (S89F)(TCT→TTT) (IU1945 X $\Delta mltG::P_c-[kan-rpsL^+]$ from IU7258)	Kan ^R	This study
IU7287	D39 $\Delta cps \Delta mltG::P_c-[kan-rpsL^+] sup3$ (<i>pbp1a</i> (ΔA at K160)(AAA→AA-)) (IU1945 X $\Delta mltG::P_c-[kan-rpsL^+]$ from IU7258)	Kan ^R	This study
IU7288	D39 $\Delta cps \Delta mltG::P_c-erm sup2$ (<i>pbp1a</i> (ΩT at F33)(TTC→TTTC))(IU1945 X $\Delta mltG::P_c-erm$ from IU7260)	Erm ^R	This study
IU7325	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-[kan-rpsL^+]$ (IU6741 X $\Delta mltG::P_c-[kan-rpsL^+]$ from K637)	Kan ^R Str ^S	This study
IU7327	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm$ (IU6741 X $\Delta mltG::P_c-erm$ from IU7260)	Erm ^R Str ^R	This study
IU7337	D39 $\Delta cps \Delta bgaA::kan-t1t2-P_{fcsK}-pbp2b$	Kan ^R	Tsui <i>et al.</i> , 2014
IU7397	D39 $\Delta cps \Delta pbp2b<>aad9//\Delta bgaA::kan-t1t2-P_{fcsK}-pbp2b$	Kan ^R Spc ^R	Tsui <i>et al.</i> , 2014
IU7399	D39 $\Delta cps mltG-HA-P_c-kan$ (IU1945 X fusion <i>mltG-HA-P_c-kan</i>)	Kan ^R	This study
IU7403	D39 $\Delta cps mltG-FLAG-P_c-erm$ (IU1945 X fusion <i>mltG-FLAG-P_c-erm</i>)	Erm ^R	This study
IU7405	D39 $\Delta cps mltG-Myc-P_c-kan$ (IU1945 X fusion <i>mltG-Myc-P_c-kan</i>)	Kan ^R	This study
IU7476	D39 $\Delta cps \Delta pbp2b<>aad9 sup1$ (IU1945 X $\Delta pbp2b<>aad9$ from IU7397)	Spc ^R	This study
IU7477	D39 $\Delta cps \Delta pbp2b<>aad9 sup2$ (IU1945 X $\Delta pbp2b<>aad9$ from IU7397)	Spc ^R	This study
IU7567	D39 $\Delta cps \Delta pbp2b<>aad9 sup3$ (IU1945 X $\Delta pbp2b<>aad9$ from IU7397)	Spc ^R	This study
IU7570	D39 $\Delta cps \Delta pbp2b<>aad9 sup4$ (IU1945 X $\Delta pbp2b<>aad9$ from IU7397)	Spc ^R	This study
IU7580	D39 $\Delta cps mreC-L-FLAG^3-P_c-erm mltG-HA-P_c-kan$ (IU4970 X <i>mltG-HA-P_c-kan</i> from IU7399)	Kan ^R Erm ^R	This study
IU7582	D39 $\Delta cps mreC-L-FLAG^3-P_c-erm mltG-Myc-P_c-kan$ (IU4970 X <i>mltG-Myc-P_c-kan</i> from IU7405)	Kan ^R Erm ^R	This study
IU7614	D39 $\Delta cps rpsL1 ftsZ^+-P_c-[kan-rpsL^+]$ (IU1824 X fusion <i>ftsZ^+-P_c-[kan-rpsL^+]</i>)	Kan ^R Str ^S	This study
IU7765	D39 $\Delta cps \Delta pbp2b<>aad9 sup5$ (IU1945 X $\Delta pbp2b<>aad9$ from IU7397)	Spc ^R	This study

IU7837	D39 $\Delta cps rpsL1 pbp1a$ (G494E) (IU6726 X <i>pbp1a</i> allele from IU7258)	Str ^R Kan ^S	This study
IU7839	D39 $\Delta cps rpsL1 pbp1a$ (ΔG at G451) (IU6726 X <i>pbp1a</i> allele from IU7260)	Str ^R Kan ^S	This study
IU7840	D39 $\Delta cps rpsL1 pbp1a$ (S89F) (IU6726 X <i>pbp1a</i> allele from IU7286)	Str ^R Kan ^S	This study
IU7843	D39 $\Delta cps rpsL1 pbp1a$ (ΔA at K160) (IU6726 X <i>pbp1a</i> allele from IU7287)	Str ^R Kan ^S	This study
IU7845	D39 $\Delta cps rpsL1 pbp1a$ (ΩT at F33) (IU6726 X <i>pbp1a</i> allele from IU7288)	Str ^R Kan ^S	This study
IU7850	D39 $\Delta cps \Delta pbp1b::P_c-[kan-rpsL^+]$ IU1824 X $\Delta pbp1b::P_c-[kan-rpsL^+]$ from K180	Kan ^R Str ^S	This study
IU7852	D39 $\Delta cps \Delta pbp2a::P_c-[kan-rpsL^+]$ IU1824 X $\Delta pbp2a::P_c-[kan-rpsL^+]$ from K166	Kan ^R Str ^S	This study
IU7931	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm$ $\Delta pbp2b \leftrightarrow aad9$ (IU7327 X $\Delta pbp2b \leftrightarrow aad9$ from IU7397)	Erm ^R Str ^R	This study
IU8549	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 5bp)$ (IU7325 X <i>mltG</i> ($\Delta 5bp$) from IU7477)	Str ^R Kan ^S	This study
IU8551	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(Y488D)$ (IU7325 X <i>mltG</i> (Y488D) from IU7567)	Str ^R Kan ^S	This study
IU8553	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 488bp)$ (IU7325 X <i>mltG</i> ($\Delta 488bp$) from IU7570)	Str ^R Kan ^S	This study
IU8555	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Omega 45bp)^2$ (IU7325 X <i>mltG</i> ($\Omega 45bp$) ² from IU7765)	Str ^R Kan ^S	This study
IU8565	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 5bp)$ $\Delta pbp2b \leftrightarrow aad9$ (IU8549 X $\Delta pbp2b \leftrightarrow aad9$ from IU7397)	Str ^R Kan ^S Spc ^R	This study
IU8567	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 488bp)$ $\Delta pbp2b \leftrightarrow aad9$ (IU8553 X $\Delta pbp2b \leftrightarrow aad9$ from IU7397)	Str ^R Kan ^S Spc ^R	This study
IU8569	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Omega 45bp)^2$ $\Delta pbp2b \leftrightarrow aad9$ (IU8555 X $\Delta pbp2b \leftrightarrow aad9$ from IU7397)	Str ^R Kan ^S Spc ^R	This study
IU8872	D39 $\Delta cps \Delta bgaA::tet-P_{Zn^-}RBS_{mltG^-}mltG^+$ (IU1945 X fusion $\Delta bgaA::tet-P_{Zn^-}$ $RBS_{mltG^-}mltG^+$)	Tet ^R	This study
IU8873	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(E428Q)$ (IU7325 X fusion <i>mltG</i> (E428Q))	Str ^R Kan ^S	This study
IU8910	D39 $\Delta cps rpsL1 \Delta pbp1a$ <i>mltG</i> (ΔDUF_1346) (IU7325 X fusion <i>mltG</i> (ΔDUF_1346))	Str ^R Kan ^S	This study

IU8964	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(E428Q) \Delta pbp2b \langle \rightarrow aad9$ (IU8873 X $\Delta pbp2b \langle \rightarrow aad9$ from IU7397)	Str ^R Kan ^S Spc ^R	This study
IU8980	D39 $\Delta cps rpsL1 P_c-[kan-rpsL^+]-mltG^+$ (IU1824 X fusion $P_c-[kan-rpsL^+]-mltG^+$)	Kan ^R Str ^S	This study
IU8982	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(E428A)$ (IU7325 X fusion $mltG(E428A)$)	Str ^R Kan ^S	This study
IU8986	D39 $\Delta cps rpsL1 mltG^+-P_c-[kan-rpsL^+]$ (IU1824 X fusion $mltG^+-P_c-[kan-rpsL^+]$)	Kan ^R Str ^S	This study
IU9025	D39 $\Delta cps rpsL1 mltG(\Delta DUF_1346) pbp1a^+$ (IU8980 X $mltG(\Delta DUF_1346)$ from IU8910)	Str ^R Kan ^S	This study
IU9041	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(E428Q)-FLAG-P_c-erm$ (IU7325 X fusion $mltG(E428Q)-FLAG-P_c-erm$)	Erm ^R Str ^R	This study
IU9043	D39 $\Delta cps rpsL1 \Delta pbp1a mltG-FLAG-P_c-erm$ (IU7325 X $mltG-FLAG-P_c-erm$ from IU7403)	Erm ^R Str ^R	This study
IU9100	D39 $\Delta cps \Delta mltG::P_c-erm//\Delta bgaA::tet-P_{Zn}-RBS_{mltG}-mltG^+$ (IU8872 X $\Delta mltG::P_c-erm$ from IU7260)	Tet ^R Erm ^R	This study
IU9102	D39 $\Delta cps \Delta mltG::P_c-aad9//\Delta bgaA::tet-P_{Zn}-RBS_{mltG}-mltG^+$ (IU8872 X fusion $\Delta mltG::P_c-aad9$)	Tet ^R Spc ^R	This study
IU9148	D39 $\Delta cps rpsL1 ftsZ-L_2-mKate2$ (IU7614 X fusion $ftsZ-L_2-mKate2$)	Str ^R Kan ^S	This study
IU9231	D39 $\Delta cps rpsL1 \Delta walk::P_c-[kan-rpsL^+]$ (IU1824 X $\Delta walk::P_c-[kan-rpsL^+]$ from K208)	Kan ^R Str ^S	This study
IU9233	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta walk::P_c-[kan-rpsL^+]$ (IU6741 X $\Delta walk::P_c-[kan-rpsL^+]$ from K208)	Kan ^R Str ^S	This study
IU9235	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta walk::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta walk::P_c-[kan-rpsL^+]$ from K208)	Kan ^R Erm ^R Str ^S	This study
IU9278	D39 $\Delta cps rpsL1 \Delta pmp23::P_c-[kan-rpsL^+]$ (IU1824 X $\Delta pmp23::P_c-[kan-rpsL^+]$ from K37)	Kan ^R Str ^S	This study
IU9290	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta pmp23::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta pmp23::P_c-[kan-rpsL^+]$ from K37)	Kan ^R Str ^S	This study
IU9292	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta spd_1874::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta spd_1874::P_c-[kan-rpsL^+]$ from K57)	Kan ^R Str ^S	This study

IU9294	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta lytC::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta lytC::P_c-[kan-rpsL^+]$ from K5)	Kan ^R Str ^S	This study
IU9296	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta spd_0873<>P_c-[kan-rpsL^+]$ (IU7327 X $\Delta spd_0873<>P_c-[kan-rpsL^+]$ from K29)	Kan ^R Str ^S	This study
IU9298	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta lytB::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta lytB::P_c-[kan-rpsL^+]$ from K27)	Kan ^R Str ^S	This study
IU9316	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta spd_0104::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta spd_0104::P_c-[kan-rpsL^+]$ from K15)	Kan ^R Str ^S	This study
IU9318	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta spd_0703::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta spd_0703::P_c-[kan-rpsL^+]$ from K489)	Kan ^R Str ^S	This study
IU9320	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta pspA::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta pspA::P_c-[kan-rpsL^+]$ from K75)	Kan ^R Str ^S	This study
IU9330	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 488bp) \Delta bgaA'::kant1t2-P_{fcsK^-} pcsB^+$ (IU8553 X $\Delta bgaA'::kant1t2-P_{fcsK^-} pcsB^+$ from IU2519)	Kan ^R Str ^R	This study
IU9382	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta lytA::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta lytA::P_c-[kan-rpsL^+]$ from K43)	Kan ^R Str ^S	This study
IU9384	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta cbpD::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta cbpD::P_c-[kan-rpsL^+]$ from K148)	Kan ^R Str ^S	This study
IU9386	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta dacA::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta dacA::P_c-[kan-rpsL^+]$ from K35)	Kan ^R Str ^S	This study
IU9388	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta dacB::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta dacB::P_c-[kan-rpsL^+]$ from K25)	Kan ^R Str ^S	This study
IU9390	D39 $\Delta cps rpsL1 \Delta pbp1a \Delta mltG::P_c-erm \Delta spd_0173::P_c-[kan-rpsL^+]$ (IU7327 X $\Delta spd_0173::P_c-[kan-rpsL^+]$ from K372)	Kan ^R Str ^S	This study
IU9445	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(E428A)-FLAG-P_c-erm$ (IU7325 X fusion $mltG(E428A)-FLAG-P_c-erm$)	Erm ^R Str ^R	This study
IU9759	D39 $\Delta cps rpsL1 \Delta pbp1a::P_c-[kan-rpsL^+] mltG(\Delta 5bp) \Delta pbp2b<>aad9$ (IU8565 X $\Delta pbp1a::P_c-[kan-rpsL^+]$ from K164)	Kan ^R Str ^S Spc ^R	This study
IU9760	D39 $\Delta cps rpsL1 mltG(Y488D)$ (IU8986)	Str ^R Kan ^S	This study

	X <i>mltG</i> (Y488D) from IU7567)		
IU9765	D39 $\Delta cps \Delta bgaA::tet-P_{Zn}-RBS_{ftsA^-}$ <i>rodZ</i> (<i>spd_2050</i>) ⁺ (IU1945 X fusion $\Delta bgaA::tet-P_{Zn}-RBS_{ftsA^-}$ <i>rodZ</i>)	Tet ^R	This study
IU9771	D39 <i>cps</i> ⁺ $\Delta mltG::P_c-erm$ (IU1690 X $\Delta mltG::P_c-erm$ from E681), <i>pbp1a</i> sequenced to contain no mutation	Erm ^R	This study
IU9772	D39 <i>cps</i> ⁺ $\Delta mltG::P_c-erm$ (IU1690 X $\Delta mltG::P_c-erm$ from E681), second isolate, <i>pbp1a</i> sequenced to contain no mutation	Erm ^R	This study
IU9777	D39 $\Delta cps rpsL1 pbp1a^+ mltG(\Delta 5bp)$ $\Delta pbp2b \leftrightarrow aad9$ (IU9759 X <i>pbp1a</i> ⁺ from IU1690)	Str ^R Kan ^S Spc ^R	This study
IU9783	D39 $\Delta cps rpsL1 mltG(Y488D)$ $\Delta pbp2b \leftrightarrow aad9$ (IU9760 X $\Delta pbp2b \leftrightarrow aad9$ from IU7397)	Str ^R Kan ^S Spc ^R	This study
IU9877	D39 $\Delta cps rpsL1 \Delta pbp1a::P_c-[kan-rpsL^+]$ <i>mltG</i> ($\Delta 488bp$) $\Delta pbp2b \leftrightarrow aad9$ (IU8567 X $\Delta pbp1a::P_c-[kan-rpsL^+]$ from K164)	Kan ^R Str ^S Spc ^R	This study
IU9879	D39 $\Delta cps rpsL1 \Delta pbp1a::P_c-[kan-rpsL^+]$ <i>mltG</i> ($\Omega 45bp$) ² $\Delta pbp2b \leftrightarrow aad9$ (IU8569 X $\Delta pbp1a::P_c-[kan-rpsL^+]$ from K164)	Kan ^R Str ^S Spc ^R	This study
IU9895	D39 $\Delta cps mltG(Y488D)-P_c-erm$ (IU1945 X fusion <i>mltG</i> (Y488D)- <i>P_c-erm</i>)	Erm ^R	This study
IU9897	D39 <i>cps</i> ⁺ $\Delta mltG::P_c-erm$ $\Delta pbp2b \leftrightarrow aad9$ (IU9711 X $\Delta pbp2b \leftrightarrow aad9$ from IU7397)	Erm ^R Spc ^R	This study
IU9899	D39 <i>cps</i> ⁺ $\Delta mltG::P_c-erm \Delta cps2E::P_c-$ $[kan-rpsL^+]$ <i>pbp1a</i> ($\Delta T@F33$) (IU9771 X $\Delta cps2E::P_c-[kan-rpsL^+]$ from IU3286), <i>pbp1a</i> sequenced to acquire a spontaneous $\Delta T@F33$ mutation	Erm ^R Kan ^R	This study
IU9905	D39 $\Delta cps rpsL1 pbp1a^+ mltG(\Delta 488bp)$ $\Delta pbp2b \leftrightarrow aad9$ (IU9877 X <i>pbp1a</i> ⁺ from IU1690)	Str ^R Kan ^S Spc ^R	This study
IU9907	D39 $\Delta cps rpsL1 pbp1a^+ mltG(\Omega 45bp)^2$ $\Delta pbp2b \leftrightarrow aad9$ (IU9879 X <i>pbp1a</i> ⁺ from IU1690)	Str ^R Kan ^S Spc ^R	This study
IU9931	D39 Δcps $\Delta rodZ$ (<i>spd_2050</i>) $\leftrightarrow aad9//\Delta bgaA::tet-$ $P_{Zn}-RBS_{ftsA^-}$ <i>rodZ</i> ⁺ (IU9765 X fusion $\Delta rodZ \leftrightarrow aad9$)	Tet ^R Spc ^R	This study
IU10021	D39 <i>cps</i> ⁺ $\Delta mltG::P_c-erm \Delta pbp1a::P_c-$	Erm ^R	This study

	[<i>kan-rpsL</i> ⁺](IU9711 X Δ <i>pbp1a</i> ::P _c -[<i>kan-rpsL</i> ⁺] from K164))	Kan ^R	
IU10048	D39 <i>cps</i> ⁺ Δ <i>mltG</i> ::P _c - <i>erm</i> Δ <i>cps2E</i> :: P _c -[<i>kan-rpsL</i> ⁺] <i>pbp1a</i> (G559stop) (IU9771 X Δ <i>cps2E</i> :: P _c -[<i>kan-rpsL</i> ⁺] from IU3286), <i>pbp1a</i> sequenced to acquire a G559stop (GGA→TGA) mutation	Erm ^R Kan ^R	This study
IU10228	D39 Δ <i>cps rpsL1 gfp-L₁-mltG</i> (IU8980 X fusion <i>gfp-L₁-mltG</i>)	Str ^R Kan ^S	This study
IU10292	D39 Δ <i>cps rpsL1</i> Δ <i>pbp1a</i> Δ <i>mltG</i> ::P _c - <i>erm</i> Δ <i>spd_1874</i> ::P _c -[<i>kan-rpsL</i> ⁺] Δ <i>spd_0104</i> ::P _c - <i>cat</i> (IU9292 X fusion Δ <i>spd_0104</i> ::P _c - <i>cat</i>)	Kan ^R Str ^S Cm ^R	This study
IU10294	D39 Δ <i>cps rpsL1</i> Δ <i>pbp1a</i> Δ <i>mltG</i> ::P _c - <i>erm</i> Δ <i>spd_0104</i> ::P _c -[<i>kan-rpsL</i> ⁺] Δ <i>spd_1874</i> ::P _c - <i>cat</i> (IU9316 X fusion Δ <i>spd_1874</i> ::P _c - <i>cat</i>)	Kan ^R Str ^S Cm ^R	This study
IU10318	D39 Δ <i>cps rpsL1 gfp-L₁-mltG ftsZ</i> ⁺ -P _c -[<i>kan-rpsL</i> ⁺] (IU10228 X <i>ftsZ</i> ⁺ -P _c -[<i>kan-rpsL</i> ⁺] from IU7614)	Kan ^R Str ^S	This study
IU10320	D39 Δ <i>cps rpsL1</i> Δ <i>pbp1a mltG</i> (Δ 5bp)-FLAG-P _c - <i>erm</i> (IU7325 X fusion <i>mltG</i> (Δ 45bp) ² -FLAG-P _c - <i>erm</i>)	Erm ^R Str ^R	This study
IU10323	D39 Δ <i>cps rpsL1</i> Δ <i>pbp1a mltG</i> (Y488D)-FLAG-P _c - <i>erm</i> (IU7325 X fusion <i>mltG</i> (Y488D)-FLAG-P _c - <i>erm</i>)	Erm ^R Str ^R	This study
IU10324	D39 Δ <i>cps rpsL1</i> Δ <i>pbp1a mltG</i> (Δ 488bp)-FLAG-P _c - <i>erm</i> (IU7325 X fusion <i>mltG</i> (Δ 488bp)-FLAG-P _c - <i>erm</i>)	Erm ^R Str ^R	This study
IU10327	D39 Δ <i>cps rpsL1</i> Δ <i>pbp1a mltG</i> (Ω 45bp) ² -FLAG-P _c - <i>erm</i> (IU7325 X fusion <i>mltG</i> (Ω 45bp) ² -FLAG-P _c - <i>erm</i>)	Erm ^R Str ^R	This study
IU10342	D39 Δ <i>cps rpsL1 mltG</i> (E428Q) (IU8986 X <i>mltG</i> (E428Q) from IU8873. This strain was sequenced to be <i>pbp1a</i> ⁺ , but grew poorly and was genetically unstable	Str ^R Kan ^S	This study
IU10353	D39 Δ <i>cps rpsL1 gfp-L₁-mltG ftsZ-L₂-mKate2</i> (IU10318 X <i>ftsZ-L₂-mKate2</i> from IU9148)	Str ^R Kan ^S	This study
IU10369	D39 Δ <i>cps pbp1a</i> ⁺ <i>mltG</i> (Y488D)-FLAG-P _c - <i>erm</i> (IU1945 X <i>mltG</i> (Y488D)-FLAG-P _c - <i>erm</i> from IU10323), <i>pbp1a</i> was sequence-confirmed to be <i>pbp1a</i> ⁺	Erm ^R	This study
IU10628	D39 Δ <i>cps</i> Δ <i>pbp1a</i>	Str ^R Kan ^S	This study

	$\Delta rodZ(sp d_{2050}) \langle \rightarrow aad9$ (IU6741 X $\Delta rodZ \langle \rightarrow aad9$ from IU9931)	Spc^R	
IU10651	D39 $\Delta cps pbp1a^+ mltG(Y488D)$ $\Delta mreCD \langle \rightarrow aad9$ (IU9760 X $\Delta mreCD \langle \rightarrow aad9$ from IU1751), $pbp1a$ was sequence-confirmed to be $pbp1a^+$	$Str^R Kan^S$ Spc^R	This study
IU10656	D39 $\Delta cps pbp1a^+ mltG(Y488D)$ $\Delta rodZ(sp d_{2050}) \langle \rightarrow aad9$ (IU9760 X $\Delta rodZ \langle \rightarrow aad9$ from IU9931), $pbp1a$ was sequence-confirmed to be $pbp1a^+$	$Str^R Kan^S$ Spc^R	This study
IU10731	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 488bp)$ $\Delta mreCD \langle \rightarrow P_c-erm$ (IU8553 X $\Delta mreCD \langle \rightarrow P_c-erm$ from IU3897)	$Str^R Kan^S$ Erm^R	This study
IU10743	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 488bp)$ $\Delta pbp2b \langle \rightarrow aad9 \Delta mreCD \langle \rightarrow P_c-erm$ (IU8567 X $\Delta mreCD \langle \rightarrow P_c-erm$ from IU3897)	$Str^R Kan^S$ $Spc^R Erm^R$	This study
IU10783	D39 $\Delta cps rpsL1 mltG(Y488D)$ $\Delta pbp2b \langle \rightarrow aad9 \Delta rodZ(sp d_{2050})::P_c-[kan-rpsL^+]$ (IU9783 X $\Delta rodZ::P_c-[kan-rpsL^+]$ from K654)	$Str^R Spc^R$ Kan^R	This study
IU10785	D39 $\Delta cps rpsL1 \Delta pbp1a mltG(\Delta 488bp)$ $\Delta mreCD \langle \rightarrow P_c-erm$ $\Delta rodZ(sp d_{2050})::P_c-[kan-rpsL^+]$ (IU10731 X $\Delta rodZ::P_c-[kan-rpsL^+]$ from K654)	$Str^R Erm^R$ Kan^R	This study
IU10829	D39 $\Delta cps rpsL1 mltG(Y488D)$ $\Delta walk::P_c-[kan-rpsL^+]$ (IU9760 X $\Delta walk::P_c-[kan-rpsL^+]$ from K208)	$Kan^R Str^S$	This study
IU10919	D39 $\Delta cps rpsL1 mltG_{Spn-Eco} P_c-erm$ (IU1824 X fusion $mltG_{Spn-Eco} P_c-erm$)	Erm^R	This study
IU10921	D39 $\Delta cps \Delta bgaA::tet-P_{Zn}-RBS_{rodA-rodA^+}$ (IU1945 X fusion $\Delta bgaA::tet-P_{Zn}-RBS_{rodA-rodA}$)	Tet^R	This study
IU10943	D39 $\Delta cps rpsL1 mltG(Y488D)$ $\Delta rodA::P_c-erm$ (IU9760 X fusion $\Delta rodA::P_c-erm$)	Erm^R	This study
IU10945	D39 $\Delta cps rpsL1 mltG(Y488D)$ $\Delta rodA::P_c-[kan-rpsL^+]$ (IU9760 X fusion $\Delta rodA::P_c-[kan-rpsL^+]$)	Kan^R	This study
IU10965	D39 $\Delta cps rpsL1 \Delta pbp1a mltG_{Spn-Eco} P_c-erm$ (IU6741 X $mltG_{Spn-Eco} P_c-erm$ from IU10919)	$Str^R Kan^S$ Erm^R	This study
IU11007	D39 $\Delta cps rpsL1 mltG_{Eco} P_c-erm$	Erm^R	This study

	(IU1824 X fusion <i>mltG</i> _{Eco} -P _c - <i>erm</i>)		
IU11009	D39 Δ <i>cps rpsL1</i> Δ <i>pbp1a</i> <i>mltG</i> _{Eco} -P _c - <i>erm</i> (IU6741 X fusion <i>mltG</i> _{Eco} -P _c - <i>erm</i>)	Erm ^R	This study
E177	D39 Δ <i>cps</i> Δ <i>pbp1a</i> ::P _c - <i>erm</i>	Erm ^R	Land <i>et al.</i> , 2013
E193	D39 Δ <i>cps</i> Δ <i>pbp1b</i> ::P _c - <i>erm</i>	Erm ^R	Land & Winkler, 2011
E655	D39 Δ <i>cps</i> Δ <i>rodZ</i> (<i>spd_2050</i>)::P _c - <i>erm</i> , (likely has suppressor) (IU1945 X fusion Δ <i>rodZ</i> ::P _c - <i>erm</i>)	Erm ^R	This study
E681	D39 Δ <i>cps</i> Δ <i>mltG</i> ::P _c - <i>erm</i> (IU1945 X fusion Δ <i>mltG</i> ::P _c - <i>erm</i>)	Erm ^R	This study
K5	D39 Δ <i>cps</i> Δ <i>lytC</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>lytC</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K15	D39 Δ <i>cps</i> Δ <i>spd_0104</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>spd_0104</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K25	D39 Δ <i>cps</i> Δ <i>dacB</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>dacB</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K27	D39 Δ <i>cps</i> Δ <i>lytB</i> >P _c -[<i>kan-rpsL</i> ⁺] (Regrowth of IU3877)	Kan ^R	This study
K29	D39 Δ <i>cps</i> Δ <i>spd_0873</i> ::P _c -[<i>kan-rpsL</i> ⁺] (Regrowth of IU3878)	Kan ^R	This study
K35	D39 Δ <i>cps</i> Δ <i>dacA</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>dacA</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K37	D39 Δ <i>cps</i> Δ <i>pmp23</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>pmp23</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K43	D39 Δ <i>cps</i> Δ <i>lytA</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>lytA</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K57	D39 Δ <i>cps</i> Δ <i>spd_1874</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>spd_1874</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K75	D39 Δ <i>cps</i> Δ <i>pspA</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>pspA</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K148	D39 Δ <i>cps</i> Δ <i>cbpD</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>cbpD</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K164	D39 Δ <i>cps</i> Δ <i>pbp1a</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 transformed with fusion Δ <i>pbp1a</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K166	D39 Δ <i>cps</i> Δ <i>pbp2a</i> ::P _c -[<i>kan-rpsL</i> ⁺] (IU1945 X fusion Δ <i>pbp2a</i> ::P _c -[<i>kan-rpsL</i> ⁺])	Kan ^R	This study
K180	D39 Δ <i>cps</i> Δ <i>pbp1b</i> ::P _c -[<i>kan-rpsL</i> ⁺]	Kan ^R	Tsui <i>et al.</i> , 2014

K208	D39 $\Delta cps \Delta walk::P_c-[kan-rpsL^+]$ (IU1945 X fusion $\Delta walk::P_c-[kan-rpsL^+]$)	Kan ^R	This study
K372	D39 $\Delta cps \Delta spd_0173::P_c-[kan-rpsL^+]$ (IU1945 X fusion $\Delta spd_0173::P_c-[kan-rpsL^+]$)	Kan ^R	This study
K489	D39 $\Delta cps \Delta spd_0703::P_c-[kan-rpsL^+]$ (IU1945 X fusion $\Delta spd_0703::P_c-[kan-rpsL^+]$)	Kan ^R	This study
K637	D39 $\Delta cps \Delta mltG::P_c-[kan-rpsL^+]$ (IU1945 X fusion $\Delta mltG::P_c-[kan-rpsL^+]$)	Kan ^R	This study
K654	D39 $\Delta cps \Delta rodZ(spdc_2050)::P_c-[kan-rpsL^+]$ (likely has suppressor) (IU1945 X fusion $\Delta rodZ::P_c-[kan-rpsL^+]$)	Kan ^R	This study

^aStrains were constructed as described in *Experimental procedures and above*.

^bPrimers used to synthesize fusion amplicons are listed in Table S2. FLAG-tagged (FLAG), c-Myc-tagged (Myc), and HA-tagged (HA) fusions were made to the carboxyl-end of MltG. The amino acid sequences of the FLAG, Myc, and HA epitope tags are DYKDDDDK (Hopp *et al.*, 1988, Wayne *et al.*, 2010), EQKLISEEDL (Evan *et al.*, 1985), and YPYDVPDYA (Wilson *et al.*, 1984), respectively. FLAG³ indicates three tandem sequences of the FLAG epitope, and L in *mreC-L-FLAG*³ refers to a 10-amino-acid spacer linker (GSAGSAAGSG) (Waldo *et al.*, 1999; Wayne *et al.*, 2010)). L₁ linker sequence in *gfp-L₁-mltG* is LEGSG (Fleurie *et al.*, 2014). The DNA template for *gfp* is pUC57-*gfp*(*Sp*) (Martin *et al.*, 2010), which was codon optimized for *S pneumoniae* and contains aa substitution (A206K) to prevent GFP dimerization. L₂-linker sequence in *ftsZ-L2-mKate2* is KLDIEFLQ (Fleurie *et al.*, 2014). mKate2 is a far red monomeric fluorescent protein with codon optimized for *S. pneumoniae* (Beilharz *et al.*, 2015).

^cAntibiotic resistance markers: Erm^R, erythromycin; Kan^R, kanamycin; Spc^R, spectinomycin; Str^R, streptomycin; Cm^R, chloramphenicol; Tet^R, tetracycline.

TABLE S2. Oligonucleotide primers used in this study (order follows Table S1)

Primer	Sequence (5' to 3')	Template ^a	Amplicon Product
For construction of IU6741 ($\Delta pbp1a$)			
P234	CCCTTGTGTTTCATAGCGAGGATAAGCA	D39	5' upstream fragment with 60 bp of 5' <i>pbp1a</i>
SV022	TCAGGGGTTGTATTTGATTGTTGCAAGCTTAAGAAGCTA ATGCTCAGATACTTG		
SV023	CTGAGCATTAGCTTCTTAAGCTTGCAACAATCAAATACA ACCCCTGATCAA	D39	60 bp of 3' <i>pbp1a</i> and 3' downstream fragment
P235	AGGCAAGCCTGCAACCATGGTCTTGAAA		
For construction of IU7399 (<i>mltG</i>-HA-P_c-<i>kan</i>)			
TT507	TCAGCAGGTGGTTACTTTGGTTACCAGTACG	D39	5' <i>mltG</i> ⁺ flanking fragment
TT519	GGTTAAGCATAATCTGGAACATCATATGGATAGTTTAAT TTGCTGTTGACATGTTTCAGCG		
TT520	CGCTGAACATGTCAACAGCAAATTAACATATCCATATGA TGTTCCAGATTATGCTTAACC	IU6929 ^b	HA- P_c - <i>kan</i>
TT517	ACATAATTTTAGTTTTGTTTACTAAAACAATTCATCCAGTA AAATATAATTTTTATTTTC		
TT518	TTACTGGATGAATTGTTTTAGTAAACAACTAAAATTATG TGATACTTCACATAATTTTC	D39	Intergenic between <i>mltG</i> and <i>greA</i> , and <i>greA</i>
TT508	GAGCAAACCTAGGAACTAGCCGCAGGTTG		
For construction of IU7403 (<i>mltG</i>-FLAG-P_c-<i>erm</i>)			
TT507	TCAGCAGGTGGTTACTTTGGTTACCAGTACG	D39	5' <i>mltG</i> ⁺ flanking fragment
TT509	CGGTTATTTATCATCATCATCTTTATAATCGTTTAATTTG CTGTTGACATGTTTCAGCG		
TT510	TGAACATGTCAACAGCAAATTAACGATTATAAAGATGA TGATGATAAATAACCGGG	IU6565 ^c	FLAG- P_c - <i>erm</i>
TT511	AGTATCACATAATTTTAGTTTTGTTTATTTCTCCCGTTAA ATAATAGATAACTATTAATAA		
TT512	ATTATTTAACGGGAGGAAATAAACAACAACTAAAATTATGT GATACTTCACATAATTTTCTT	D39	Intergenic between <i>mltG</i> and <i>greA</i> , and <i>greA</i>
TT508	GAGCAAACCTAGGAACTAGCCGCAGGTTG		
For construction of IU7405 (<i>mltG</i>-Myc-P_c-<i>kan</i>)			
TT507	TCAGCAGGTGGTTACTTTGGTTACCAGTACG	D39	5' <i>mltG</i> ⁺ flanking fragment
TT515	GATCTTCTTCAGAAATAAGTTTTTGTTCGTTTAATTTGCT GTTGACATGTTTCAGCG		
TT516	TGAACATGTCAACAGCAAATTAACGAACAAAACTTAT TTCTGAAGAAGATCTTTAACC	IU6962 ^d	Myc- P_c - <i>kan</i>
TT517	ACATAATTTTAGTTTTGTTTACTAAAACAATTCATCCAGTA AAATATAATTTTTATTTTC		
TT518	TTACTGGATGAATTGTTTTAGTAAACAACTAAAATTATG TGATACTTCACATAATTTTC	D39	Intergenic between <i>mltG</i> and <i>greA</i> , and <i>greA</i>
TT508	GAGCAAACCTAGGAACTAGCCGCAGGTTG		

For construction of IU7614 (<i>ftsZ</i>⁺-P_c-[<i>kan-rpsL</i>⁺])			
TT165	AGTGGTGCCGATATGGTCTTCATCACTGCT	D39	<i>ftsZ</i> ⁺
TT583	CATTATCCATTAATAAATCAAACGGATCCTATTAACGATTT TTGAAAAATGGAGGTGTATC		
Kan <i>rpsL</i> forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c -[<i>kan-rpsL</i> ⁺] cassette ^e	P _c -[<i>kan-rpsL</i> ⁺]
Kan <i>rpsL</i> reverse	GGGCCCTTTCCCTTATGCTTTTG		
TT584	CAAAAGCATAAGGAAAGGGCCCGCCCAATTTCAAA GATGAAGATG	D39	60 bp of 3' <i>ftsZ</i> - <i>ylmE</i> ⁺
TT166	TCATTGGGAGAGCCGGTTCCTGTGAAGAAT		
For construction of IU8872 (Δ<i>bgaA</i>::<i>tet</i>-P_{<i>czcD</i>}-RBS_{<i>mltG</i>}-<i>mltG</i>)			
TT657	CGCCCCAAGTTCATCACCAATGACATCAAC	pJWV25 ^f	5' <i>bgaA</i> ':: <i>tet</i> -P _{<i>czcD</i>}
TT681	AGTTTTTCCTCCTTGTTGATAATTTGTTATAATAGATTTA TGAACACCTTGTTCAATATC		-
TT682	ACAAGGTGTTTCATAAATCTATTATAACAAATTATCAACAA GGAGGAAAACTTTTGAGTG	D39	RBS(_{<i>mltG</i>})- <i>mltG</i>
TT679	CAACTGGTTTATGAGAAAGTAAGTTCCTTTAGTTTAATTT GCTGTTGACATGTTTCAGC		
TT680	GAACATGTCAACAGCAAATTAACATAAAGAACTTACTT TCTCATAAACAGTTGCTG	D39	3' <i>bgaA</i> ' flanking
CS121	GCTTTCTTGAGGCAATTCACCTGGTGC		
For construction of IU8873 (<i>mltG</i>(E428Q))			
P1348	TCTTCTTGACGCCCTTGAAAGAGGTGGCAGT	D39	<i>spd_1347</i> -5' <i>mltG</i> fragment with <i>mltG</i> (E428Q)(GA A to CAA)
TT706	TCTGTCTTGGCACCTTGTTTTTCGACCAAGGAAGCAATG G		
TT707	CTTGGTGCAAAAACAAGGTGCCAAGACAGAAGATCGTA AG	D39	3' <i>mltG</i> fragment with <i>mltG</i> (E428Q)(GA A to CAA)- <i>greA</i>
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		
For construction of IU8910 (<i>mltG</i>(Δ DUF_1346))			
P1348	TCTTCTTGACGCCCTTGAAAGAGGTGGCAGT	D39	5' upstream fragment with 98 bp of 5' <i>mltG</i>
TT708	CGTGATGTATCTCTGATGATATCCACTACTTTTTCTAAAT CTCTCAGAATCTGCTCTTT		
TT709	GCAGATTCTGAGAGATTTAGAAAAAGTAGTGGATATCAT CAGAGATACATCACGTCG	D39	1213 bp of 3' <i>mltG</i> and 3' downstream fragment
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		
For construction of IU8980 (P_c-[<i>kan-rpsL</i>⁺]-<i>mltG</i>⁺)			
P1348	TCTTCTTGACGCCCTTGAAAGAGGTGGCAGT	D39	<i>spd_1347</i> ⁺
TT730	CATTATCCATTAATAAATCAAACGGATCCTATTATTTTTTC ATTTCCAGACCATATCATA		
Kan <i>rpsL</i> forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c -[<i>kan-rpsL</i> ⁺] cassette ^e	P _c -[<i>kan-rpsL</i> ⁺]
Kan <i>rpsL</i> reverse	GGGCCCTTTCCCTTATGCTTTTG		

TT731	CAAAAGCATAAGGAAAGGGGCCCGTTAGGAAAAGTATC ATAGATTGCTTTTTTTGGACTT	D39	80 bp upstream of <i>mltG</i> and 5' of <i>mltG</i> ⁺
TT412	CGACCAAGGAAGCAATGGTCAACAACATCAT		
For construction of IU8982 (<i>mltG</i>(E428A))			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	D39	<i>spd</i> _1347 ⁻ -5' <i>mltG</i> fragment with <i>mltG</i> (E428A)(GA A to GCA)
TT728	TCTGTCTTGGCACCTGCTTTTTCGACCAAGGAAGCAAT GG		
TT729	CTTGGTCGAAAAAGCAGGTGCCAAGACAGAAGATCGTA AG	D39	3' <i>mltG</i> fragment with <i>mltG</i> (E428A)(GA A to GCA)- <i>greA</i>
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		
For construction of IU8986 (<i>mltG</i>⁺-P_c-[<i>kan-rpsL</i>⁺])			
TT415	AAGCAGAACAAGCAGGTCCAGAAACACCTACG	D39	3' <i>mltG</i>
TT726	CATTATCCATTAATAAATCAAACGGATCCTATTAGTTTAAT TTGCTGTTGACATGTTTCAGC		
Kan <i>rpsL</i> forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c -[<i>kan- rpsL</i> ⁺] cassette ^e	P _c -[<i>kan-rpsL</i> ⁺]
Kan <i>rpsL</i> reverse	GGGCCCTTTCCTTATGCTTTTG		
TT727	CAAAAGCATAAGGAAAGGGGCCACAACTAAAATTAT GTGATACTTCACATAATTTTCT	D39	Intergenic between <i>mltG</i> and <i>greA</i> , and <i>greA</i>
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		
For construction of IU9041 (<i>mltG</i>(E428Q)-FLAG-P_c-<i>erm</i>)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	D39	<i>spd</i> _1347-5' <i>mltG</i> fragment with <i>mltG</i> (E428Q)(GA A to CAA)
TT706	TCTGTCTTGGCACCTTGTTTTTCGACCAAGGAAGCAATG G		
TT707	CTTGGTCGAAAAACAAGGTGCCAAGACAGAAGATCGTA AG	IU7403 ^g	3' <i>mltG</i> fragment with <i>mltG</i> (E428Q)- FLAG-P _c - <i>erm</i> - <i>greA</i>
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		
For construction of IU9102 (Δ<i>mltG</i>::P_c-<i>aad9</i>)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	E681 ^g	5' upstream fragment with 90 bp of 5' <i>mltG</i> and P _c
TT688	GTATTCAAATATATCCTCCTCATTTATTATTTTCTTCTCCTC TTTTCTACAGTATTTAAAGA		
TT689	ACTGTAGAAAAGAGGAAGGAAATAATAATGAGGAGGA TATATTTGAATACATACGAACA	IU7397 ^g	<i>aad9</i>
TT690	TTTAGTACCGTATTATAATTTTTTTAATCTGTTATTTAAAT AGTTTATAGTTAAATTTAC		
TT691	CTATTTAAATAACAGATTAATAAATAATAATACGGTACT AAACGTCCAAAAGCATAAAGG	E681 ^g	90 bp of 3' <i>mltG</i> and 3' downstream fragment
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		

For construction of IU9148 (<i>ftsZ</i>-L₂-<i>mKate2</i>)			
TT165	AGTGGTGCCGATATGGTCTTCATCACTGCT	IU8845 ^h	3' <i>ftsZ</i> -L ₂
TT746	TGTGCATATTTTCCTTGATAAGTTCTGACATCTGCAGGA ACTCGATGTCTAGTTTACG		
TT747	AAACTAGACATCGAGTTCCTGCAGATGTCAGAACTTATC AAGGAAAATATGCACA	pKB01_mK ate2 ⁱ	<i>mkate2</i>
TT748	TTAACGGTGTCCCAATTTACTAGGCAAATCAC		
TT749	TTGCCTAGTAAATTGGGACACCGTTAACATTTTCAAAA ATCGTTAAGTAAATGAATGTA	D39	17 bp of 3' <i>ftsZ</i> - <i>ylmE</i> ⁺
TT166	TCATTGGGAGAGCCGTTCTGTGAAGAAT		
For construction of IU9445 (<i>mltG</i>(E428A)-FLAG-P_c-<i>erm</i>)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	D39	<i>spd_1347</i> -5' <i>mltG</i> fragment with <i>mltG</i> (E428A)
TT728	TCTGTCTTGGCACCTGCTTTTTTCGACCAAGGAAGCAAT GG		
TT729	CTTGGTGCAGAAAAGCAGGTGCCAAGACAGAAGATCGTA AG	IU7403 ^g	3' <i>mltG</i> fragment with <i>mltG</i> (E428A)- FLAG-P _c - <i>erm</i> - <i>greA</i>
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		
For construction of IU9765 (Δ<i>bgaA</i>::<i>tet</i>-P_{Zn}-RBS_{<i>ftsA</i>}-<i>rodZ</i>(<i>spd_2050</i>)⁺)			
TT657	CGCCCCAAGTTCATCACCAATGACATCAAC	IU8906 ^l	5' <i>bgaA</i> :: <i>tet</i> -P _{<i>czcD</i>} -RBS _{<i>ftsA</i>}
TT769	CCTCTCCAATTGTTTTTTTTCTCATTACATCGCTTCCTCT CTATCTTCCTTGT		
TT770	GGAAGATAGAGAGGAAGCGATGTAATGAGAAAAAAAC AATTGGAGAGGTTTTAC	D39	<i>rodZ</i>
TT771	ACTGGTTTATGAGAAAGTAAGTTCTTTAATTTTATAGTAA AGGTTACAGTGATTTGTCCA		
TT772	AAATCACTGTAACCTTTACTAAAAATTAAGAAGACTTACT TTCTCATAAACCAGTTGCTG	D39	3' <i>bgaA</i> ' flanking
CS121	GCTTTCTTGAGGCAATTCATTGGTGC		
For construction of IU9895 (<i>mltG</i>(Y488D)-P_c-<i>erm</i>)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	IU7567 ^g	upstream of <i>mltG</i> and <i>mltG</i> (Y488D)
TT726	CATTATCCATTAATAAATCAAACGGATCCTATTAGTTTAAT TTGCTGTTGACATGTTTCAGC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c - <i>erm</i> cassette ^e	P _c - <i>erm</i>
Kan rpsL reverse	GGGCCCTTTCTTATGCTTTTG		
TT727	CAAAAGCATAAGGAAAGGGCCACAACTAAAATTAT GTGATACTTCACATAATTTTCT	D39	Intergenic between <i>mltG</i> and <i>greA</i> , and <i>greA</i>
P1349	AGAGCAAACACTAGGAAACTAGCCGCAGGTTG		
For construction of IU9931 (Δ<i>rodZ</i>(<i>spd_2050</i>)<><i>aad9</i>)			
P1384	GAGGTAAGCGAGAAGTTTCTGAAGCGGATTGC	D39	5' upstream fragment
TT311	GTATGTATTCAAATATATCCTCCTCACACTTGTATCCC TTCTTTCTAGTGTCTATTATG		

TT312	GACACTAGAAAGAAGGGATGACAAGTGTGAGGAGGATA TATTTGAATACATACGAACA	IU7397 ^g	<i>aad9</i>
TT313	CTTTTTTCATTCGTTTTTCCTTATAATTTTTTAAATCTGTT ATTTAAATAGTTTATAGTT		
TT314	CTATTTAAATAACAGATTAATAAATTATAAGGAAAAACG AATGAAAAAAGAACAAATTC	D39	3' downstream fragment
P1385	ACAACACCTGCAATGGCCACACGTTGCTTT		
For construction of IU10228 (<i>gfp-L₁-mltG</i>)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	D39	<i>spd_1347</i>
TT854	TAAACAATTCTTCACCTTTAGAAATCATAAGTTTTTCCTC CTTGTTGATAATCC		
TT855	ATCAACAAGGAGGAAAACTTATGATTTCTAAAGGTGAA GAATTGTTTACAGGTGT	IU9020 ^l	<i>gfp-L₁</i> with ATG start codon
TT757	TCCGGATCCCTCGAGTTTATAACAATTCATCC		
TT853	GAATTGTATAAACTCGAGGGATCCGGAAGTGAAAAGTC AAGAGAAGAAGAGAAATTAAGC	D39	5' <i>mltG</i> starting from second codon of <i>mltG</i>
TT412	CGACCAAGGAAGCAATGGTCAACAATCAT		
For construction of IU10292 (Δ<i>spd_0104::P_c-cat</i>)			
P108	ACGAGTGACATTCATCTGGGCGAT	K15 ^g	5' upstream fragment with 60 bp of 5' <i>spd_0104-P_c</i>
MB12	AAATCAATTTTATTAAGTTCATTTATTATTTCTTCCTCT TTTCTACAGTATTTAAAGA		
MB11	TGTAGAAAAGAGGAAGGAAATAATAAATGAACTTTAATA AAATTGATTTAGACAATTGGA	IU3373 ^k	<i>cat</i> ORF
TT869	GCCCCTTTCTTATGCTTTTGTATAAAAGCCAGTCATT AGGCCTATCTGA		
TT870	AGGCCTAATGACTGGCTTTTATAACAAAAGCATAAGGAA AGGGGCC	K15 ^g	60 bp of 3' <i>spd_0104</i> and 3' downstream fragment
P111	ACCACCTCATCACCCTGTCTGTT		
For construction of IU10294 (Δ<i>spd_1874::P_c-cat</i>)			
P174	ATGTGGTGTATCCGCATTGGGACAGGAT	K57 ^g	5' upstream fragment with 60 bp of 5' <i>spd_1874-P_c</i>
MB12	AAATCAATTTTATTAAGTTCATTTATTATTTCTTCCTCT TTTCTACAGTATTTAAAGA		
MB11	TGTAGAAAAGAGGAAGGAAATAATAAATGAACTTTAATA AAATTGATTTAGACAATTGGA	IU3373 ^k	<i>cat</i> ORF
TT869	GCCCCTTTCTTATGCTTTTGTATAAAAGCCAGTCATT AGGCCTATCTGA		
TT870	AGGCCTAATGACTGGCTTTTATAACAAAAGCATAAGGAA AGGGGCC	K57 ^g	60 bp of 3' <i>spd_1874</i> and 3' downstream fragment
P175	AGCCGTAAGTCGCAGCACCAATCACAAA		
For construction of IU10320 (<i>mltG</i>(Δ5bp)-FLAG-<i>P_c-erm</i>)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	IU7477 ^g	<i>spd_1347</i> , <i>mltG</i> (Δ 5bp)
TT509	CGGTTATTTATCATCATCTTTATAATCGTTTAAATTTG CTGTTGACATGTTACAGCG		

TT510	TGAACATGTCAACAGCAAATTAACGATTATAAAGATGA TGATGATAAATAACCGGG	IU7403 ⁹	FLAG-P _c -erm, and greA
P1349	AGAGCAAACCTAGGAAACTAGCCGCAGGTTG		
For construction of IU10323 (mltG(Y488D)-FLAG-P_c-erm)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	IU7567 ⁹	spd_1347, mltG(Y488D)
TT509	CGGTTATTTATCATCATCATCTTTATAATCGTTTAATTTG CTGTTGACATGTTTCAGCG		
TT510	TGAACATGTCAACAGCAAATTAACGATTATAAAGATGA TGATGATAAATAACCGGG	IU7403 ⁹	FLAG-P _c -erm, and greA
P1349	AGAGCAAACCTAGGAAACTAGCCGCAGGTTG		
For construction of IU10324 (mltG(Δ488bp)-FLAG-P_c-erm)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	IU7570 ⁹	spd_1347, mltG(Δ488bp)
TT509	CGGTTATTTATCATCATCATCTTTATAATCGTTTAATTTG CTGTTGACATGTTTCAGCG		
TT510	TGAACATGTCAACAGCAAATTAACGATTATAAAGATGA TGATGATAAATAACCGGG	IU7403 ⁹	FLAG-P _c -erm, and greA
P1349	AGAGCAAACCTAGGAAACTAGCCGCAGGTTG		
For construction of IU10327 (mltG(Ω45bp)²-FLAG-P_c-erm)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	IU7765 ⁹	spd_1347, mltG(Ω45bp) ²
TT509	CGGTTATTTATCATCATCATCTTTATAATCGTTTAATTTG CTGTTGACATGTTTCAGCG		
TT510	TGAACATGTCAACAGCAAATTAACGATTATAAAGATGA TGATGATAAATAACCGGG	IU7403 ⁹	FLAG-P _c -erm, and greA
P1349	AGAGCAAACCTAGGAAACTAGCCGCAGGTTG		
For construction of IU10919 (mltG_{Spn-Eco}-P_c-erm)			
TT413	GAGCCTGGTGGTTATATCTCTGTGACCTGT	D39	Upstream of <i>S.pn</i> mltG and (N) _{Spn} - aa(1-209)
TT890	GGCAAGATGGCGAACCTTCTGGTAACCAAAGTAACCAC CTGCTG		
TT891	CAGGTGGTACTTTGGTTACCAGAAGGTTCCGCATCTT GCCGA	<i>E. coli</i> K12 genomic DNA	aa(25-341)-(C) _{Eco}
TT892	CATTATCCATTAATAAATCAAACGGATCCTATTACTGCGC ATTTTTTTCCTTAAGCA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	IU9895 ⁹	P _c -erm- downstream mltG
P1349	AGAGCAAACCTAGGAAACTAGCCGCAGGTTG		
For construction of IU10921 (ΔbgaA::tet-P_{czcD}-RBS_{rodA}-rodA)			
TT657	CGCCCCAAGTTCATCACCAATGACATCAAC	IU8872 ⁹	5' bgaA::tet-P _{czcD} -
TT895	TGTAACCTTATACTTTTCATATTGTTATAATAGATTTA TGAACACCTTGTTTCATTATC		
TT896	TGTTCATAAATCTATTATAACAATATGAAAGTATAAGGTT AGTACATATGAAACGTTCTC	D39	RBS _(rodA) -rodA
TT897	AACTGGTTTATGAGAAAGTAAGTTCTTTTATTTAATTTGT TTTAATACAACCTTTTTCCG		
TT898	AAAAGGTTGTATTAACAATAAATAAAGAAGTACT TTCTCATAAACCAGTTGCTG	D39	3' bgaA' flanking

CS121	GCTTTCTTGAGGCAATTCACCTTGGTGC		
For construction of IU10943 ($\Delta rodA::P_cerm$)			
P1543	CAGGCCGTA CTCTTCTGTCCTCTTTACTTCC	D39	5' upstream fragment with 81 bp of 5' <i>rodA</i>
P1545	CATTATCCATTA AAAATCAAACGGATCCTAAGCCACCAC ACCGATGACCA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P_cerm^e	P_cerm
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P1546	CAAAGCATAAGGAAAGGGGCCCTCGATGAGTTACCAG ACTAATCTAGCTGAA	D39	87 bp of 3' <i>rodA</i> and 3' downstream fragment
P1544	CGGGTGTTCAAGCTCTCTGGCTTCATTTTC		
For construction of IU10945 ($\Delta rodA::P_c[kan-rpsL^+]$)			
P1543	CAGGCCGTA CTCTTCTGTCCTCTTTACTTCC	D39	5' upstream fragment with 81 bp of 5' <i>rodA</i>
P1545	CATTATCCATTA AAAATCAAACGGATCCTAAGCCACCAC ACCGATGACCA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c[kan-rpsL^+]$ cassette ^e	$P_c[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P1546	CAAAGCATAAGGAAAGGGGCCCTCGATGAGTTACCAG ACTAATCTAGCTGAA	D39	87 bp of 3' <i>rodA</i> and 3' downstream fragment
P1544	CGGGTGTTCAAGCTCTCTGGCTTCATTTTC		
For construction of IU11007 and IU11009 ($mltG_{Eco}-P_cerm$)			
P1348	TCTTCTTG CAGCCTTGAAAGAGGTGGCAGT	D39	Upstream of <i>S.pn mltG</i>
TT903	TAACAAGATTATCAATAACACTTTTTTCATAAGTTTTTCC TCCTTGTTGATAATCCATAA		
TT904	GGATTATCAACAAGGAGGAAAACTTATGAAAAAGTGT TATTGATAATCTTGTTATTGC	<i>E. coli</i> K12 genomic DNA	<i>E. coli mltG</i> ORF
TT892	CATTATCCATTA AAAATCAAACGGATCCTATTACTGCGC ATTTTTTTCCTTAAGCA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	IU9895 ^g	P_cerm - downstream <i>mltG</i>
P1349	AGAGCAA ACTAGGAAACTAGCCGCAGGTTG		
For construction of E655 ($\Delta rodZ(sp d_2050)::P_cerm$)			
TT329	CAACTGATATAGTTGGAAGTGAGGAGTCCATTTCCC	D39	5' upstream fragment with 60 bp of 5' <i>rodZ</i>
P1386	CATTATCCATTA AAAATCAAACGGATCCTAACTCAATCC CTGATTGATTCTAGCTAATCG		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P_cerm^e	P_cerm
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P1387	CAAAGCATAAGGAAAGGGCCCGATTTATCGAAATTA ACAGCTCAGACTGG	D39	60 bp of 3' <i>rodZ</i> and 3'

P1385	ACAACACCTGCAATGGCCACACGTTGCTTT		downstream fragment
For construction of E681 ($\Delta mltG::P_c erm$)			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	D39	5' upstream fragment with 90 bp of 5' <i>mltG</i>
P1350	CATTATCCATTA AAAATCAAACGGATCCTAACTTCATC ATAGCCTTTTACTTTTTCTAA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c erm^e$	$P_c erm$
Kan rpsL reverse	GGGCCCTTTCCCTTATGCTTTTG		
P1351	CAAAAGCATAAGGAAAGGGGCCCGATGTCACAGAAGG CAAGGTCTACTATG	D39	90 bp of 3' <i>mltG</i> and 3' downstream fragment
P1349	AGAGCAAACCTAGGAACTAGCCGCAGGTTG		
For construction of K5 ($\Delta lytC::P_c-[kan-rpsL^+]$)			
P13	TTTGAGAACTGGCGCCATGAAGG	D39	5' upstream fragment with 60 bp of 5' <i>lytC</i>
P17	CATTATCCATTA AAAATCAAACGGATCCTAGACATGACT AGTTGCCAAGCCTAGTAAAC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCCTTATGCTTTTG		
P18	CAAAAGCATAAGGAAAGGGGCCACCTCTTCTGAATAC ATGAAAGGAATCCA	D39	60 bp of 3' <i>lytC</i> and 3' downstream fragment
P14	AAACCAGGTGCTTGCCAAGTTTCG		
For construction of K15 ($\Delta spd_0104::P_c-[kan-rpsL^+]$)			
P108	ACGAGTGACATTCATCTGGGCGAT	D39	5' upstream fragment with 60 bp of 5' <i>spd_0104</i>
P120	CATTATCCATTA AAAATCAAACGGATCCTATGCAAACAA GGCAGCTACTCCTG		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCCTTATGCTTTTG		
P121	CAAAAGCATAAGGAAAGGGGCCCGGACGTTACGGTTC ATGGACTGC	D39	60 bp of 3' <i>spd_0104</i> and 3' downstream fragment
P111	ACCACCTCATCACCCTGTCTGTT		
For construction of K25 ($\Delta dacB::P_c-[kan-rpsL^+]$)			
P136	ACTGCCCATGTAGATACGCTTGGTGCTA	D39	5' upstream fragment with 60 bp of 5' <i>dacB</i>
P140	CATTATCCATTA AAAATCAAACGGATCCTATGAACAAGC TGCTAGACTCAAGGCTAG		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCCTTATGCTTTTG		
P141	CAAAAGCATAAGGAAAGGGGCCCGAGAGTGGTCTCAG TTTGGAAGAATACTATG	D39	60 bp of 3' <i>dacB</i> and 3'

P137	ACCGTTTGGCAATACCATTGCTCCACGG		downstream fragment
For construction of K35 ($\Delta dacA::P_c-[kan-rpsL^+]$)			
P150	AGCCTGCAATATGCAAGCGATCCCTCTT	D39	5' upstream fragment with 60 bp of 5' <i>dacA</i>
P152	CATTATCCATTA AAAATCAAACGGATCCTAAGCAGTAGA AACACCCCCTAAAAGAGAGAC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P153	CAAAAGCATAAGGAAAGGGGCCCTTCTTCTTAAAAGTTT GGTGAATCAGTTTG	D39	60 bp of 3' <i>dacA</i> and 3' downstream fragment
P151	TATCGTTGATGAGGGAGCAAGCGTCCACTA		
For construction of K37 ($\Delta pmp23::P_c-[kan-rpsL^+]$)			
P154	AGTTCAGTCCGGATGATACCGAGCTGTT	D39	5' upstream fragment with 60 bp of 5' <i>pmp23</i>
P156	CATTATCCATTA AAAATCAAACGGATCCTATTTATAGCC AGCAAAAAGGAAGACTGCTAG		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P157	CAAAAGCATAAGGAAAGGGCCCGTACGACTTAACCTT TACATCATCAAATGTTTC	D39	60 bp of 3' <i>pmp23</i> and 3' downstream fragment
P155	ACTGTCGCCAGCTTGTGATACGATGCTT		
For construction of K43 ($\Delta lytA::P_c-[kan-rpsL^+]$)			
P166	CCTTTGCCCTTCTTCCTATGACCGCTAT	D39	5' upstream fragment with 60 bp of 5' <i>lytA</i>
P168	CATTATCCATTA AAAATCAAACGGATCCTAATATGGTTG CACGCCGACTTGAGGC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P169	CAAAAGCATAAGGAAAGGGCCCTGGCAGACAGGCC AGAATTCACAGTAGAG	D39	60 bp of 3' <i>lytA</i> and 3' downstream fragment
P167	CCTCAACCATCCTATACAGTGAAGATGGGA		
For construction of K57 ($\Delta spd_1874::P_c-[kan-rpsL^+]$)			
P174	ATGTGGTGTATCCGCATTGGGACAGGAT	D39	5' upstream fragment with 60 bp of 5' <i>spd_1874</i>
P176	CATTATCCATTA AAAATCAAACGGATCCTATGCCAATAC TGGGGCAAATGACAAGGC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P177	CAAAAGCATAAGGAAAGGGCCCGTGGTAGTGTGAC AGAAAATCACTATGATCAG	D39	60 bp of 3' <i>spd_1874</i> and 3'

P175	AGCCGTAAGTCGCAGCACCAATCACAAA		downstream fragment
For construction of K75 (Δ<i>pspA</i>::P_c-[<i>kan-rpsL</i>⁺])			
P70	ACCGTTCGGCAATTCATGGTGACATGGACA	D39	5' upstream fragment with 60 bp of 5' <i>pspA</i>
P72	CATTATCCATTA AAAATCAAACGGATCCTAACCAGCCCC TAAGATAGCGACGCTGGC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c -[<i>kan-rpsL</i> ⁺] cassette ^e	P _c -[<i>kan-rpsL</i> ⁺]
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P73	CAAAAGCATAAGGAAAGGGGCCCTTGCAGTCAACACA ACTGTAGATGGCTAT	D39	60 bp of 3' <i>pspA</i> and 3' downstream fragment
P71	TCCTTGTTGCACATAACGTCCGGATTTGGC		
For construction of K148 (Δ<i>cbpD</i>::P_c-[<i>kan-rpsL</i>⁺])			
P202	TATCTTAGCAGCTCGTCCAGCAGTTGGT	D39	5' upstream fragment with 60 bp of 5' <i>cbpD</i>
P204	CATTATCCATTA AAAATCAAACGGATCCTATTTAACTGA CATCTTCAAGTAATAACTTGT		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c -[<i>kan-rpsL</i> ⁺] cassette ^e	P _c -[<i>kan-rpsL</i> ⁺]
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P205	CAAAAGCATAAGGAAAGGGGCCCTTAGCTATTAATACG ACGGTGGATGGCTACAG	D39	60 bp of 3' <i>cbpD</i> and 3' downstream fragment
P203	AGCTATGGCAATGGTCGGAATGGTCTGA		
For construction of K164 (Δ<i>pbp1a</i>::P_c-[<i>kan-rpsL</i>⁺])			
P234	CCCTTGTTTCATAGCGAGGATAAGCA	D39	5' upstream fragment with 60 bp of 5' <i>pbp1a</i>
P236	CATTATCCATTA AAAATCAAACGGATCCTACAAGCTTAA GAAGCTAATGCTCAGATACTT		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c -[<i>kan-rpsL</i> ⁺] cassette ^e	P _c -[<i>kan-rpsL</i> ⁺]
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P237	CAAAAGCATAAGGAAAGGGGCCCAACAATCAAATACA ACCCCTGATCAACAAAATC	D39	60 bp of 3' <i>pbp1a</i> and 3' downstream fragment
P235	AGGCAAGCCTGCAACCATGGTCTTGAAA		
For construction of K166 (Δ<i>pbp2a</i>::P_c-[<i>kan-rpsL</i>⁺])			
P226	GGTACGACAACGAAATGTCATACACTGCAC	D39	5' upstream fragment with 60 bp of 5' <i>pbp2a</i>
P228	CATTATCCATTA AAAATCAAACGGATCCTATTCACCTTGT TCTTTTTTAAAAGAGAAAAG		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	P _c -[<i>kan-rpsL</i> ⁺] cassette ^e	P _c -[<i>kan-rpsL</i> ⁺]
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P229	CAAAAGCATAAGGAAAGGGGCCCGGAAGATTAAGGA AAAGGCTCAAACAATATG	D39	60 bp of 3' <i>pbp2a</i> and 3'

P227	TCTGTTCCCGTGTGATCCGACAAATCCT		downstream fragment
For construction of K208 ($\Delta walk::P_c-[kan-rpsL^+]$)			
TT163	AGCAGTTTGACCTCTTTCATCAGGTTGCGG	D39	5' upstream fragment with 60 bp of 5' <i>walk</i>
P508	CATTATCCATTA AAAATCAAACGGATCCTACAAAATCAG GATAAAGATAAAAATCTCTGGT		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P509	CAAAAGCATAAGGAAAGGGGCCCGTACTCCCTTATGAT AAGGATGCAGTGAAA	D39	60 bp of 3' <i>walk</i> and 3' downstream fragment
TT164	TGTCAATGGTGTGCGGTATCTGGTGAGGT		
For construction of K372 ($\Delta spd_0173::P_c-[kan-rpsL^+]$)			
P830	TATACGGCGCACCAACTCATTGGGCTCATA	D39	5' upstream fragment with 60 bp of 5' <i>spd_0173</i>
P832	CATTATCCATTA AAAATCAAACGGATCCTAGACAAAATC CTCTCCGATAATGCCA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P833	CAAAAGCATAAGGAAAGGGGCCCATGCTCATGTAGAT GCACAGGAACA	D39	60 bp of 3' <i>spd_0173</i> and 3' downstream fragment
TT831	AACGCCCAAGCCTTCTACAGTTACAGGCA		
For construction of K489 ($\Delta spd_0703::P_c-[kan-rpsL^+]$)			
P1063	TTTCCAGCGAGTAAAGCCATGCTCCACCAA	D39	5' upstream fragment with 60 bp of 5' <i>spd_0703</i>
P1065	CATTATCCATTA AAAATCAAACGGATCCTACTGCTGGTT TAATTCTTCTTGATAGTCTAC		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P1066	CAAAAGCATAAGGAAAGGGGCCCGTTCAACGGTCTATT ATCAAACGAAAACGT	D39	39 bp of 3' <i>spd_0703</i> and 3' downstream fragment
P1064	CCCTGCTTGAGAGTATGCAGAAGCAACAGT		
For construction of K637 ($\Delta mltG::P_c-[kan-rpsL^+]$)			
P1348	TCTTCTTGACGCTTGAAGAGGTGGCAGT	D39	5' upstream fragment with 90 bp of 5' <i>mltG</i>
P1350	CATTATCCATTA AAAATCAAACGGATCCTAACTTCATC ATAGCCTTTTACTTTTTCTAA		
Kan rpsL forward	TAGGATCCGTTTGATTTTTAATGGATAATG	$P_c-[kan-rpsL^+]$ cassette ^e	$P_c-[kan-rpsL^+]$
Kan rpsL reverse	GGGCCCTTTCCTTATGCTTTTG		
P1351	CAAAAGCATAAGGAAAGGGGCCCGATGTACAGAAGG CAAGGTCTACTATG	D39	90 bp of 3' <i>mltG</i> and 3'

P1349	AGAGCAAAGCTAGGAACTAGCCGCAGGTTG		downstream fragment
For construction of K654 ($\Delta rodZ(spd_2050)::P_c-[kan-rpsL^+]$)			
TT329	CAACTGATATAGTTGGAAGTGAGGAGTCCATTTCCC	D39	5' upstream fragment with 60 bp of 5' <i>rodZ</i>
P1386	CATTATCCATTAATAAATCAAACGGATCCTAACTCAATCCCTGATTGATTCTAGCTAATCG		
Kan <i>rpsL</i> forward	TAGGATCCGTTTGATTTTTAATGGATAATG	<i>P_c-[kan-rpsL⁺]</i> cassette ^e	<i>P_c-[kan-rpsL⁺]</i>
Kan <i>rpsL</i> reverse	GGGCCCTTTCTTATGCTTTTG		
P1387	CAAAAGCATAAGGAAAGGGGCCCGATTTATCGAAATTAACAGCTCAGACTGG	D39	60 bp of 3' <i>rodZ</i> and 3' downstream fragment
P1385	ACAACACCTGCAATGGCCACACGTTGCTTT		

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For transformation assays			
P1348	TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT	K637	$\Delta mltG::P_c-erm$
P1349	AGAGCAAAGCTAGGAACTAGCCGCAGGTTG		
TT452	GGAGGGTTGGCTGTGGGTGGCTACAAGAAC	IU7397	$\Delta pbp2b \rightarrow aad9$
TT352	TGAAGGACTGGAAAGACCACTGCACCTTCT		
P1543	CAGGCCGTA CTCTTCTGTCCTCTTTACTTCC	IU10943	$\Delta rodA::P_c-erm$
P1544	CGGGTGTTC AAGCTCTCTGGCTTCATTTTC		
P1543	CAGGCCGTA CTCTTCTGTCCTCTTTACTTCC	IU10945	$\Delta rodA::P_c-[kan-rpsL^+]$
P1544	CGGGTGTTC AAGCTCTCTGGCTTCATTTTC		
AL35	GGGGGGCAAACCAAGTGATGTC	IU3897	$\Delta mreCD \rightarrow P_c-erm$
AL17	TGCTCCA ACTTGAGGTGTTGAACC		
AL35	GGGGGGCAAACCAAGTGATGTC	IU1751	$\Delta mreCD \rightarrow aad9$
AL17	TGCTCCA ACTTGAGGTGTTGAACC		
TT329	CAACTGATATAGTTGGAAGTGAGGAGTCCATTTCCC	IU9931	$\Delta rodZ \rightarrow aad9$
P1385	ACAACACCTGCAATGGCCACACGTTGCTTT		
TT329	CAACTGATATAGTTGGAAGTGAGGAGTCCATTTCCC	E655	$\Delta rodZ::P_c-erm$
P1385	ACAACACCTGCAATGGCCACACGTTGCTTT		
TT329	CAACTGATATAGTTGGAAGTGAGGAGTCCATTTCCC	K654	$\Delta rodZ::P_c-[kan-rpsL^+]$
P1385	ACAACACCTGCAATGGCCACACGTTGCTTT		
P222	CGTTCGTGTGGCGCTGCTTCAAATTGTT	E193	$\Delta pbp1b::P_c-erm$
P522	AACGGCAACCACCAAGGAGAAACCAAGGA		

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Primers used for QRT-PCR		
		gene
AL031	CTGCGACAGCAGATTTGACCACTA	<i>spd_1874</i>
AL032	TTCCTGAGGAGCTTCTTCTGCAAC	
AL023	GGAGTAGCTGCCTTGTTTGCAGTA	<i>spd_0104</i>
AL024	CAGGGCCATCGATAACCAACTCTT	
AL018	GTAGTGCTCAAACAAATGGAGCCG	<i>pcsB</i>
AL019	GTCAATGCTTGAGCATCATCAGCC	
KK489	AAAGGTCGTGGTGGTAAGGGAATG	<i>gyrA</i>
KK490	GCATCTTGATCCAGGCGCATTACT	

^aGenomic DNA of the indicated *S. pneumoniae* strains was used as templates for PCR reactions, except for P_c-*erm* and P_c-[*kan-rpsL*⁺] cassettes.

^bGenotype of IU6929 is D39 Δ *cps pbp2x*-HA-P_c-*kan* (Land *et al.*, 2013).

^cGenotype of IU6565 is D39 Δ *cps ftsZ*-FLAG-P_c-*erm* (Land *et al.*, 2013).

^dGenotype of IU6962 is D39 Δ *cps ftsZ*-Myc-P_c-*kan* (Land *et al.*, 2013).

^eP_c-*erm* and P_c-*kan-rpsL*⁺ cassettes are described in (Tsui *et al.*, 2011).

^fpJWV25 (Eberhardt *et al.*, 2009).

^gStrain genotypes are listed in Supplemental Table S1.

^hGenotype of IU8845 is D39 Δ *cps rpsL1 ftsZ*-L₂-*gfp* (Winkler lab collection, unpublished), L₂-linker sequence is KLDIEFLQ (Fleurie *et al.*, 2014).

ⁱpKB01_mKate2 (Beilharz *et al.*, 2015).

^jGenotype of IU9020 is D39 Δ *cps rpsL1 gfp*-L₁-*pbp2x* (Winkler lab collection, unpublished). L₁ linker sequence is LEGSG The DNA template for *gfp* is pUC57-*gfp*(*Sp*) (Martin *et al.*, 2010).

^kGenotype of IU3373 is D39 *rpsL*⁺-*rpsG*⁺-*cat* (Tsui *et al.*, 2010).

^lIU8906 is an unpublised strain containing Δ *bgaA::tet*-P_{Zn} followed by 24 bp upstream of *ftsA* (RBS_{*ftsA*}). Δ *bgaA::tet*-P_{Zn} was obtained from pJWV25 (Eberhardt *et al.*, 2009).

Table S3. Structural similarity of the extracellular domain of MltG_{Spn} to known protein structures

Aligned crystalized structure (organism, PDB ID) ^a	3D alignment coverage (MltG _{Spn} residues) ^{a,b}	Match confidence ^c (%)	RMSD (Å) ^d	Z-score ^e	2D domain ^f	Confirmed function
Lmo14992 (<i>L. monocytogenes</i> , 4IIW)	222-541	100	0.9	38.4	YceG-like (aa 87-248, cd08010)	no
MltG (<i>E. coli</i> , 2RIF)	266-547	100	3.7	31.4	YceG-like (aa 82-327, cd08010)	Lytic transglycosylase (Yunck <i>et al.</i> 2015)
SleB (<i>B. anthracis</i> , 4FET)	411-533	97.1	1.5	7.6	Hydrolase_2 (aa 153-253, pfam07486)	Lytic transglycosylase (Heffron <i>et al.</i> , 2011)
SleB (<i>B. cereus</i> , 4F55)	412-460	94.7	3.6	8.1	Hydrolase_2 (aa 159-259, pfam07486)	no

^aResults were obtained from Phyre2 (Kelley & Sternberg, 2009) analysis by inputting amino acid sequence of the entire MltG_{Spn} and ran on intensive mode. Structures listed are >90% in match confidence.

^bAmino acid residues of MltG_{Spn} aligned by Phyre2 analysis to protein structures in column 1. For Lmo14992, MltG_{Eco}, and SleB_{Ban}, the alignments cover the entire sequence of the crystalized peptide chains, which are aa 42-349, aa 81-340, and aa 131-253, respectively, of these proteins. In contrast, only 49 residues of MltG_{Spn} align with aa 133-181 of the crystalized 123-residue SleB_{Bce} peptide.

^cConfidence scores were obtained from Phyre2 analysis.

^dRMSD values were determined via PyMOL alignment of the PDB modelling file generated from Phyre2 input of MltG sequence aligning with each homolog of *L.*

358 *monocytogenes*, *E.coli*, and *B. anthracis* using only the residues sequences shown in
359 column 2. Alignment of the *B. cereus* homolog was obtained with aa 411-533 of MltG_{Spn}
360 and aa 131-253 of the SleB_{Bce} peptide.

361 ^eZ-score was determined via input of the MltG PDB modeling file generated from
362 Phyre2 into the DALI server. Amino acid sequences used for the alignment and
363 generation of scores are the same as for RMSD.

364 ^fConserved domains identified by NCBI search
365 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) by inputting the complete amino
366 acid sequence of each protein listed in column 1. Domain names (YceG-like or
367 hydrolase_2) are followed in parenthesis by the residue numbers of each protein and
368 accession numbers of the domains. Residues 267 to 542 of MltG_{Spn} are identified as
369 YceG-like and cd08010).

370 **TABLE S4.** Genes encoding proteins with known or putative PG lytic domains and WalRK-regulated genes in *S.*
 371 *pneumoniae* serotype 2 strain D39^a, observed hydrolase activities in zymogram assays, and phenotypes of deletion
 372 mutants in a $\Delta pbp1a \Delta mltG$ background.

Gene or tag	Hydrolase class ^b	MW (kDa)	Missing band in zymogram ^c	Constructed deletion strain in IU7327 ($\Delta pbp1a \Delta mltG$) background ^e	Doubling time of triple deletion strain (min) ^f
				IU7327	50.5 ± 5.5 (5)
<i>walk</i>	None, histidine kinase	51.7	ND ^d	IU9235	125.5 ± 0.5(2)
WalRK _{Spn} regulon					
<i>pcsB</i>	Putative <i>N</i> -acetylmuramoyl-l-alanine amidase/endopeptidase	41.8	ND ^d	Essential in WT background ^g	
<i>lytB</i>	<i>N</i> -Acetylglucosaminidase	81.9	~80 kDa	IU9298	58.3 ± 15.5 (2)
<i>spd_1874</i>	Putative <i>N</i> -acetylmuramidase (LysM domain)	40.6	ND ^d	IU9292	55.3 (1)
<i>spd_0703</i>	None (putative SEDS protein)	11.0	ND ^d	IU9318	44 (1)
<i>spd_0104</i>	None (LysM domain)	17.8	ND ^d	IU9316	40.2 (1)
<i>spd_0126 (pspA)</i>	None	68.6	ND ^d	IU9320	51.1 ± 5.7 (2)
Cell division					
<i>pmp23</i>	Putative lytic transglycosylase	23.0	ND ^d	IU9290	62.9 ± 10.5 (3)
<i>dacA</i>	d,d-Carboxypeptidase	45.2	ND ^d	IU9386	ND ^h
<i>dacB (spd_0549)</i>	l,d-Carboxypeptidase	26.0	ND ^d	IU9388	ND ^h
Fratricide and autolysis during growth					
<i>lytA</i>	<i>N</i> -Acetylmuramoyl-l-alanine amidase	36.6	~36 kDa	IU9382	ND ^h
<i>cbpD</i>	Putative <i>N</i> -acetylmuramoyl-l-alanine amidase/endopeptidase	50.4	ND ^d	IU9384	40.4 (1)
<i>lytC</i>	<i>N</i> -Acetylmuramidase	57.4	~57 kDa	IU9294	53.1 ± 7.8 (2)

Other putative hydrolases that do not affect cell division					
<i>spd_0873</i>	Putative <i>N</i> -acetylmuramidase	30.1	ND ^d	IU9296	62.3 ± 12.6 (3)
<i>spd_0173</i>	Putative l,d-carboxypeptidase	38.8	ND ^d	IU9390	ND ^h

^aGrouping of genes based on (Barendt *et al.*, 2011).

^bBased on (Layec *et al.*, 2008).

^cZymogram assays were performed as described in *Supplemental experimental procedures*. Bands of indicated molecular weights were absent in the single Δ *lytA*, Δ *lytB* or Δ *lytC* mutants when compared to the wild-type parent (See Fig. S8).

^dND, not determined

^eConstructed mutants with deleted gene in column 1 in IU7327 (Δ *pbp1a* Δ *mltG*) background. Strains IU10292 and IU10294 (Δ *pbp1a* Δ *mltG* Δ *spd_0104* and *spd_1874*) deleted for both LysM-domain proteins (Spd_0104 and Spd_1874) were also constructed. Only IU9235 (Δ *pbp1a* Δ *mltG* Δ *walkK*) among all the triple mutants showed significantly smaller colony size compared to the parent strain IU7327 after 24h incubation.

^fGrowth curves and doubling time determinations in BHI broth were performed as described in *Experimental procedures*. Most doubling times are the average and SEM of 2 to 5 independent growth experiments, indicated by n. Among the constructed triple mutants tested, only the triple Δ *pbp1a* Δ *mltG* Δ *walkK* mutant grew with a significantly longer doubling time than the Δ *pbp1a* Δ *mltG* *walkK*⁺ parent.

388 ^g Since *pcsB* is essential for pneumococcal growth, it was not feasible to construct a $\Delta pbp1a \Delta mltG \Delta pcsB$ strain. We
389 investigated whether controlling expression of the essential *pcsB*⁺ gene from a promoter not regulated by the WalRK TCS
390 would improve the poor growth of the $\Delta pbp1a \Delta mltG \Delta walk$ mutant. We utilized the construct $\Delta bgaA':::kant1t2-P_{fcsK^-} pcsB^+$,
391 which expresses *pcsB* from a fucose-controlled promoter (Barendt *et al.*, 2009) to a level similar to the expression from
392 the native *walk*-dependent promoter. We transformed a $\Delta walk$ amplicon into IU9330 ($\Delta pbp1a mltG(\Delta 488bp) P_{fcsK^-} pcsB^+$)
393 in the presence or absence of 1 % fucose. We reasoned that if the poor growth of the $\Delta pbp1a mltG(\Delta 488bp) \Delta walk$ strain
394 was due solely to the under-expression of *pcsB*, the ectopic expression of *pcsB* from $P_{fcsK^-} pcsB^+$ in the presence of fucose
395 would be able to overcome the growth defect. To the contrary, we observed that the transformants grew very poorly under
396 both inducing and non-inducing conditions. This result indicates that under-expression of *pcsB* alone does not account for
397 the poor growth of the $\Delta pbp1a \Delta mltG \Delta walk$ mutant.

398 ^hGrowth curves and of doubling time determinations in BHI broth were not determined; but colony sizes of the triple
399 mutants on TSAII-BA plates were similar to the control strain ($\Delta pbp1a \Delta mltG \Delta bgaA$).

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Model of PG biosynthesis in ovococcus bacteria, such as *S. pneumoniae*, and topology of proteins involved in peripheral PG synthesis. (A) Top. Ovococci divide perpendicularly to their long axis (Zapun *et al.*, 2008). Unencapsulated derivatives of serotype 2 strain D39 *S. pneumoniae* form mostly diplococci and chains of two cells, whereas capsulated D39 strains form short chains of 8–10 cells (Barendt *et al.*, 2009). Bottom. Formation of prolate-ellipsoid-shaped bacteria requires two modes of PG synthesis, peripheral (sidewall-like) and septal PG synthesis, that occur in the midcell region of dividing *S. pneumoniae* cells (Tsui *et al.*, 2014, Massidda *et al.*, 2013, Pinho *et al.*, 2013, Sham *et al.*, 2012, Zapun *et al.*, 2008). At the start of a division cycle, components of both peripheral PG synthesis complexes (orange ovals) and septal synthesis complexes (green rectangles) locate to the equators of cells (bottom). Peripheral PG synthesis (light blue; top) occurs between the future equator and septum of dividing cells and may commence before septal synthesis (Massidda *et al.*, 2013, Wheeler *et al.*, 2011, Zapun *et al.*, 2008). At some point, septal PG synthesis (medium blue) commences to divide the cell in two. The complexes that carry out peripheral and septal PG synthesis locate to a large constricting ring throughout the division cycle, with the exception of PBP2x, which moves to the centers of septa in mid-to-late divisional cells (Tsui *et al.*, 2014). The grey Pac-Man symbol corresponds to PG hydrolases that remodel the PG and allow septal separation. Reproduced from (Tsui *et al.*, 2014). (B) Topology of proteins (not drawn to scale) known or speculated to be involved in peripheral PG synthesis in ovococci (Philippe *et al.*, 2014, Massidda *et al.*, 2013). Involvement of MreC, MreD, PBP2b, and PBP1a in peripheral PG synthesis in

423 *Streptococcus pneumoniae* was shown experimentally in previous studies (Philippe *et*
424 *al.*, 2014, Tsui *et al.*, 2014, Berg *et al.*, 2013, Massidda *et al.*, 2014, Land & Winkler,
425 2011). This study shows that the MltG endo-LT is in the peripheral PG synthesis
426 machine, that MreCD and/or RodZ regulate PBP1a (arrows) and/or MltG activity and/or
427 localization, and that RodA controls activity of PBP2b (arrow), likely by direct interaction
428 (*Results and Discussion*). Figure is based on Philippe *et al.*, 2014 and Massidda *et al.*,
429 2013 and work reported in this study.

430 **Fig. S2.** Phenotypes and Western blots of cells expressing epitope-tagged MltG
431 derivatives. Full genotypes of strains are listed in Table S1. Growth curves and Western
432 blotting were performed as described in *Experimental procedures and Supplemental*
433 *experimental procedures*. (A) and (B) Quantitative Western-blot analyses of strains
434 expressing MltG-FLAG (expected molecular mass = 62 kDa). Equal amounts (12 μ g for
435 A, 26.5 μ g for B) of total proteins from each strain with genotype indicated in the tables
436 were loaded onto each lane. Chemiluminescent signal intensity of each band was
437 normalized to the integrated intensity value obtained for IU9043 (Δ *pbp1a mltG-FLAG*) in
438 right panel of A, or B, or IU7403 (*mltG-FLAG*, left panel of A). Relative signal intensities
439 (average \pm SEM) were obtained from two independent experiments. (C) Representative
440 growth curves of IU1945 parent, IU7399 (*mltG-HA*) and IU7405 (*mltG-Myc*). (D and E)
441 Western-blot analysis of untagged parent strain IU1945 (lane 1), and *mltG-HA* (D,
442 IU7399, expected molecular mass = 62 kDa), and *mltG-Myc* (E, IU7405, expected
443 molecular mass = 62 kDa).

444 **Fig. S3.** Domain architecture of YceG-domain proteins in bacteria of various cell
445 shapes. YceG-domain proteins of ovococci, including streptococci (*S. pneumoniae*, *S*

446 *mitis*, *S. pyogenes*, *S. mutans*, and *S. agalactiae*) and *Lactococcus lactis*, have a similar
447 domain architecture consisting of an intracellular domain of 150 to 200 aa, a
448 transmembrane (TM) domain of ≈ 24 aa, and the extracellular YceG-like domain. The
449 intracellular domain of MltG_{Spn} (DUF_1346) has weak aa similarity to an intracellular
450 Mid-1 related chloride channel (MCLC) domain and to other DUFs (see *Results*). The
451 intracellular domain of *S. agalactiae* MltG has an DUF different from DUF_1346. The
452 intracellular domains of the MltG homologues of other ovoid species are predicted to be
453 disordered by Phyre2 analysis. Gram-positive rod-shaped bacteria, such as *Bacillus*
454 *subtilis* and *Listeria monocytogenes*, and Gram-negative rod-shaped bacterium *E. coli*
455 contain a short (<20 aa) intracellular domain. YceG domain proteins are absent in the
456 spherical bacterium *Staphylococcus aureus*. M designates membrane region. L
457 indicates a conserved LysM-like structure with a $\beta_1\alpha_1\alpha_2\beta_2\alpha_3$ fold that is a putative PG
458 binding domain. Strains and protein IDs used to generate this figure are: *S. pneumoniae*
459 D39 (ABJ53954.1), *S. mitis* B6 (YP_003446601), *S. pyogenes* A20 (YP_006932381.1),
460 *S. mutans* NN2025 (YP_003484312.1), *S. agalactiae* A909 (YP_330231), *Lactococcus*
461 *lactis* II1403 (NP_266791), *Bacillus subtilis* 168 (NP_390615), and *E. coli* K-12
462 (NP_415615.1).

463 **Fig. S4.** Scheme of experimental reconstruction of $\Delta pbp2b$ suppressor strains in
464 D39 $\Delta cps rpsL1 pbp1a^+$ background. See Supplemental *experimental procedures* for
465 details.

466 **Fig. S5.** Summary of stabilities and viabilities of strains containing frame-shift *mltG*
467 suppressor alleles. Strains containing frameshift *mltG* alleles (*sup2*, *sup4* and *sup5*) are
468 viable, but not stable in *pbp1a^+* or *pbp1a^+ \Delta pbp2b* genetic backgrounds. The following

469 $\Delta cps\ pbp1a^+$ strains showed non-uniform-sized colonies when streaked from ice stocks
470 onto TSAII BA plates: IU10342 ($pbp1a^+ mltG(E428Q)$), the original suppressor strains
471 IU7477, IU7570, and IU7765, and reconstructed suppressor strains IU9777 ($pbp1a^+$
472 $mltG(\Delta 5bp)\ \Delta pbp2b$), IU9905 ($pbp1a^+ mltG(\Delta 488bp)\ \Delta pbp2b$) and IU9907 ($pbp1a^+$
473 $mltG(\Omega 45bp)^2\ \Delta pbp2b$). In contrast, strains containing $\Delta mltG$ or frameshift $mltG$ alleles
474 are viable and stable in $\Delta pbp1a$ mutants (IU7325, IU7327, IU8549, IU8553 and
475 IU8555), or $\Delta pbp1a\ \Delta pbp2b$ (IU7931, IU8565, IU8567, and IU8569) mutants (see Table
476 S1 for constructions and *Results* for additional details).

477 **Fig. S6.** Clustal Omega alignment of the transmembrane and extracellular domains
478 of MltG_{Eco}, MltG_{Spn}, and YceG_{Lmo} (the MltG homologue in *L. monocytogenes*). Letter
479 colors indicate the following aa properties: red: small, hydrophobic or aromatic; blue:
480 acidic; magenta: basic; green: hydroxyl, sulfhydryl, or amine. The MltG_{Eco} sequence (gi
481 accession 687676267) was aligned with MltG_{Spn} (gi: 116076234) and YceG_{Lmo} (gi:
482 16803539) using Clustal Omega (Sievers *et al.*, 2011) with default parameters. Symbols
483 indicate the following: asterisks (*) single fully conserved aa; colons (:), conservation
484 between groups with strongly similar properties; periods (.), conservation between
485 groups with weakly similar properties. The catalytic glutamate (E218 of MltG_{Eco} and
486 E428 of MltG_{Spn}) is indicated as E218/E428. Y488 of MltG_{Spn}, the aa changed in the
487 $\Delta pbp2b\ sup3$ strain, aligns with Y274 of MltG_{Eco} and is indicated as Y274/Y488.
488 Secondary structure information diagramed below the alignment was obtained from the
489 DSSP annotation in the sequence display feature of the PDB file (PDB ID: 4IIW). Start
490 and end sites mark the protein sequences used in the crystal structure determination of
491 YceG_{Lmo} (aa 26 to 349). See *Results*, *Discussion*, and Table S3 for additional details.

492 **Fig. S7.** (A) Superposition of crystalized region of YceG_{Lmo} (red, aa 42 to 349) and
493 MltG_{Spn} (blue, aa 266 to 547) backbones. Two distinct subdomains are in the YceG
494 domain: the N-terminal/membrane-proximal subdomain contains a LysM-like domain
495 with a $\beta_1\alpha_1\alpha_2\beta_2\alpha_3$ fold (inset) that is a putative PG binding site; and the C-terminal
496 catalytic subdomain with endo-LT activity. (B). Degree of conservation of each residue
497 in the LysM-like $\beta_1\alpha_1\alpha_2\beta_2\alpha_3$ subdomain (top line) and in the endo-LT catalytic subdomain
498 (bottom line), where asterisks mark the catalytic E aa and the Y aa changed in the *sup3*
499 allele. Color coded box at each residue was determined using the ConSurf server
500 (Ashkenazy *et al.*, 2010) using amino acid sequences from MltG_{Lmo}. The residues within
501 $\beta_1\alpha_1\alpha_2\beta_2\alpha_3$ folds are mostly conserved. The catalytic E and Y residues are highly
502 conserved. See *Results, Discussion*, and Table S3 for additional details.

503 **Fig. S8.** Zymogram of membrane extracts from strain IU1945 (wild-type
504 unencapsulated parent) and isogenic PG hydrolase mutants K43 (Δ lytA), K27 (Δ lytB),
505 and K5 (Δ lytC). Electrophoresis of membrane extracts was performed in an SDS-12%
506 polyacrylamide gel containing a lysed-cell preparation of IU1945 (D39 Δ cps) as PG
507 substrate. Membrane extracts from the strains and lysed-cell PG substrate were
508 prepared and subjected to zymography as described in *Supplemental experimental*
509 *procedures*. Equal amounts of proteins (35 μ g) were loaded into each lane. Lane
510 molecular weight (MW) markers (PageRuler prestained protein ladder, Thermo
511 Scientific) calibrated by the vendor are shown at left. Arrows to the right of the gel
512 indicate the migration positions of LytA, LytB, and LytC as inferred by absence of bands
513 in the corresponding deletion mutants. MltG endo-LT activity is expected to overlap the
514 upper intact LytC band and would be visible in the Δ lytC lane.

515 **Fig. S9.** Growth phenotypes and cell morphologies of *mltG*(Δ DUF_1346) mutant
516 strain IU9025 compared to that of its *mltG*⁺ parent strain IU1824. Experiments were
517 carried out as described in *Supplemental experimental procedures*. (A) Slight growth
518 defect of IU9025 (*mltG*(Δ DUF_1346)) compared to IU1824 (*mltG*⁺ parent) under high
519 salt conditions. (B) Marginal difference in sensitivity of IU9025 (*mltG*(Δ DUF_1346)) to
520 penicillin G compared to IU1824 (*mltG*⁺ parent). (C) No difference in growth of IU1824
521 (*mltG*⁺) and IU9025 (*mltG*(Δ DUF_1346)) in BHI broth at neutral or low pH conditions.
522 (D) IU9025 (*mltG*(Δ DUF_1346)) and IU1824 (*mltG*⁺) cells have the same length and
523 width within experimental error.

524 **Fig. S10.** Growth curves of *mltG*⁺ parent strain IU1945 (D39 Δ *cps*) and merodiploid
525 strain IU9102 (Δ *mltG*// Δ *bgaA*::P_{Zn}-*mltG*) in the absence or presence of different
526 concentrations of the inducer Zn²⁺. IU1945 and IU9102 were grown overnight in BHI
527 broth containing 0.2 mM ZnCl₂ and 0.02 mM MnSO₄, centrifuged to remove Zn²⁺ and
528 Mn²⁺, and resuspended to OD₆₂₀ \approx 0.005 in BHI broth containing 0.2 mM ZnCl₂ and 0.02
529 mM MnSO₄, 0.1 mM ZnCl₂ and 0.01 mM MnSO₄, or 0.05 mM ZnCl₂ and 0.005 mM
530 MnSO₄, or no Zn²⁺ and Mn²⁺ (0 mM Zn) as described in *Experimental procedures*. Tenth
531 the concentration of MnSO₄ was added to prevent effects of zinc toxicity (Jacobsen *et*
532 *al.*, 2011).

533 **Fig. S11.** Representative growth curves and morphological changes of Δ *pbp1a*,
534 Δ *pbp1a* Δ *mltG* and Δ *pbp1a* Δ *mltG* Δ *pbp2b* strains. Strains IU1824 (1, D39 Δ *cps* parent),
535 IU6741 (2, Δ *pbp1a*), IU7327 (3, Δ *pbp1a* Δ *mltG*) and IU7931 (4, Δ *pbp1a* Δ *mltG* Δ *pbp2b*)
536 were grown overnight in BHI and diluted to OD₆₂₀ \approx 0.003 in BHI to start growth cultures
537 as described in *Experimental procedures*. (A) Representative growth curves. Doubling

538 times (mean \pm SEM) for IU1824, IU6741, IU7327 and IU7931 were 38 ± 1 (n=4), 48 ± 3
539 (n=3), 46 ± 4 (n=2), and 48 ± 3 (n=2) respectively, and were not statistically different
540 from each other by one-way ANOVA analysis (GraphPad Prism, nonparametric Kruskal-
541 Wallis test)). (B) Phase-contrast images of strains growing in exponential phase (OD_{620}
542 ≈ 0.15). Micrographs are at the same magnification (scale bars = 1 μ m). (C) Box-and-
543 whisker plots (whiskers, 5 and 95 percentile) of cell lengths, widths, aspect ratios
544 (length to width ratio) and relative volumes. Fifty or more cells from two independent
545 experiments were measured as described in *Experimental procedures* for each strain. P
546 values were obtained by one-way ANOVA analysis (GraphPad Prism, nonparametric
547 Kruskal-Wallis test). *** denotes $p < 0.001$.

548 **Fig. S12.** (A) Appearance of colonies on TSAII-BA plates of strains IU1690 (D39
549 *cps*⁺), IU9771 (*cps*⁺ Δ *mltG*), IU9897 (*cps*⁺ Δ *mltG* Δ *pbp2b*), and IU10021 (*cps*⁺ Δ *mltG*
550 Δ *pbp1a*). Frozen stocks of strains were streaked onto TSAII-BA plates and
551 photographed after 17 h of incubation at 37°C. (B) Transformation of a Δ *cps2E*
552 amplicon into IU9771 (D39 *cps*⁺ Δ *mltG*) to obtain an unencapsulated strain results in
553 accumulations of spontaneous mutations in *pbp1a*. Two isolates (IU9899 and IU10048)
554 obtained from two independent transformations were sequenced for the *pbp1a* region.
555 IU9899 contained *pbp1a*(Δ T@F33) and IU10048 contained *pbp1a*(G559stop
556 (GGA \rightarrow TGA)) mutations.

557 **Fig. S13.** Larger width of MltG-HA rings compared to FL-V rings detected by 2D and
558 3D-SIM IFM. Strain IU7399 (*mltG*-HA) was grown in BHI broth and processed for FL-V
559 staining, IFM, and DAPI labeling as described in *Experimental procedures*. (A)
560 Representative images of a field of cells stained with FL-V and subjected to 2D IFM to

561 detect MltG-HA localization. (B) Representative 3D-SIM IFM and FL-V images of cells
562 at division stages 1-4, (C) averaged 2D IFM images and fluorescence intensity traces,
563 and (D) scatter plots of FL-V labeling width versus MltG width at midcell equators and
564 septa at division stages 1-4 in (C) were obtained and processed as described for Fig.
565 10, except FL-V images are colored green. In D, *** denotes $p < 0.001$. Representative
566 images in (A) and (B), and data in (C) and (D) were obtained from two independent
567 biological replicates.

568 **Fig. S14.** Representative growth curves and doubling times of mutants containing
569 $\Delta walk$ or single deletion mutations in known and putative PG hydrolase genes in (A)
570 wild-type $pbp1a^+$ strain IU1824, and in (B) $\Delta pbp1a \Delta mltG$ mutant strain IU7327 (see
571 Table S1). Doubling times are the averages \pm SEM from 2 to 5 independent growths for
572 most strains or from one growth (n=1) in some cases.

573 **Fig. S15.** Induction of WalRK regulon members in strains containing $mltG(\Delta 488bp)$
574 or $mltG(Y488D)$ mutations. RNA preparation and QRT-PCR procedures to determine
575 relative transcript amounts of spd_{1874} , spd_{0104} , and $pcsB$ were performed as
576 described in *Supplemental experimental procedures* for isogenic strains IU1824 (D39
577 $\Delta cps rpsL1$, wild-type (WT) parent), IU6741 ($\Delta pbp1a$), IU8553 ($\Delta pbp1a mltG(\Delta 488bp)$),
578 IU8567 ($\Delta pbp1a mltG(\Delta 488bp) \Delta pbp2b$), IU9760 ($mltG(Y488D)$), and IU9783
579 ($mltG(Y488D) \Delta pbp2b$). Numbers at top of each bar indicate the average fold changes
580 of transcript amounts relative to the WT parent based on three independent QRT-PCR
581 experiments from three independent biological replicates. *, **, *** indicate p values
582 < 0.05 , < 0.01 and < 0.001 , respectively using a one-sample t test to determine if the

583 mean was significantly different from a hypothetical value of 1. See *Results* and
584 *Discussion* for additional details.

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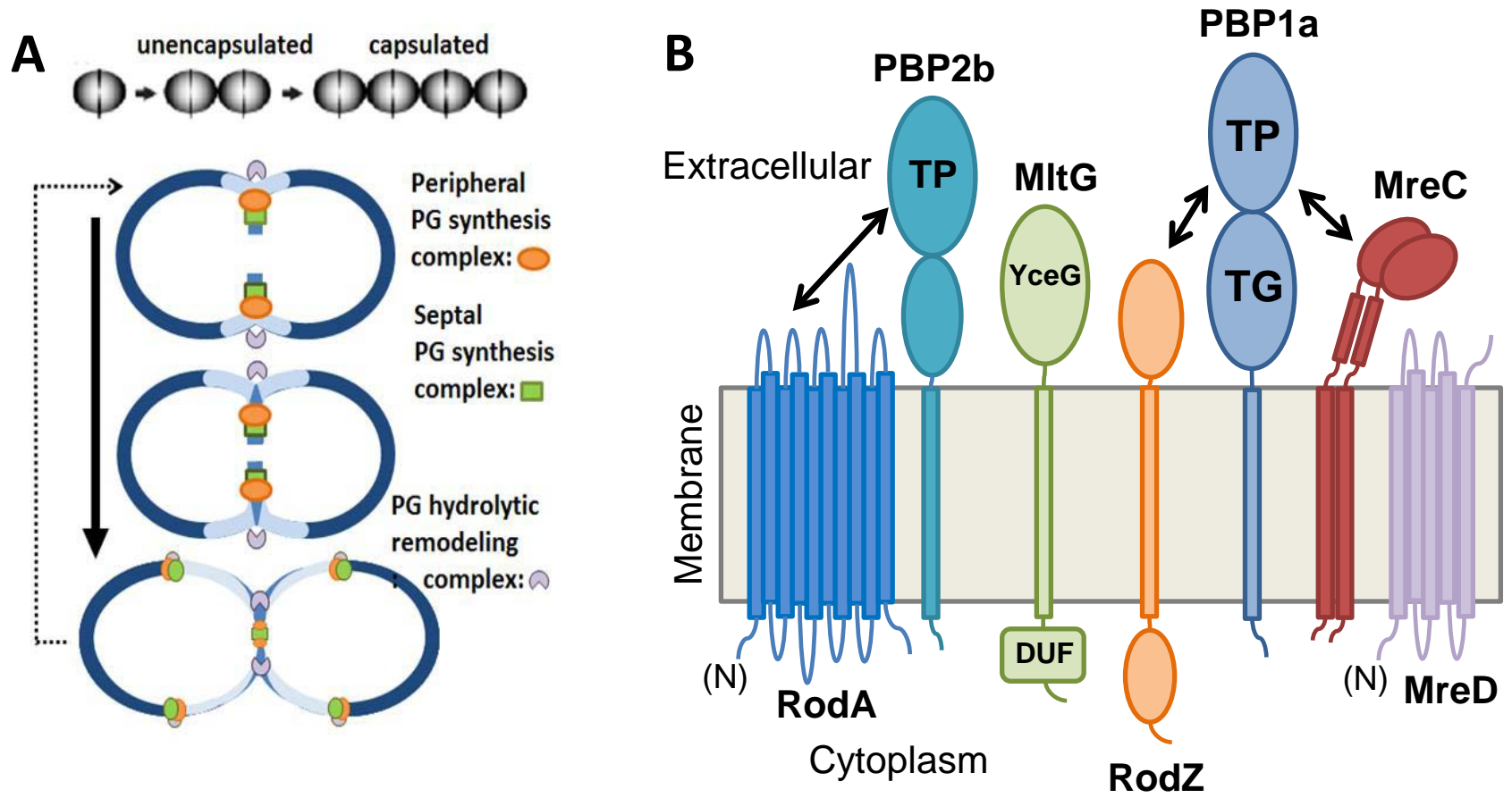
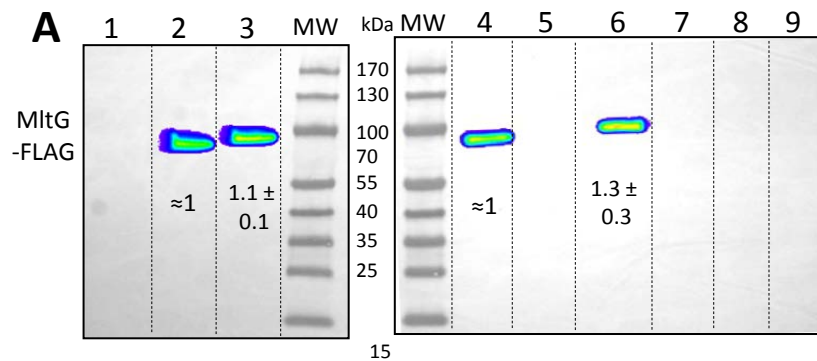
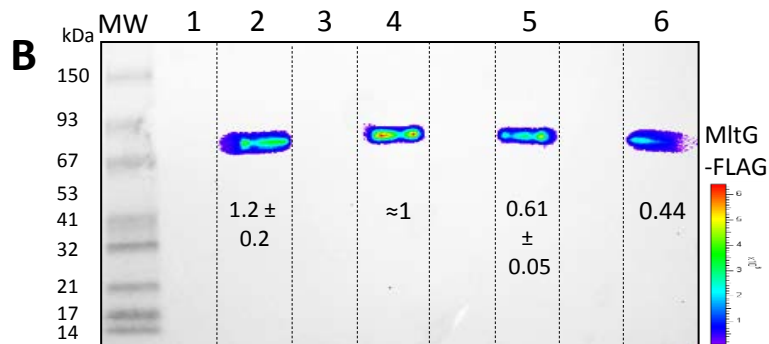


Fig. S1



Lane	Strain #	Genotype
1	IU1945	WT = D39 Δcps
2	IU7403	<i>mltG</i> -FLAG
3	IU10369	<i>mltG</i> (Y488D)-FLAG
4	IU9043	$\Delta pbb1a$ <i>mltG</i> -FLAG
5	IU10320	$\Delta pbb1a$ <i>mltG</i> ($\Delta 5bp$)-FLAG
6	IU10323	$\Delta pbb1a$ <i>mltG</i> (Y488D)-FLAG
7	IU10324	$\Delta pbb1a$ <i>mltG</i> ($\Delta 488bp$)-FLAG
8	IU10327	$\Delta pbb1a$ <i>mltG</i> ($\Omega 45bp$)-FLAG
9	IU6741	$\Delta pbb1a$



Lane	Strain #	Genotype
1	IU1945	WT = D39 Δcps
2	IU7403	<i>mltG</i> -FLAG
3	IU6741	$\Delta pbb1a$
4	IU9043	$\Delta pbb1a$ <i>mltG</i> -FLAG
5	IU9041	$\Delta pbb1a$ <i>mltG</i> (E428Q)-FLAG
6	IU9445	$\Delta pbb1a$ <i>mltG</i> (E428A)-FLAG

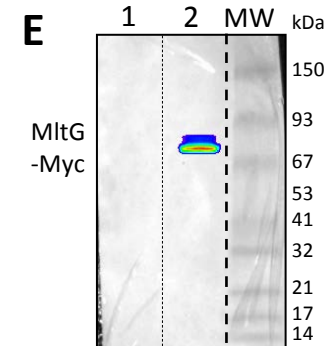
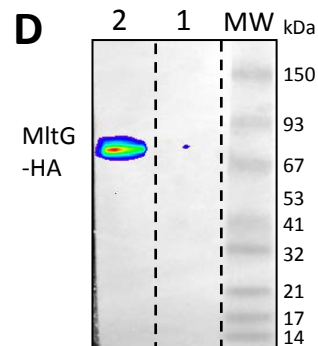
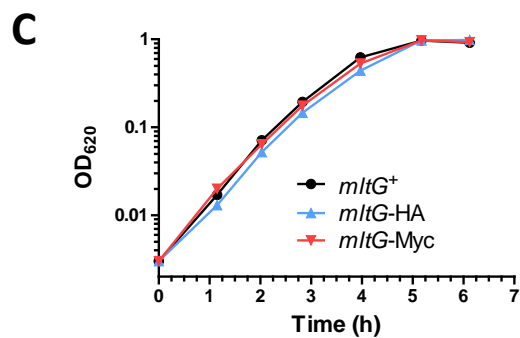


Fig. S2

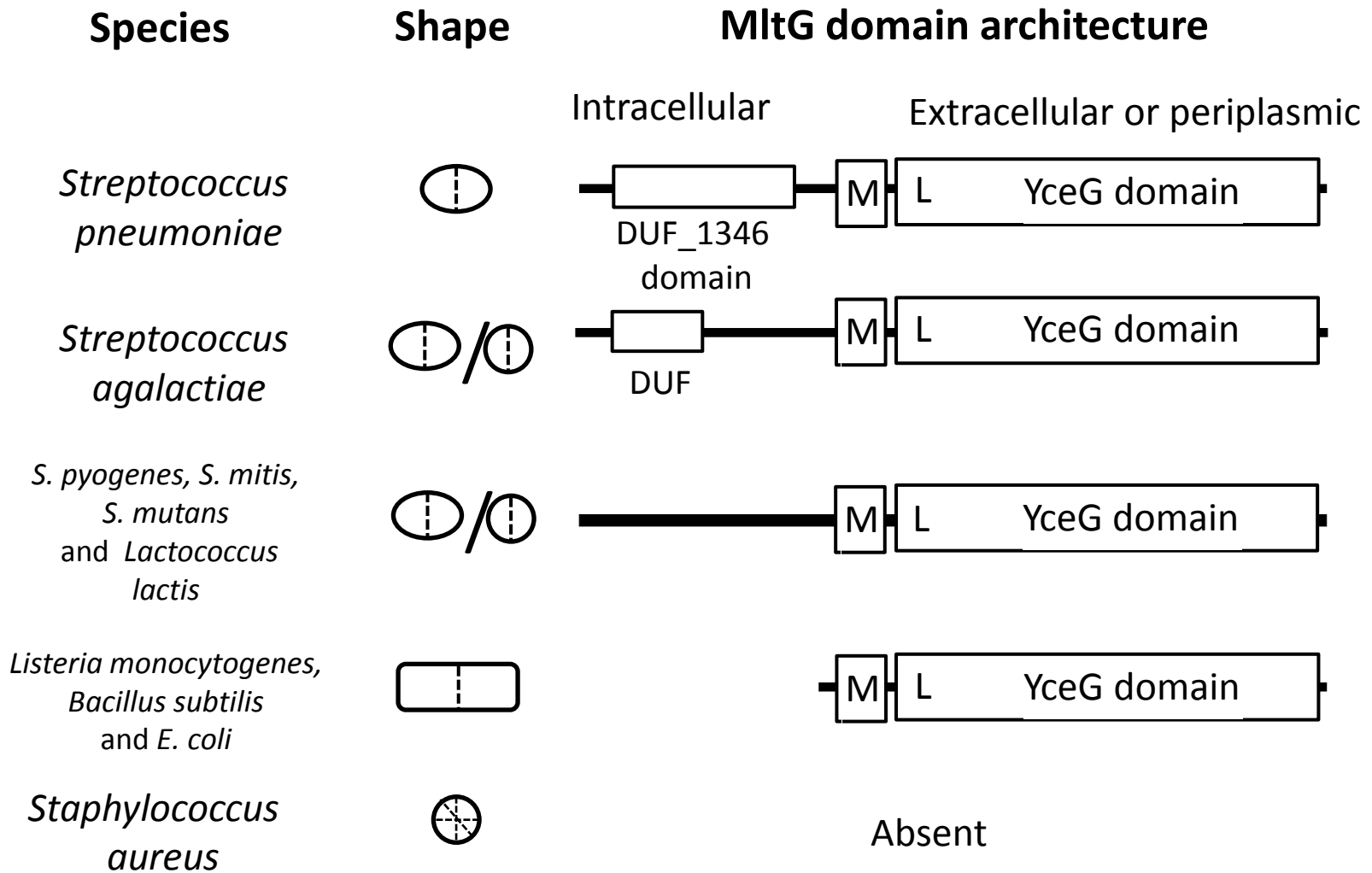


Fig. S3

Viable, but not stable (picks up suppressors):

mltG(E428Q) pbp1a⁺

[*mltG*(Δ 5bp), *mltG*(Δ 488bp), or *mltG*(Ω 45bp)²] Δ *pbp2b*

Viable and stable:

Δ *mltG* Δ *pbp1a*

[*mltG*(Δ 5bp), *mltG*(Δ 488bp), or *mltG*(Ω 45bp)²] Δ *pbp1a*

Δ *mltG* Δ *pbp1a* Δ *pbp2b*

mltG(Δ 5bp) Δ *pbp1a* Δ *pbp2b*

mltG(Δ 488bp) Δ *pbp1a* Δ *pbp2b*

mltG(Ω 45bp)² Δ *pbp1a* Δ *pbp2b*

sup2 = *mltG*(Δ 5bp)

sup4 = *mltG*(Δ 488bp)

sup5 = *mltG*(Ω 45bp)²

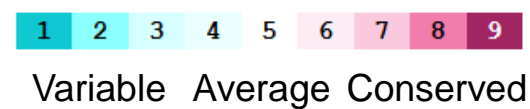
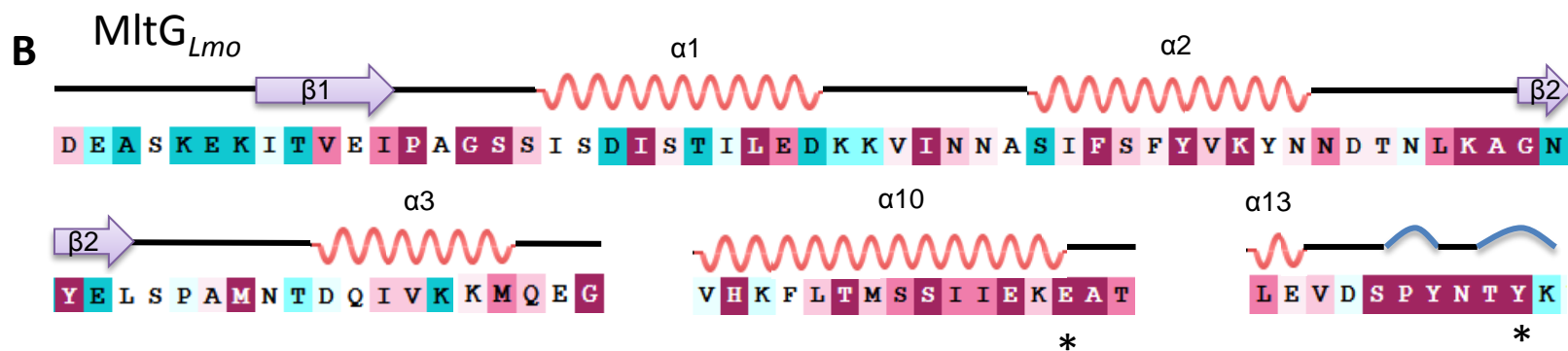
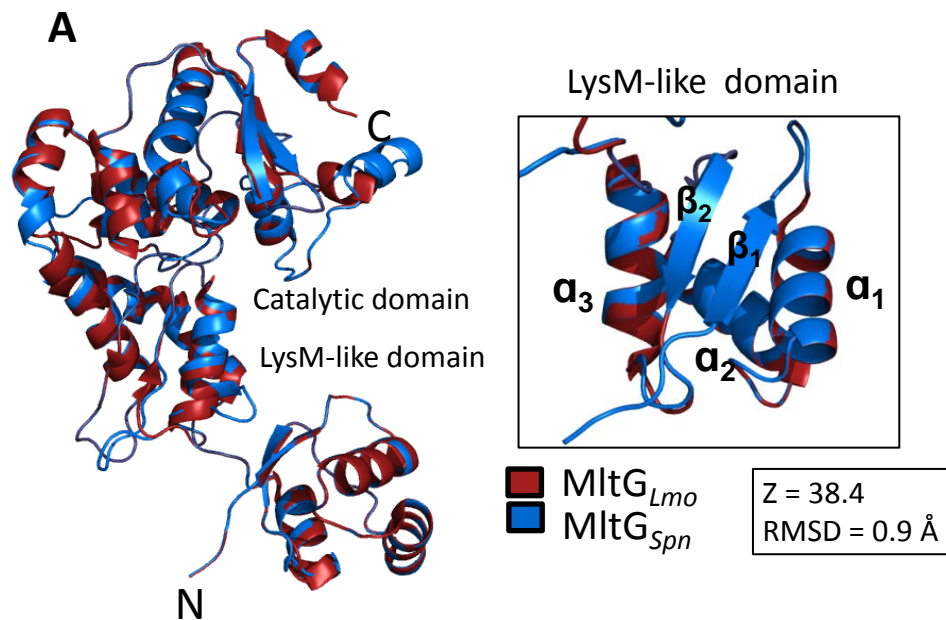


Fig. S7

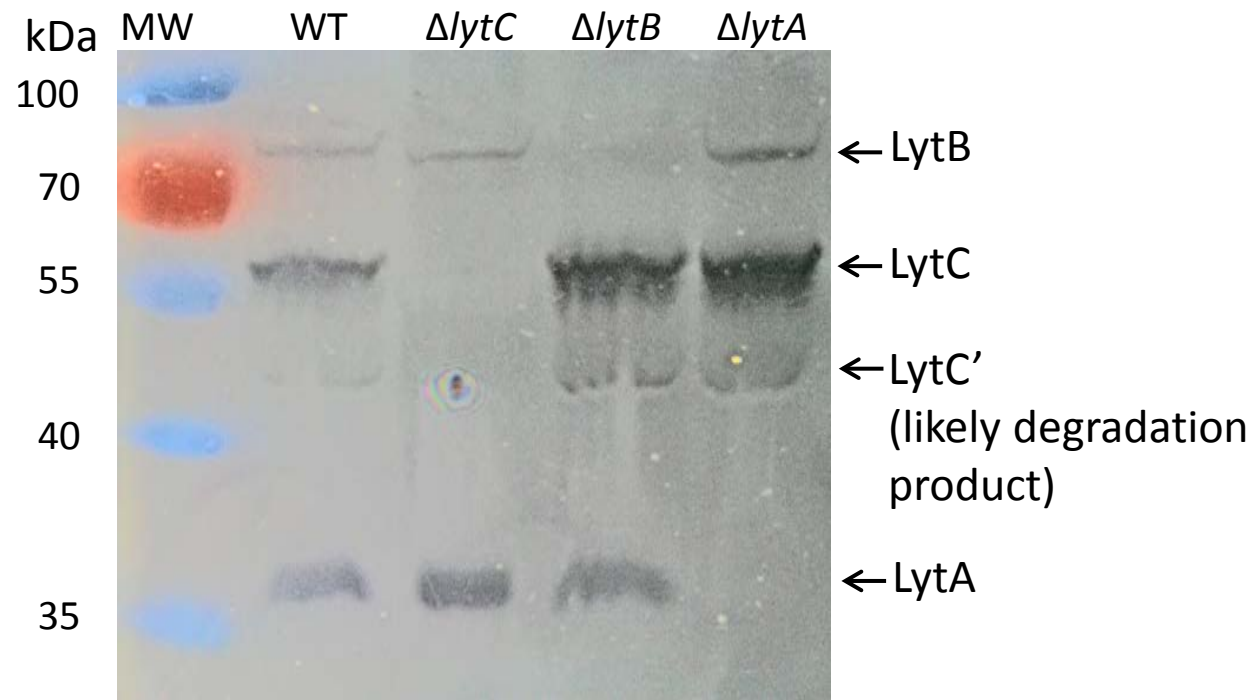


Fig. S8

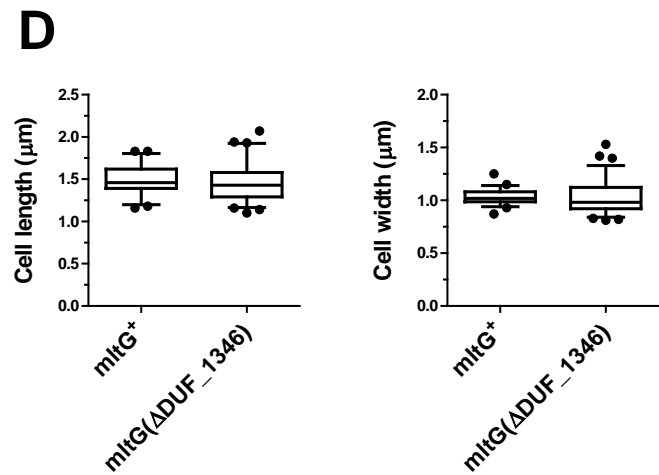
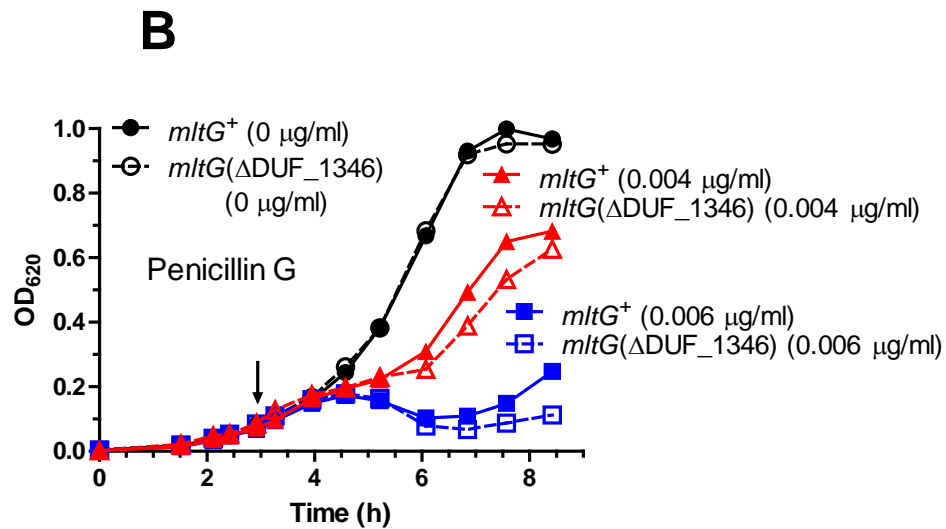
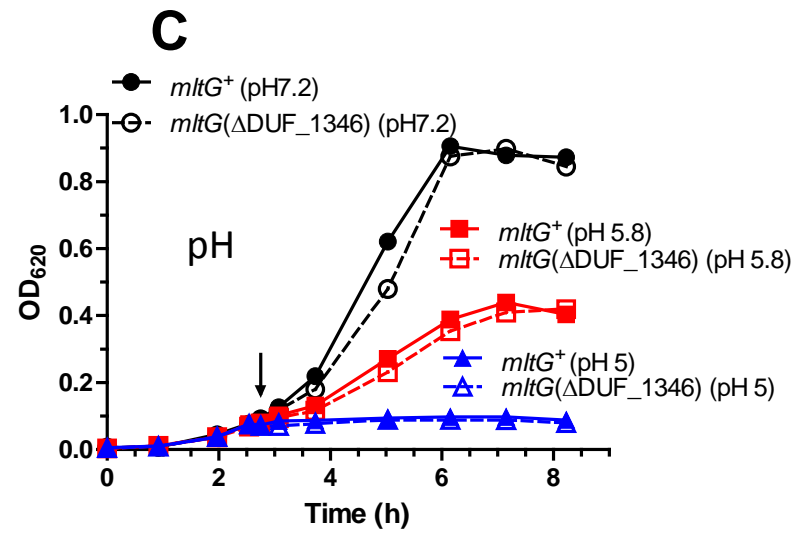
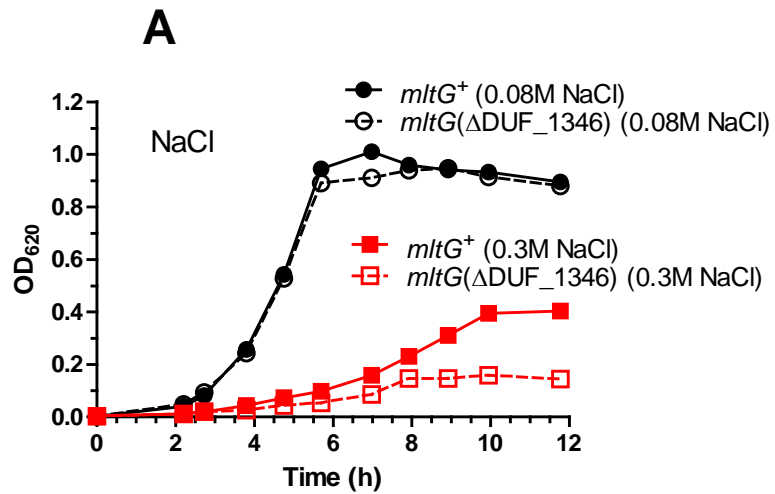


Fig. S9

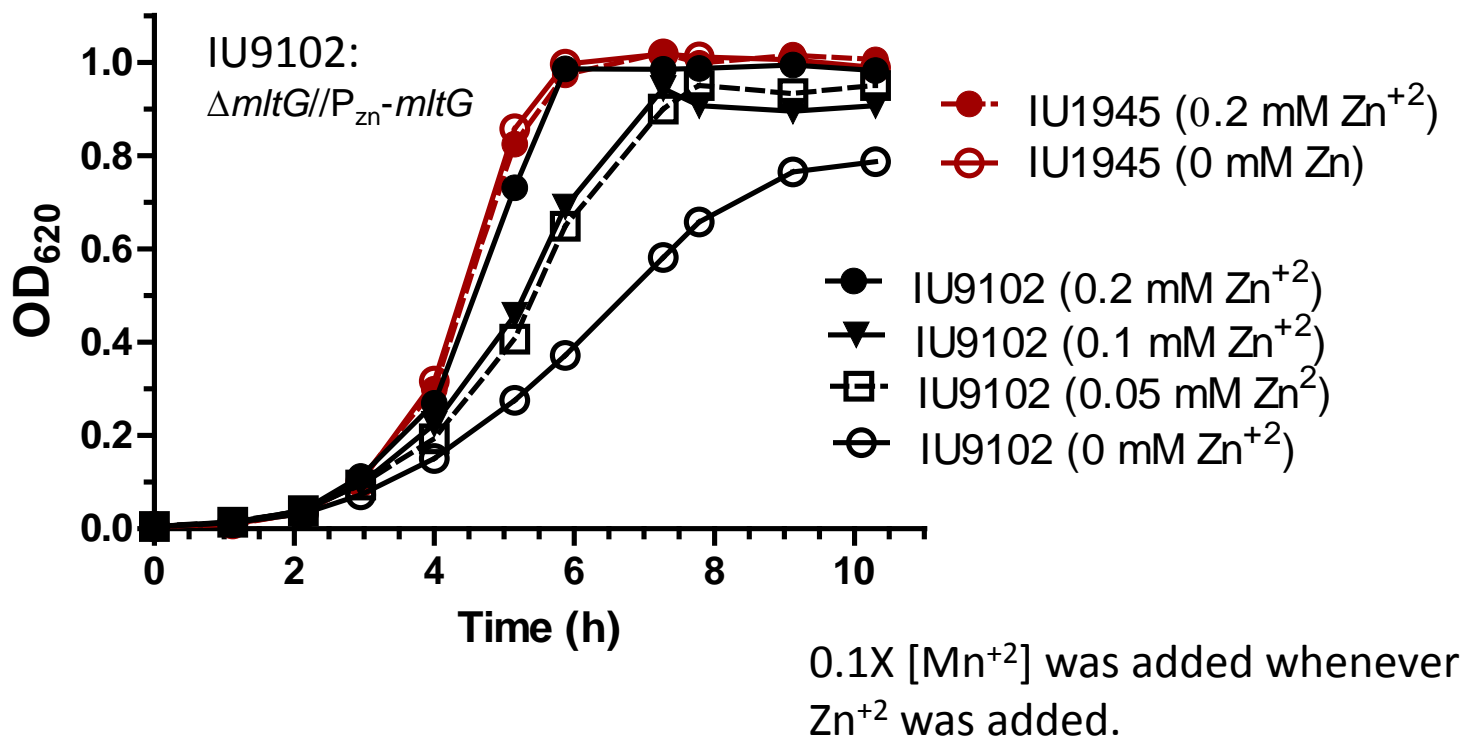


Fig. S10

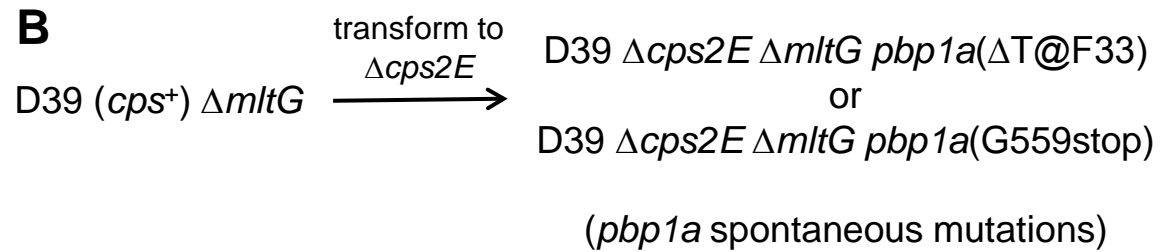
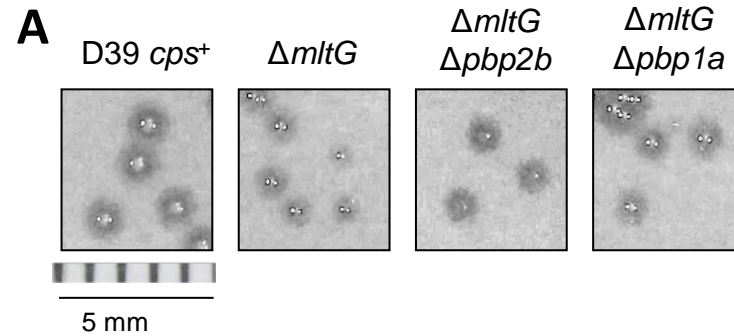


Fig. S12

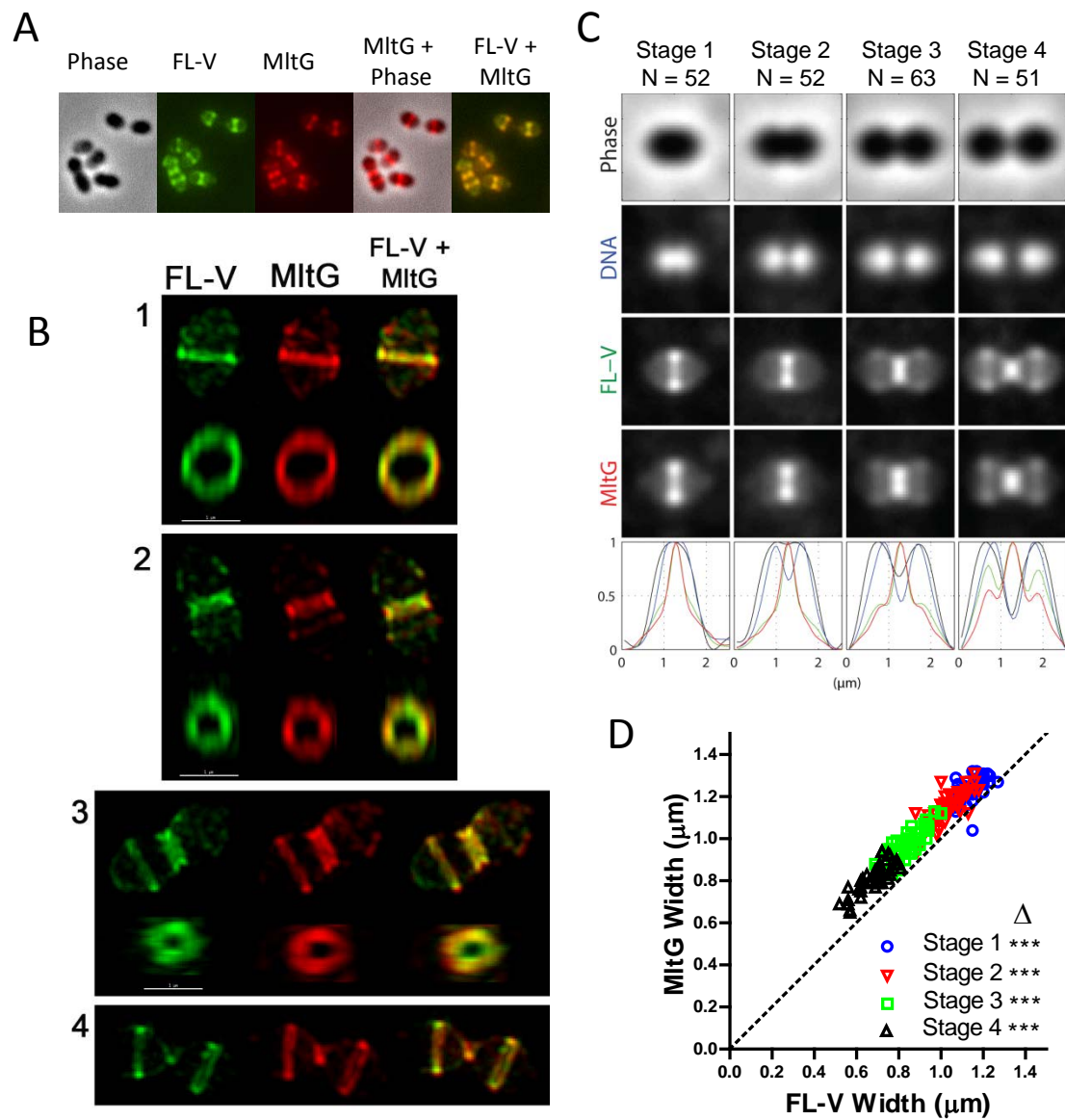


Fig. S13

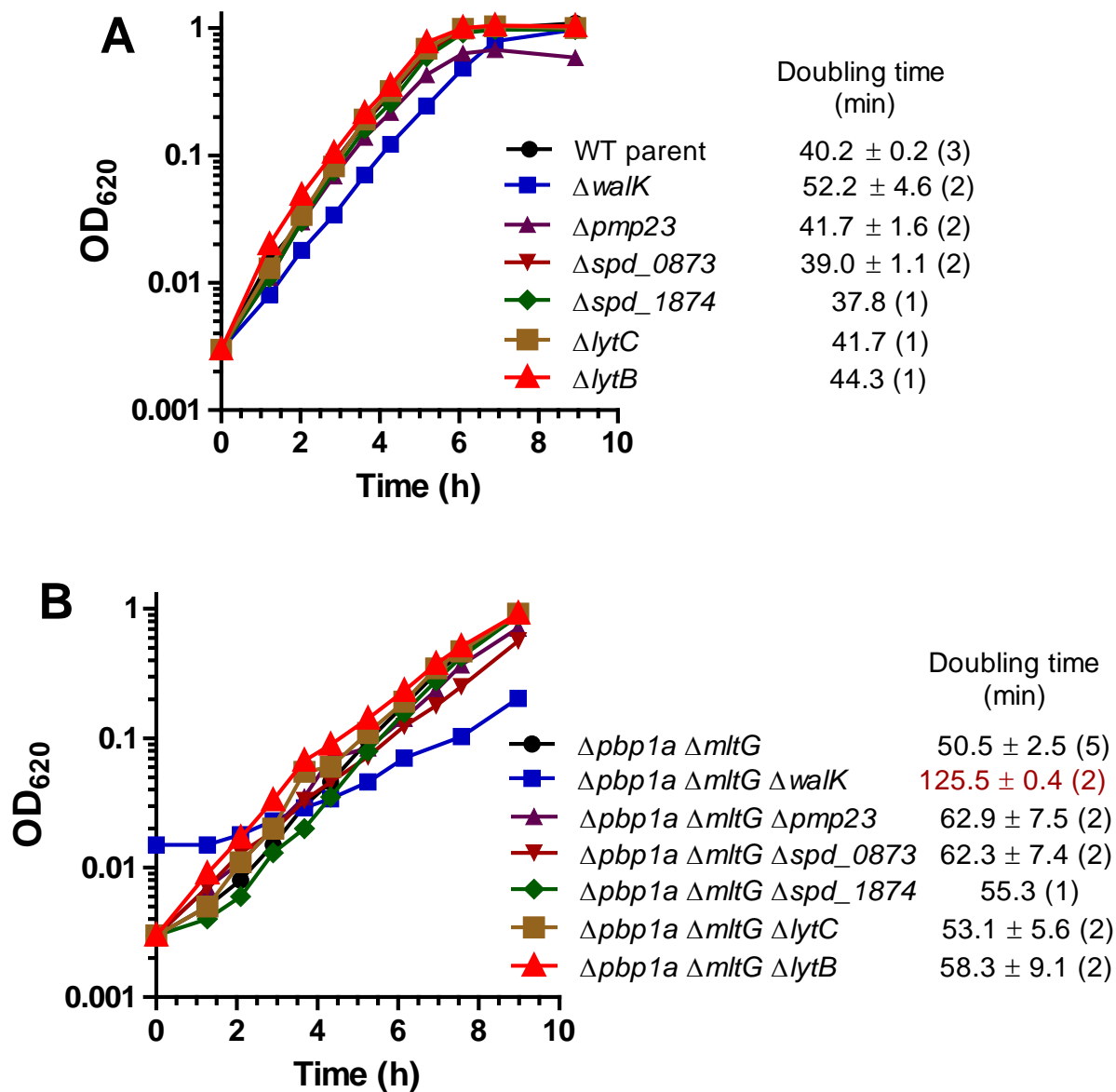
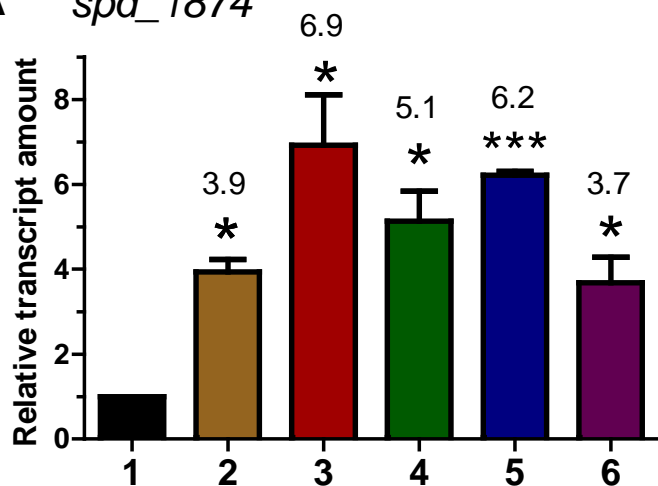
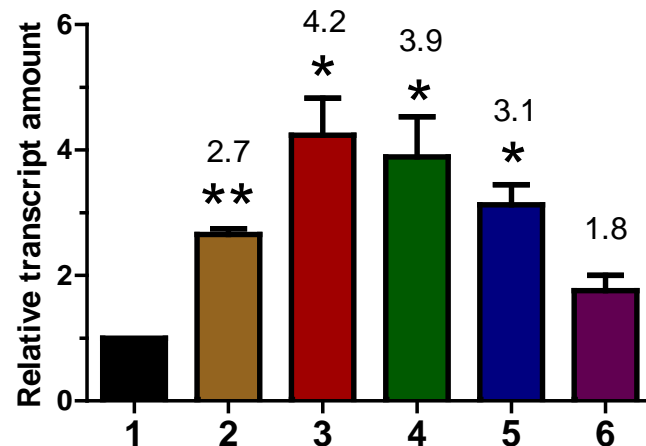
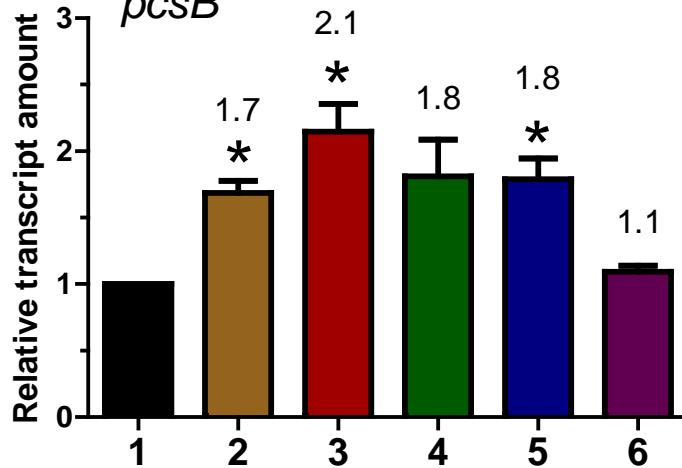


Fig. S14

A *spd_1874***B** *spd_0104***C***pcsB*

- 1 WT parent
- 2 $\Delta pbp1a$
- 3 $\Delta pbp1a mltG(\Delta 488bp)$
- 4 $\Delta pbp1a mltG(\Delta 488bp) \Delta pbp2b$
- 5 *mltG*(Y488D)
- 6 *mltG*(Y488D) $\Delta pbp2b$

Fig. S15