# SUPPLEMENTARY INFORMATION

## Biochemical reconstitution defines new functions for membrane-bound glycosidases in assembly of the bacterial cell wall

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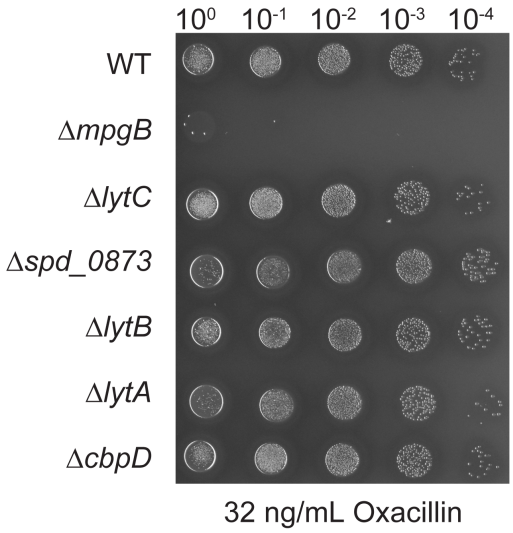


**Figure S1. Δ*mpgB* cells are hypersensitive to oxacillin.** Spot dilution series of *S. pneumoniae* strains with the indicated deletion were plated on agar containing 32 ng/mL oxacillin. A representative result from two independent experiments is shown.

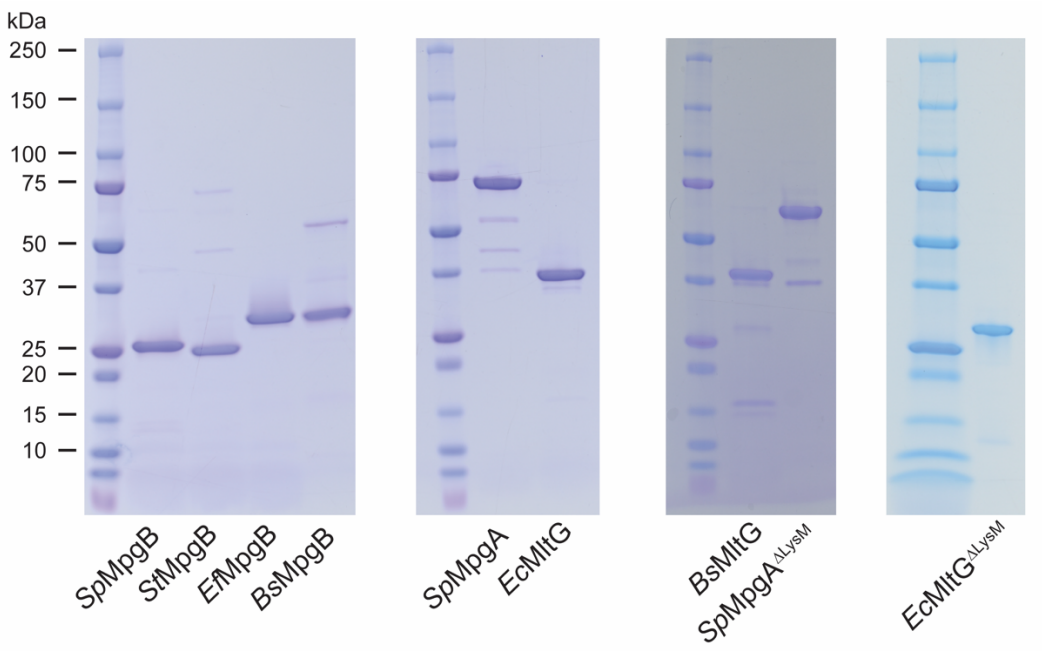


**Figure S2. Coomassie stained gels of purified glycosidases used in this study.** ~2 µg protein was loaded per lane.
*Sp*MpgB: *S. pneumoniae* SPD_0912
*St*MpgB: *S. thermophilus* stu0757
*Ef*MpgB: *E. faecalis* EF1518
*Bs*MpgB: *B. subtilis* BSU19130
*Sp*MpgA: *S. pneumoniae* SPD_1346
*Ec*MltG: *E. coli* b1097
*Bs*MltG: *B. subtilis* BSU27370
*Sp*MpgA$^{\Delta LysM}$: *Sp*MpgA$^{\Delta D219\text{-}P295}$
*Ec*MltG$^{\Delta LysM}$: *Ec*MltG$^{\Delta K33\text{-}F110}$

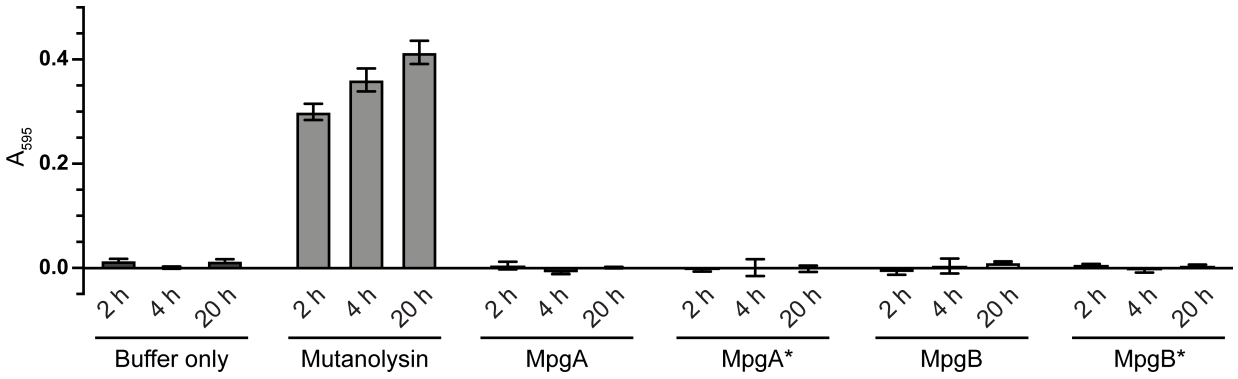


**Figure S3. MpgA and MpgB do not cleave isolated *S. pneumoniae* sacculi.** Isolated *S. pneumoniae* sacculi labeled with Remazol Brilliant Blue (RBB: ~500 µg) were incubated with 5 µM MpgA, MpgB, or their catalytically inactive variant indicated with an asterisk. Peptidoglycan cleavage activity was assessed by measuring the released RBB from the sacculi, which was determined by measuring the $A_{595\ nm}$ of the reaction supernatant after pelleting the intact peptidoglycan by centrifugation (1, 2). Mutanolysin (5 µM), a well-characterized muramidase capable of digesting *S. pneumoniae* sacculi, was used as a positive control (Fig. S7). Error bars represent mean ± SD from triplicates. A representative result from two independent experiments is shown.

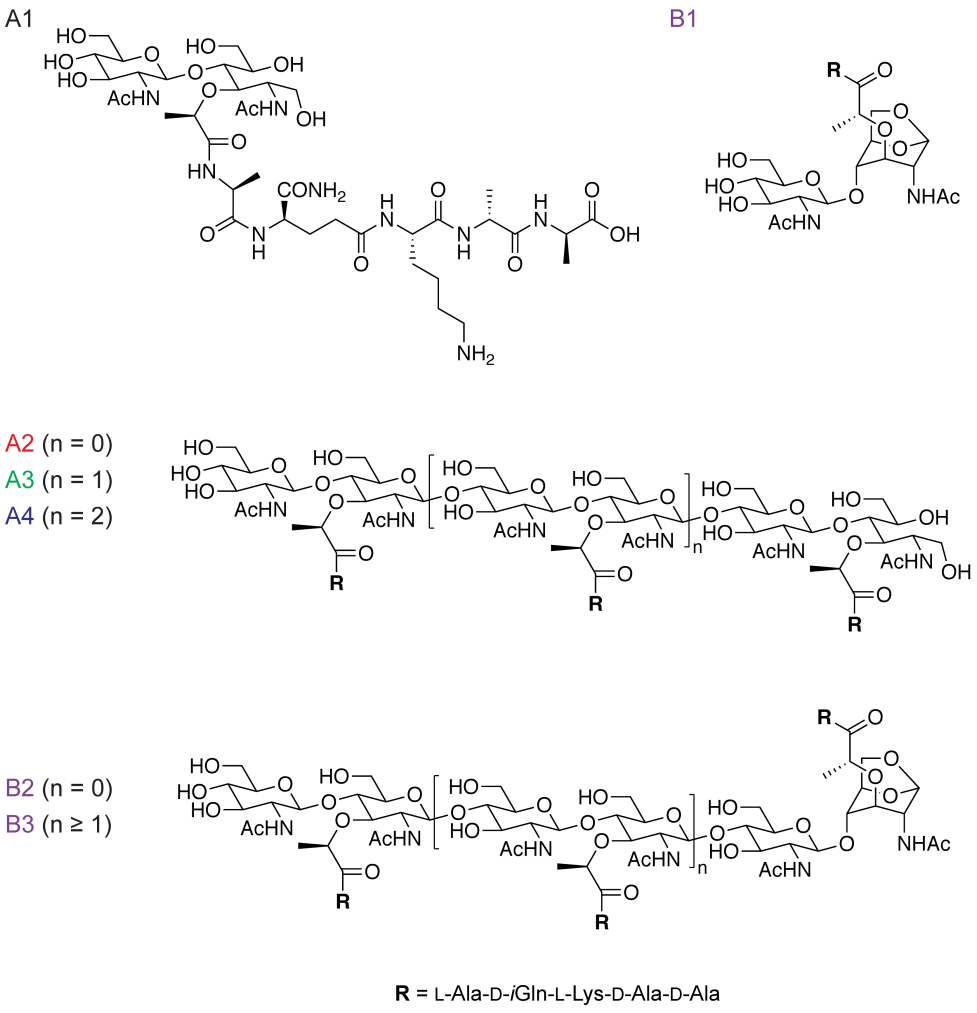


**Figure S4. Chemical structures of muropeptide products detected in digestion reactions following sodium borohydride reduction.** A1-A4 are peptidoglycan muramidase products and B1-B3 are lytic transglycosylase products with an 1,6-anhydroMurNAc end. See Fig. 4A for the schematic of LC-MS assay used to detect cleavage products.

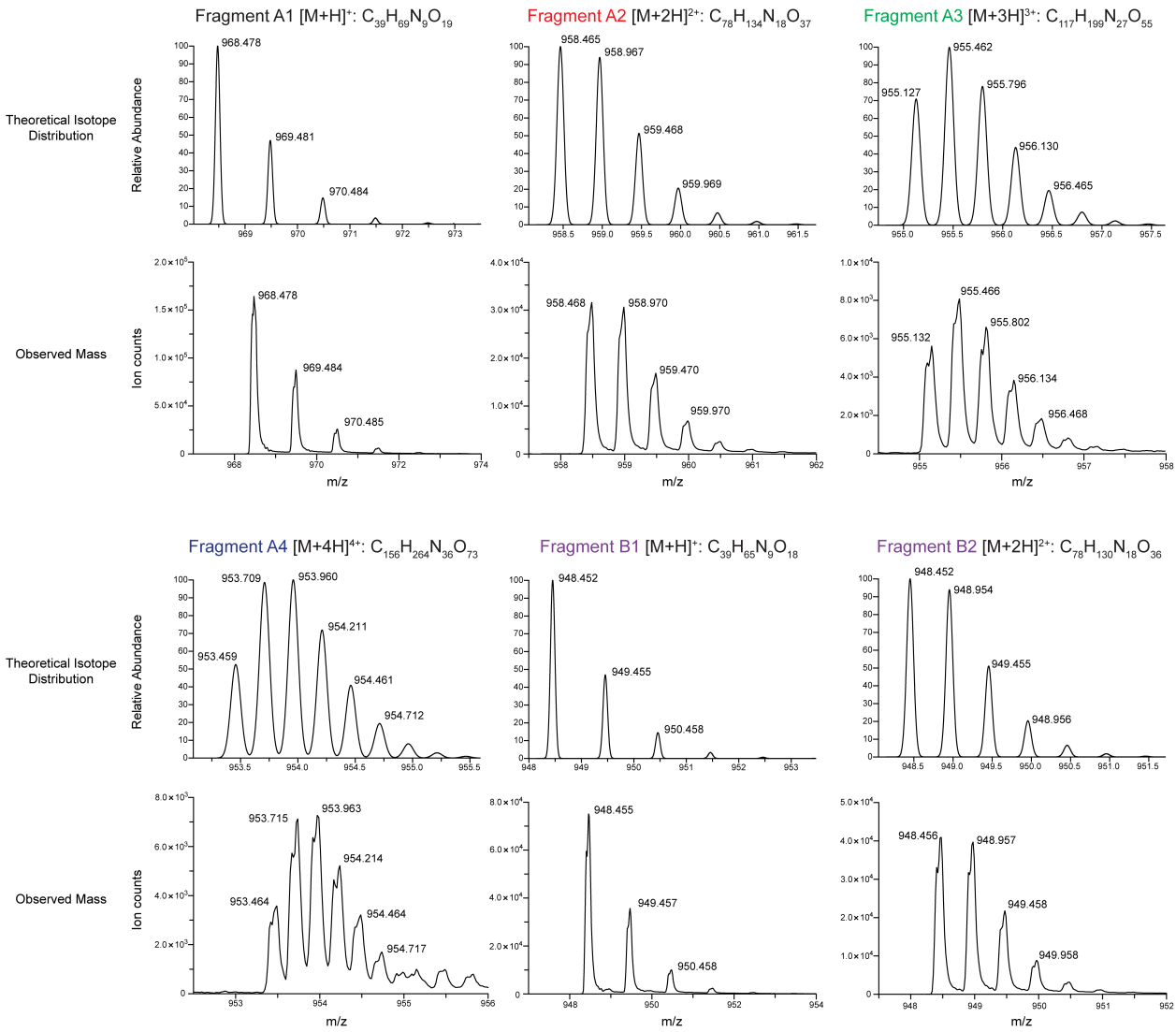


**Figure S5. Representative mass spectra of muropeptide products.** See Fig. S4 for the chemical structure of each product.

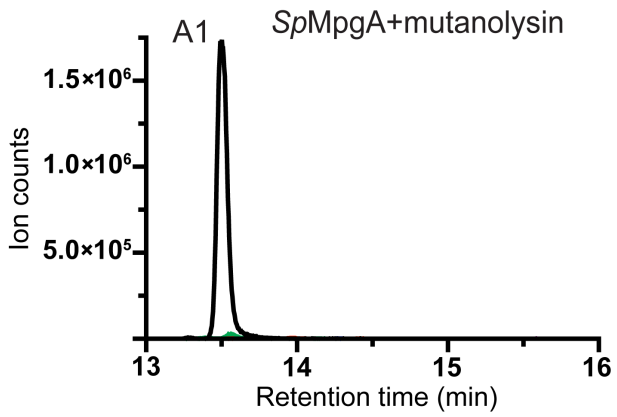


**Figure S6. Extracted ion chromatogram for the MpgA sample treated with mutanolysin.** Addition of mutanolysin to the MpgA-treated sample results in the disappearance of peaks A2-A4 and the increase in peak A1 compared to the MpgA-only sample (Fig 4C, panel 1). See Fig 4A for the peak assignment.

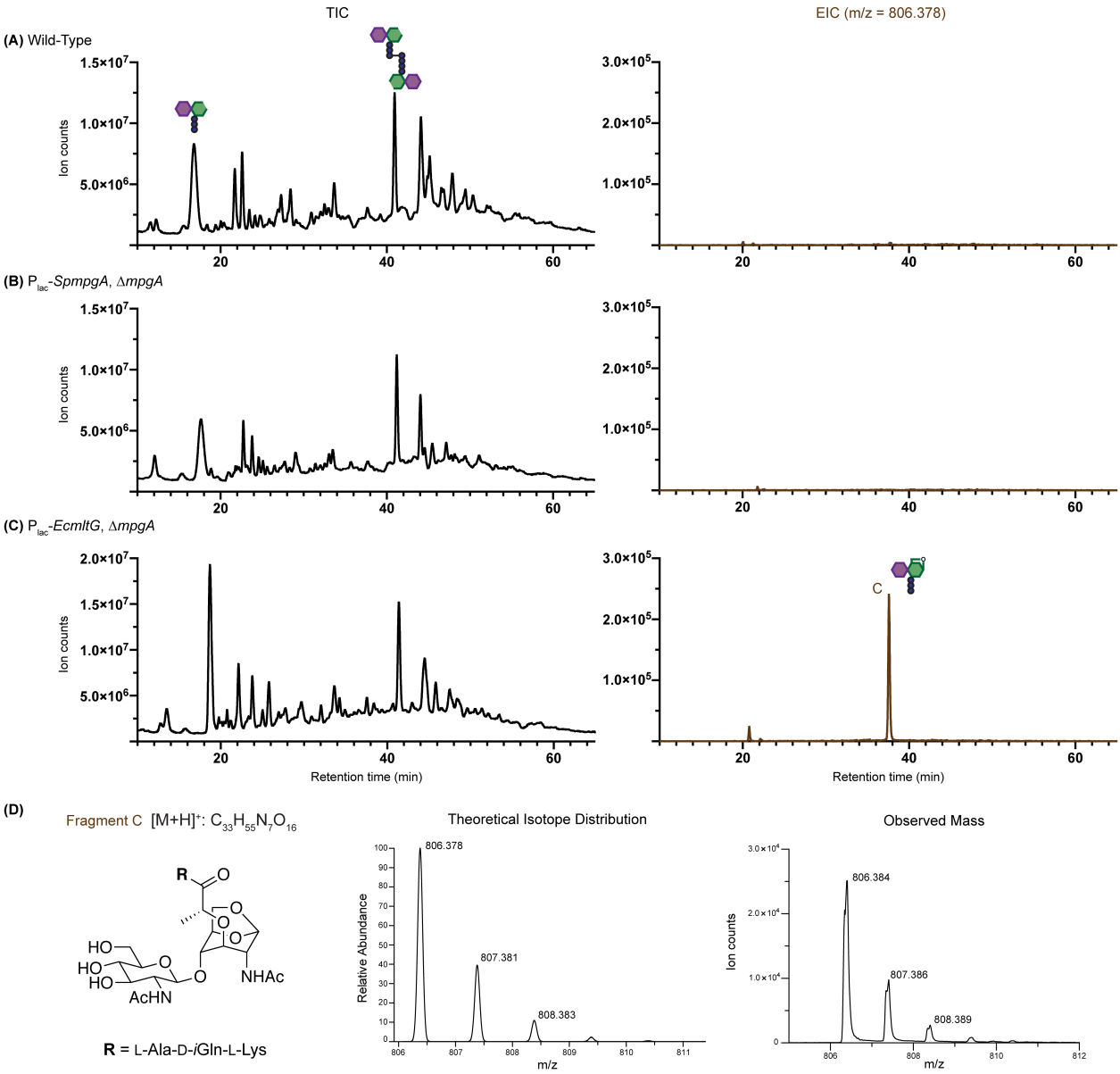


**Figure S7. Muropeptides with anhydro-MurNAc ends are detected in peptidoglycan isolated from *S. pneumoniae* complemented with *Ec*MltG, but not in peptidoglycan complemented with *Sp*MpgA.** Isolated peptidoglycan from unencapsulated *S. pneumoniae* D39 (A), $P_{lac}$-*SpmpgA* Δ*mpgA* (B), or $P_{lac}$-*EcmltG* Δ*mpgA* (C) was digested with mutanolysin. The resulting muropeptide products were separated and detected by LC-MS. The total ion chromatogram (TIC) and the extracted ion chromatogram (EIC: m/z = 806.378) are shown for each sample. Major peaks at ~18 min and ~41 min correspond to a tripeptide monomer and crosslinked dimer with unbranched stem peptides, respectively. A peak corresponding to a tripeptide monomer with an anhydro-MurNAc end was only detected in peptidoglycan isolated from cells expressing *Ec*MltG. Note that $P_{lac}$ is a constitutive promoter in the absence of LacI (3). (D) Chemical structure and mass spectrum of the anhydro-MurNAc containing muropeptide species. Representative results from two independent experiments are shown.

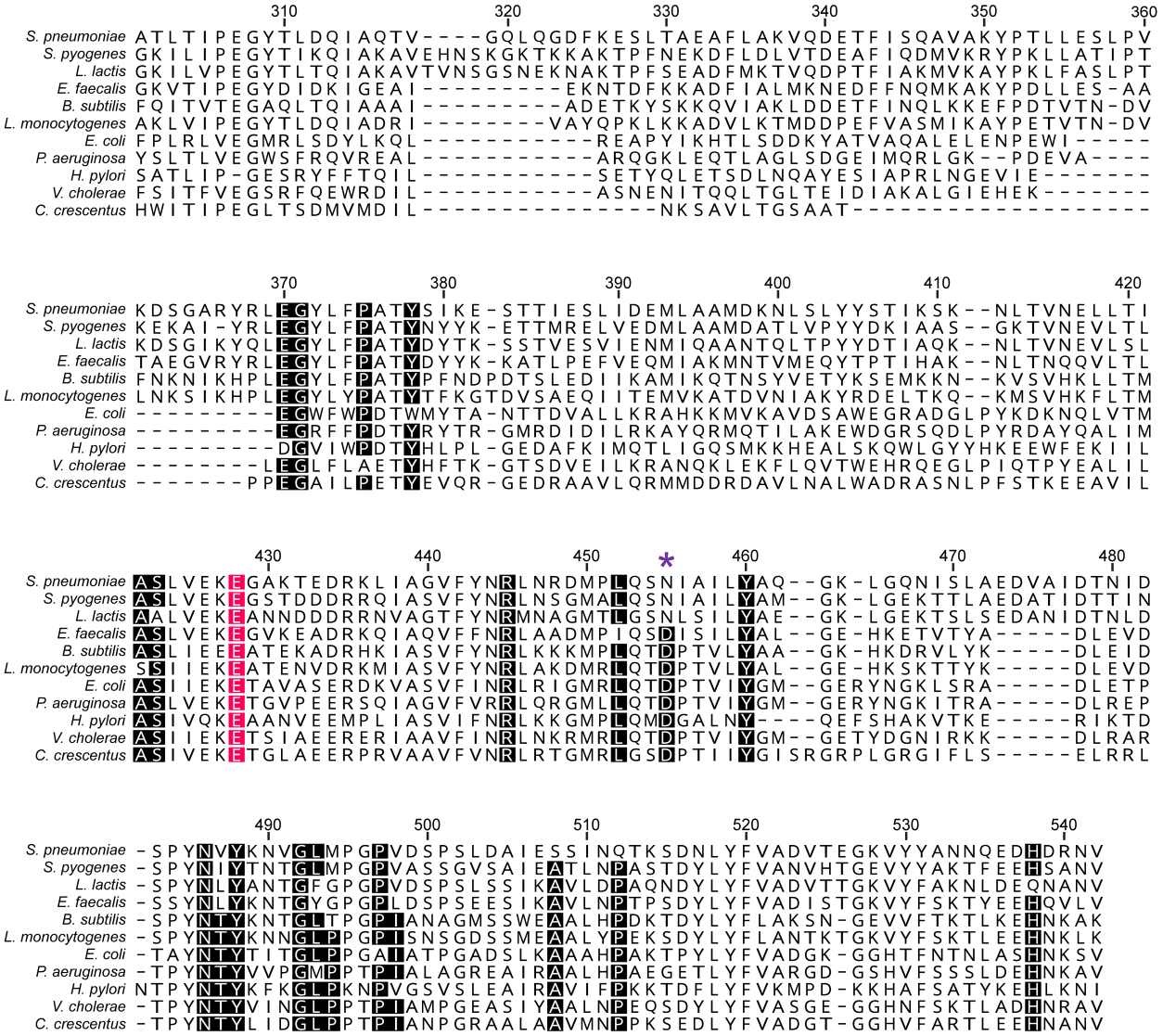


**Figure S8. Sequence alignment of the YceG catalytic subdomain.** Sequence conservation analysis of ~15000 sequences containing the YceG domain was performed using the EVcouplings server (4). The catalytic glutamate (~100% conserved) is highlighted in red. Residues conserved in >90% of the analyzed sequences are highlighted in black. The purple asterisk denotes the residue investigated in this study. Representative examples from 11 species (*S. pneumoniae*: SPD_1346; *S. pyogenes*: Spy_0348; *L. lactis*: L24228; *E. faecalis*: EF2915; *B. subtilis*: BSU27370; *L. monocytogenes*: lmo1499; *E. coli*: b1097; *P. aeruginosa*: PA2963; *H. pylori*: HP0587; *V. cholerae*: VC2017; *C. crescentus*: CCNA_01751) are shown.

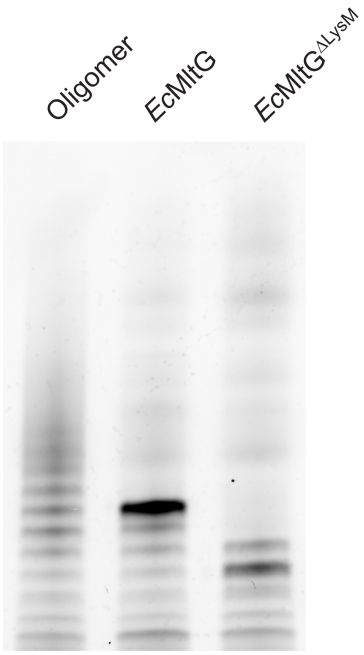


**Figure S9. *Ec*MltG$^{\Delta LysM}$ produces shorter lipid-linked cleavage products compared to *Ec*MltG.** See Fig 3A for the schematic of the SDS-PAGE assay used to assess cleavage products. A representative image from two independent blots is shown.

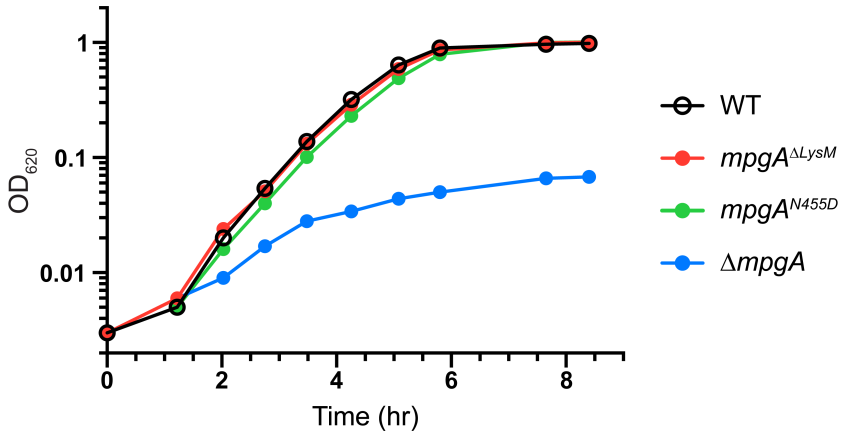


**Figure S10. Cells lacking the LysM-like domain of MpgA have growth rates similar to wild-type in BHI broth.** *S. pneumoniae* D39 Δ*cps rpsL1* strains containing the indicated copy of *mpgA* at the native locus and a zinc-inducible wild-type *mpgA* at the *bgaA* locus were grown in the absence of zinc. Representative growth curves from three independent experiments are shown.

| *mpgA* | # of colonies after transformation with Δ*pbp2b* amplicon |
|---|---|
| WT | 0 |
| ΔLysM | >500 |
| N455D | >500 |
| Y488D | >500 |

**Figure S11. PBP2b is dispensable in MpgA$^{\Delta LysM}$ cells and in cells with catalytic domain defect.** *S. pneumoniae* D39 Δ*cps rpsL1* strains containing the indicated copy of *mpgA* at the native locus and a zinc-inducible wild-type *mpgA* at the *bgaA* locus were transformed with the *pbp2b* deletion amplicon in the absence of zinc. Y488D is an MpgA variant that was previously characterized to be partially defective (5). The numbers of transformants were adjusted to the numbers obtained with 1 mL of transformation mixture. Two independent transformation experiments were performed with similar results.

**Supplementary Methods**

**Plasmid construction for protein expression**

*Sp*MpgB. The *SPD_0912 (M1-G204)* gene encoding MpgB was amplified from *S. pneumoniae* D39 Δ*cps* genomic DNA using primers oAT101/oAT102. After digestion with NdeI and BamHI, the PCR product was ligated into pET28b. The resulting plasmid pATPL240 expresses $His_6$-*Sp*MpgB. Plasmid pATPL368, expressing $His_6$-*Sp*MpgB$^{D68N}$ (catalytically inactive mutant *Sp*MpgB*) was PCR generated from pATPL240 with oAT103/oAT104 containing the desired mutation.

*St*MpgB. The *stu0757 (A2-L221)* gene encoding MpgB was amplified from *S. thermophilus* LMG18311 genomic DNA using primers oAT105/oAT106, and this fragment was ligated to a linearized pMS211 plasmid backbone (oAT107/oAT108) via InFusion Cloning (TakaraBio). The resulting plasmid pATPL424 expresses $His_6$-SUMO-FLAG-*St*MpgB.

*Ef*MpgB. The *EF1518 (D2-N209)* gene encoding MpgB was amplified from *E. faecalis* V583 genomic DNA using primers oAT109/oAT110 and this fragment was ligated to a linearized pMS211 plasmid backbone (oAT107/oAT108) via InFusion Cloning. The resulting plasmid pATPL425 expresses $His_6$-SUMO-FLAG-*Ef*MpgB.

*Bs*MpgB. The *BSU19130 (K2-E225)* gene encoding MpgB was amplified from *B. subtilis* PY79 genomic DNA using primers oAT111/oAT112 and this fragment was ligated to a linearized pMS211 plasmid backbone (oAT107/oAT108) via InFusion Cloning. The resulting plasmid pATPL426 expresses $His_6$-SUMO-FLAG-*Bs*MpgB.

*Sp*MpgA. The *SPD_1346 (M1-N551)* gene encoding MpgA was amplified from *S. pneumoniae* D39 Δ*cps* genomic DNA using primers oAT113/oAT114. After digestion with NdeI and XhoI, the PCR product was ligated into pET28b. The resulting plasmid pATPL434 expresses $His_6$-*Sp*MpgA. Primer pairs oAT115/oAT116, and oAT117/oAT118 were used to generate expression plasmids pATPL472 ($His_6$-*Sp*MpgA$^{E428Q}$; catalytically inactive mutant *Sp*MpgA*) and pATPL473 ($His_6$-*Sp*MpgA$^{N455D}$), respectively, from pATPL434 via InFusion Cloning. Plasmid pATPL488, expressing $His_6$-*Sp*MpgA$^{\Delta D219\text{-}P295}$ ($His_6$-*Sp*MpgA$^{\Delta LysM}$), was generated from pATPL434 with oAT119/120.

*Ec*MltG. The *b1097 (M1-Q340)* gene encoding MltG was amplified from *E. coli* BL21(DE3) genomic DNA using primers oAT121/oAT122. After digestion with NdeI and XhoI, the PCR product was ligated into pET28b. The resulting plasmid pATPL474 expresses $His_6$-*Ec*MltG. Primer pairs oAT123/oAT124 and

oAT125/oAT126 were used to construct expression plasmids pATPL475 ($His_6$-*Ec*MltG$^{E224Q}$; catalytically inactive mutant *Ec*MltG*) and pATPL476 ($His_6$-*Ec*MltG$^{D245N}$), respectively, from pATPL474 via InFusion Cloning. Plasmid pJP121, expressing $His_6$-*Ec*MltG$^{\Delta K33\text{-}F110}$ ($His_6$-*Ec*MltG$^{\Delta LysM}$), was generated from pATPL474 with oJP202/203.

*Bs*MltG. The *BSU27370 (M1-K360)* gene encoding MltG was amplified from *B. subtilis* PY79 genomic DNA using primers oAT127/oAT128. After digestion with NdeI and XhoI, the PCR product was ligated into pET28b. The resulting plasmid pATPL489 expresses $His_6$-*Bs*MltG.

***S. pneumoniae* strain construction**

AT513 (Δ*lytA*::*kan*): The ~1kb upstream and downstream regions of *lytA* were amplified using primer pairs oAT129/oAT130 and oAT131/oAT132. The *kan* gene was amplified by oAT133/oAT134 from D39 Δ*cps* Δ*bgaA*::*kan* genomic DNA. These PCR fragments were assembled by overlap extension PCR. The resulting PCR cassette was transformed into D39 Δ*cps* and transformants were selected with kanamycin. Integration into the genome was confirmed by diagnostic PCR using primers oAT135/oAT134.

AT514 (Δ*lytB*::*kan*): The ~1kb upstream and downstream regions of *lytB* were amplified using primer pairs oAT136/oAT137 and oAT138/oAT139. These PCR fragments and *kan* were assembled by overlap extension PCR. The resulting PCR cassette was transformed into D39 Δ*cps* and transformants were selected with kanamycin. Integration into the genome was confirmed by diagnostic PCR using primers oAT140/oAT134.

AT515 (Δ*lytC*::*kan*): The ~1kb upstream and downstream regions of *lytC* were amplified using primer pairs oAT141/oAT142 and oAT143/oAT144. These PCR fragments and *kan* were assembled by overlap extension PCR. The resulting PCR cassette was transformed into D39 Δ*cps* and transformants were selected with kanamycin. Integration into the genome was confirmed by diagnostic PCR using primers oAT145/oAT134.

AT516 (Δ*cbpD*::*kan*): The ~1kb upstream and downstream regions of *cbpD* were amplified using primer pairs oAT146/oAT147 and oAT148/oAT149. These PCR fragments and *kan* were assembled by overlap extension PCR. The resulting PCR cassette was transformed into D39 Δ*cps* and transformants were selected with kanamycin. Integration into the genome was confirmed by diagnostic PCR using primers oAT150/oAT134.

AT520 (Δ*spd_0873*::*erm*): The ~1kb upstream and downstream regions of *spd_0873* were amplified using primer pairs oAT151/oAT152 and oAT153/oAT154. The *erm* gene was amplified by oAT133/oAT134 from D39 Δ*cps* Δ*bgaA*::*erm* genomic DNA. These PCR fragments were assembled by overlap extension PCR. The resulting PCR cassette was transformed into D39 Δ*cps* and transformants were selected with erythromycin. Integration into the genome was confirmed by diagnostic PCR using primers oAT155/oAT134.

AT446 (Δ*mpgB*): $P_{96}$ (*spd_0104* promoter region) and *B. subtilis sacB* gene were amplified using primer pairs oAT156/oAT157 and oAT158/oAT159 and combined to make a $P_{96}$-*sacB* DNA fragment (6). The ~1kb upstream and downstream regions of *mpgB* were amplified using primer pairs oAT160/oAT161 and oAT162/oAT163. These PCR fragments and *erm* were assembled by overlap extension PCR. The resulting PCR cassette was transformed into D39 Δ*cps* and transformants were selected with erythromycin (AT428). For making a markerless deletion, the ~1kb upstream and downstream regions of *mpgB* were amplified using primer pairs oAT160/oAT164 and oAT165/oAT163. These PCR fragments were combined by overlap extension PCR, and the resulting PCR cassette was transformed into AT428. Transformants were selected with 10% sucrose, and the loss of resistance marker was assessed by diagnostic PCR using primers oAT166 and oAT167. Markerless deletion of *mpgB* was confirmed by DNA sequencing.

AT563 & AT564: Wild-type (AT563) and Δ*mpgB* (AT564) constitutively expressing LacI were constructed by transforming pPEPY-PF6-*lacI* to D39 Δ*cps* or AT446 and selecting transformants with gentamicin.

AT580-584: Plasmids containing $P_{lac}$-*mpgB* were constructed by first linearizing the pPEPZ-$P_{lac}$ vector using primers oAT168/oAT169. This linearized vector was ligated to *SpmpgB* (oAT170/oAT171), *StmpgB* (oAT172/oAT173), *EfmpgB* (oAT174/oAT175) or *BsmpgB* (oAT176/oAT177) via InFusion Cloning to generate pATPL461 ($P_{lac}$-*SpmpgB*), pATPL462 ($P_{lac}$-*StmpgB*), pATPL463 ($P_{lac}$-*EfmpgB*) or pATPL464 ($P_{lac}$-*BsmpgB*). These plasmids were transformed into AT564 and transformants were selected with spectinomycin.

AT600 & AT635: Plasmids containing $P_{lac}$-*EcmltG* or $P_{lac}$-*SpmpgA* were constructed by ligating the linearized pPEPZ-$P_{lac}$ vector to *EcmltG* (oAT178/oAT179) or *SpmpgA* (oAT180/oAT181) via InFusion Cloning. The resulting plasmids pATPL471 ($P_{lac}$-*EcmltG*) and pATPL492 ($P_{lac}$-*SpmpgA*) were transformed into D39 Δ*cps* to generate AT592 and AT634, respectively. A *mpgA* deletion cassette was assembled by first amplifying the ~1kb upstream and downstream regions of *mpgA* using primer pairs

oAT182/oAT183 and oAT184/oAT185. These PCR fragments and *kan* were assembled by overlap extension PCR. The resulting PCR cassette was transformed into AT592 or AT634 and transformants were selected with kanamycin. Integration of the deletion cassette into the genome was confirmed by diagnostic PCR using primers oAT186/oAT134.

IU15898 (*rpsL1*, Δ*bgaA*::$P_{zn}$-*mpgA*): A PCR fragment containing *tet* and a zinc-inducible *mpgA* flanked by the upstream and downstream regions of *bgaA* was amplified from IU8872 genomic DNA using primers oTT657/oCS121. This PCR fragment was transformed into D39 *cps rpsL1* (IU1824) and the transformants were selected with tetracycline. Integration of the fragment was confirmed by diagnostic PCR using primers oBR05/oSK27 and the construct was confirmed by DNA sequencing.

IU18052, IU18054, IU18056 and IU18058: A PCR fragment containing *kan* and *rpsL* flanked by the upstream and downstream regions of *mpgA* was amplified from K637 genomic DNA using primers oP1348/oP1349. This PCR fragment was transformed into IU15898 and the transformants were selected with kanamycin (IU15922). IU18052 was constructed by first amplifying the 5' fragment and 3' fragment using primer pairs oP1348/oTT1406 and oTT1407/oP1349, respectively. These fragments were assembled by overlap extension PCR. The resulting $mpgA^{N455D}$ PCR cassette was transformed into IU15922 and transformants were selected with streptomycin. IU18054 was constructed by first amplifying the 5' fragment and 3' fragment using primer pairs oP1348/oTT1408 and oTT1409/oP1349, respectively. These fragments were assembled by overlap extension PCR. The resulting $mpgA^{\Delta LysM}$ ($mpgA^{\Delta D219\text{-}P295}$) PCR cassette was transformed into IU15922 and transformants were selected with streptomycin. IU18056 was constructed by first amplifying the 5' fragment and 3' fragment using primer pairs oP1348/oTT1410 and oTT1411/oP1349, respectively. These fragments were assembled by overlap extension PCR. The resulting Δ*mpgA* PCR cassette was transformed into IU15922 and transformants were selected with streptomycin. IU18058 was constructed by first amplifying the $mpgA^{Y488D}$ PCR cassette from IU9760 genomic DNA using primers oP1348 and oP1349. The resulting $mpgA^{Y488D}$ PCR cassette was transformed into IU15922 and transformants were selected with streptomycin. Construction of these strains were carried out in the presence of 0.2 mM $Zn^{2+}$ and 0.02 mM $Mn^{2+}$. Constructs were confirmed by DNA sequencing.

**Table S1. Bacterial strains used in this study**

| Strain | Description* | Reference |
|---|---|---|
| *E. coli* | | |
| XL1-Blue | Host strain for plasmid cloning | Stratagene |
| Stellar | Host strain for plasmid cloning | TakaraBio |
| C43(DE3) | BL21(DE3) derivative strain for protein production | (7) |
| *S. pneumoniae* | | |
| D39 Δ*cps* | Unencapsulated D39 derivative strain (wild-type) | (8) |
| AT030 | D39 Δ*cps*, Δ*bgaA*::*erm*; $Erm^R$ | (9) |
| AT031 | D39 Δ*cps*, Δ*bgaA*::*kan*; $Kan^R$ | (9) |
| AT513 | D39 Δ*cps*, Δ*lytA*::*kan*; $Kan^R$ | This study |
| AT514 | D39 Δ*cps*, Δ*lytB*::*kan*; $Kan^R$ | This study |
| AT515 | D39 Δ*cps*, Δ*lytC*::*kan*; $Kan^R$ | This study |
| AT516 | D39 Δ*cps*, Δ*cbpD*::*kan*; $Kan^R$ | This study |
| AT520 | D39 Δ*cps*, Δ*spd_0873*::*erm*; $Erm^R$ | This study |
| AT446 | D39 Δ*cps*, Δ*mpgB* | This study |
| AT563 | D39 Δ*cps*, Δ*prsA*::(PF6-*lacI*, *gent*); $Gent^R$ | This study |
| AT564 | D39 Δ*cps*, Δ*prsA*::(PF6-*lacI*, *gent*), Δ*mpgB*; $Gent^R$ | This study |
| AT580 | D39 Δ*cps*, Δ*prsA*::(PF6-*lacI*, *gent*), Δ*mpgB*, Δ*spd_1735*::($P_{lac}$, *spec*); $Gent^R$, $Spec^R$ | This study |
| AT581 | D39 Δ*cps*, Δ*prsA*::(PF6-*lacI*, *gent*), Δ*mpgB*, Δ*spd_1735*::($P_{lac}$-*SpmpgB*, *spec*); $Gent^R$, $Spec^R$ | This study |
| AT582 | D39 Δ*cps*, Δ*prsA*::(PF6-*lacI*, *gent*), Δ*mpgB*, Δ*spd_1735*::($P_{lac}$-*StmpgB*, *spec*); $Gent^R$, $Spec^R$ | This study |
| AT583 | D39 Δ*cps*, Δ*prsA*::(PF6-*lacI*, *gent*), Δ*mpgB*, Δ*spd_1735*::($P_{lac}$-*EfmpgB*, *spec*); $Gent^R$, $Spec^R$ | This study |
| AT584 | D39 Δ*cps*, Δ*prsA*::(PF6-*lacI*, *gent*), Δ*mpgB*, Δ*spd_1735*::($P_{lac}$-*BsmpgB*, *spec*); $Gent^R$, $Spec^R$ | This study |
| AT600 | D39 Δ*cps*, Δ*mpgA*::*kan,* Δ*spd_1735*::($P_{lac}$-*mpgA*, *spec*); $Spec^R$, $Kan^R$ | This study |
| AT635 | D39 Δ*cps*, Δ*mpgA*::*kan,* Δ*spd_1735*::($P_{lac}$-*EcmltG*, *spec*); $Spec^R$, $Kan^R$ | This study |
| K637 | D39 Δ*cps*, Δ*mpgA*::($P_c$-*kan*,*rpsL*); $Kan^R$ | (5) |
| IU1824 | D39 Δ*cps*, *rpsL1*; $Str^R$ | (8) |
| IU8872 | D39 Δ*cps*, Δ*bgaA*::($P_{zn}$-*mpgA*, *tet*), $Tet^R$ | (5) |
| IU9760 | D39 Δ*cps*, *rpsL1*; $mpgA^{Y488D}$; $Str^R$ | (5) |
| IU15898 | D39 Δ*cps*, *rpsL1*, Δ*bgaA*::($P_{zn}$-*mpgA*, *tet*); $Str^R$, $Tet^R$ | This study |
| IU18052 | D39 Δ*cps*, *rpsL1*, Δ*bgaA*::($P_{zn}$-*mpgA*, *tet*), $mpgA^{N455D}$; $Str^R$, $Tet^R$ | This study |
| IU18054 | D39 Δ*cps*, *rpsL1*, Δ*bgaA*::($P_{zn}$-*mpgA*, *tet*), $mpgA^{\Delta D219\text{-}P295}$; $Str^R$, $Tet^R$ | This study |
| IU18056 | D39 Δ*cps*, *rpsL1*; Δ*bgaA*::($P_{zn}$-*mpgA*, *tet*), Δ*mpgA*; $Str^R$, $Tet^R$ | This study |
| IU18058 | D39 Δ*cps*, *rpsL1*; Δ*bgaA*::($P_{zn}$-*mpgA*, *tet*), $mpgA^{Y488D}$; $Str^R$, $Tet^R$ | This study |

*Abbreviations: $Amp^R$, ampicillin/carbenicillin resistance; $Cm^R$, chloramphenicol resistance; $Erm^R$, erythromycin resistance; $Gent^R$, gentamicin resistance; $Kan^R$, kanamycin resistance; $Spec^R$, spectinomycin resistance; $Str^R$, streptomycin resistance; $Tet^R$, tetracycline resistance

**Table S2. Plasmids used in this study**

| Plasmid | Description* | Reference |
|---|---|---|
| pET28b(+) | IPTG-inducible protein expression vector; $Kan^R$ | Novagen |
| pATPL240 | $His_6$-*Sp*MpgB expression vector; $Kan^R$ | This study |
| pATPL368 | $His_6$-*Sp*MpgB$^{D68N}$ expression vector; $Kan^R$ | This study |
| pATPL434 | $His_6$-*Sp*MpgA expression vector; $Kan^R$ | This study |
| pATPL472 | $His_6$-*Sp*MpgA$^{E428Q}$ expression vector; $Kan^R$ | This study |
| pATPL473 | $His_6$-*Sp*MpgA$^{N455D}$ expression vector; $Kan^R$ | This study |
| pATPL488 | $His_6$-*Sp*MpgA$^{\Delta D219\text{-}P295}$ expression vector; $Kan^R$ | This study |
| pATPL474 | $His_6$-*Ec*MltG expression vector; $Kan^R$ | This study |
| pATPL475 | $His_6$-*Ec*MltG$^{E224Q}$ expression vector; $Kan^R$ | This study |
| pATPL476 | $His_6$-*Ec*MltG$^{D245N}$ expression vector; $Kan^R$ | This study |
| pJP121 | $His_6$-*Ec*MltG$^{\Delta K33\text{-}F110}$ expression vector; $Kan^R$ | This study |
| pATPL489 | $His_6$-*Bs*MltG expression vector; $Kan^R$ | This study |
| pMS211 | $His_6$-SUMO-FLAG-*Tt*RodA expression vector; $Amp^R$ | (10) |
| pATPL424 | $His_6$-SUMO-FLAG-*St*MpgB expression vector; $Amp^R$ | This study |
| pATPL425 | $His_6$-SUMO-FLAG-*Ef*MpgB expression vector; $Amp^R$ | This study |
| pATPL426 | $His_6$-SUMO-FLAG-*Bs*MpgB expression vector; $Amp^R$ | This study |
| pAM174 | Encodes arabinose-inducible Ulp1$^{L403\text{-}K621}$ protease; $Cm^R$ | (11) |
| pMgt1 | *S. aureus* SgtB-$His_6$ expression vector; $Amp^R$ | (12) |
| pET24bSgtBY181D | *S. aureus* SgtB$^{Y181D}$-$His_6$ expression vector; $Kan^R$ | (13) |
| pPEPY-PF6-lacI | *S. pneumoniae prsA::*PF6-*lacI* integration vector; $Gent^R$, $Kan^R$ | (14), Addgene |
| pPEPZ-$P_{lac}$ | *S. pneumoniae spd_1735* integration vector containing IPTG-inducible $P_{lac}$ promoter; $Spec^R$ | (3), Addgene |
| pATPL461 | *S. pneumoniae spd_1735*::$P_{lac}$-*SpmpgB* integration vector; $Spec^R$ | This study |
| pATPL462 | *S. pneumoniae spd_1735*::$P_{lac}$-*StmpgB* integration vector; $Spec^R$ | This study |
| pATPL463 | *S. pneumoniae spd_1735*::$P_{lac}$-*EfmpgB* integration vector; $Spec^R$ | This study |
| pATPL464 | *S. pneumoniae spd_1735*::$P_{lac}$-*BsmpgB* integration vector; $Spec^R$ | This study |
| pATPL471 | *S. pneumoniae spd_1735*::$P_{lac}$-*EcmltG* integration vector; $Spec^R$ | This study |
| pATPL492 | *S. pneumoniae spd_1735*::$P_{lac}$-*SpmpgA* integration vector; $Spec^R$ | This study |

*Abbreviations: $Amp^R$, ampicillin/carbenicillin resistance; $Cm^R$, chloramphenicol resistance; $Erm^R$, erythromycin resistance; $Gent^R$, gentamicin resistance; $Kan^R$, kanamycin resistance; $Spec^R$, spectinomycin resistance; $Str^R$, streptomycin resistance; $Tet^R$, tetracycline resistance

**Table S3. Oligonucleotide primers used in this study**

| Primer | Sequence (5'-3')* |
|---|---|
| oAT101 | GTACATATGTTTAAACGAATTCGAAGAGTGCTTGT |
| oAT102 | ACTGGATCCCTAGCCAGATGTTGAAAA |
| oAT103 | AATGTTATGCAGTCTAGTGAGTCT |
| oAT104 | GCCTTCTTTTCCTTTTGTTTCAGTATA |
| oAT105 | CCTGGGGGGTCATCCTTTAAATGGATAAGACGTCTGGTGGT |
| oAT106 | TGCAGTCACCCGGGCTTAGAGACGAGAGAATATTCGTATCAGAAAG |
| oAT107 | GCCCGGGTGACTGCAGGA |
| oAT108 | GGATGACCCCCCAGGGCC |
| oAT109 | CCTGGGGGGTCATCCGATGATTCGATGCGTAGAGTAAGA |
| oAT110 | TGCAGTCACCCGGGCTTAATTTAACTTTTCAATAAACCAACGATTC |
| oAT111 | CCTGGGGGGTCATCCAAGAAAAAGAGAAAAGGCTGTTTC |
| oAT112 | TGCAGTCACCCGGGCTTATTCATGAGCCTTGGATTCC |
| oAT113 | GTACATATGAGTGAAAAGTCAAGAGAAGAAGAGA |
| oAT114 | ACTCCTCGAGTTAGTTTAATTTGCTGTTGACATGTTCAG |
| oAT115 | TCGAAAAACAAGGTGCCAAGACAGAAGATCG |
| oAT116 | CACCTTGTTTTTCGACCAAGGAAGCAATGG |
| oAT117 | TTCAAAGTGATATTGCAATCTTGTATGCCCAAGG |
| oAT118 | CAATATCACTTTGAAGTGGCATATCACG |
| oAT119 | GATAGGTAATAAGGAATCTAGCACGTACT |
| oAT120 | CAAGAACCTGTACTTGCGACTTTG |
| oAT121 | AGTACATATGAAAAAAGTGTTATTGATAATCTTGTTATT |
| oAT122 | ACTCCTCGAGTTACTGCGCATTTTTTTCCTTAAG |
| oAT123 | TCGAAAAACAAACCGCCGTTGCCAGTG |
| oAT124 | CGGTTTGTTTTTCGATAATTGATGCCATCGTC |
| oAT125 | TGCAGACCAACCCGACCGTGATTTACGGG |
| oAT126 | TCGGGTTGGTCTGCAGGCGCATACC |
| oAT127 | AGTACATATGTATATCAATCAGCAAAAAAAATCGTTT |
| oAT128 | ACTCCTCGAGTTATTTCTCATTTTTTGAGGAAATGTATTTTTC |
| oAT129 | ATGAGTTCAATTGTATCTATCGGCAG |
| oAT130 | TCCTGCCTTTCCTCCCTCATTCTACTCCTTATCAATTAAAACAACTC |
| oAT131 | GAGAGCACAGATACGGCGTAATGGAATGTCTTTCAAATCAGAACAG |
| oAT132 | TCCTCAATCTATATAACATAGCTTTATGAC |
| oAT133 | GAGGGAGGAAAGGCAGGA |
| oAT134 | CGCCGTATCTGTGCTCTC |
| oAT135 | CGGTTCTCTGCTTTTATTATATTCG |
| oAT136 | CACAGGAACAGTTGTATTATAAGGAG |
| oAT137 | TCCTGCCTTTCCTCCCTCATTACAAACTAATAAAAAATCAAGAACAGAT |
| oAT138 | GAGAGCACAGATACGGCGTACTATAAGTGAATATGATTTGAGTGAATAG |
| oAT139 | ATGCTTGACTGAGACTTCCTTCA |

* Underlined sequences are restriction sites introduced in primers.

**Table S3. Oligonucleotide primers used in this study (cont.)**

| Primer | Sequence (5'-3') |
|---|---|
| oAT140 | TCTCTGGAATCTCGTATGGC |
| oAT141 | AGAAAGAGAAGCAAGCAATCCTCA |
| oAT142 | TCCTGCCTTTCCTCCCTCCTGAATGCTGTTCCACCTAG |
| oAT143 | GAGAGCACAGATACGGCGGCGATGATTTGAAAGAGGGATGT |
| oAT144 | ATTTCTTACAAACCAGGTGCTTG |
| oAT145 | GATGGAACCAGTGCTGACTTGA |
| oAT146 | CTCGAAATGGGTGCGGAAAG |
| oAT147 | TCCTGCCTTTCCTCCCTCTCTTCCTCCTTAAAAAATAATATAAAGCGAT |
| oAT148 | GAGAGCACAGATACGGCGAAAATTGGAGTAGGAGAAATTTCC |
| oAT149 | CATAAAAGTAAGGCAGGCTAACC |
| oAT150 | GAATATCGCTCAGAATATTGGGA |
| oAT151 | CCTTTCAAGATAACATTGGCTTC |
| oAT152 | TCCTGCCTTTCCTCCCTCATCTTATAATTCTACCCTAAAAATCAAAAAAAAT |
| oAT153 | GAGAGCACAGATACGGCGCATTTGTTTATCATTTGCTTTTTCTTTTTG |
| oAT154 | TGATGAGGGATAGCTGCTTTAG |
| oAT155 | CCTTTCACCTCAACAAAGTTACC |
| oAT156 | TCCGATGATATCAAAGACAGATTGAAA |
| oAT157 | ATTCGAAAATTCTCCTTCTTTCTATAGTT |
| oAT158 | ATAGAAAGAAGGAGAATTTTCGAATATGAACATCAAAAAGTTTGCAAAAC |
| oAT159 | TCCTGCCTTTCCTCCCTCTTATTTGTTAACTGTTAATTGTCCTTG |
| oAT160 | ATTCAAGACAAGTGGGATTCGT |
| oAT161 | TTTCAATCTGTCTTTGATATCATCGGATTATTTACTTTGGATATCCTCGATATTTTTGA |
| oAT162 | GAGAGCACAGATACGGCGACCAGGTGTTTTTGTTATAAGTTTTCT |
| oAT163 | CGTTACGGTTACCATCCATTATACC |
| oAT164 | TTATTTACTTTGGATATCCTCGATATTTTTGA |
| oAT165 | TCAAAAATATCGAGGATATCCAAAGTAAATAACCAGGTGTTTTTGTTATAAGTTTTCT |
| oAT166 | TTGCTGGATGAAGTCAATATTACCC |
| oAT167 | CAACTTCTACAATATCATTTTTCTTTAAC |
| oAT168 | TATTTTTCCTCCTTATTTATTTAGATCTTAATTGTG |
| oAT169 | GATCCCTCCAGTAACTCGAG |
| oAT170 | TAAGGAGGAAAAATAATGTTTAAACGAATTCGAAGAGTGCTT |
| oAT171 | GTTACTGGAGGGATCCTAGCCAGATGTTGAAAAGAGAGTG |
| oAT172 | TAAGGAGGAAAAATAATGTTTAAATGGATAAGACGTCTGGTG |
| oAT173 | GTTACTGGAGGGATCTTAGAGACGAGAGAATATTCGTATCAGAAA |
| oAT174 | TAAGGAGGAAAAATAATGGATGATTCGATGCGTAGAGTAA |
| oAT175 | GTTACTGGAGGGATCTTAATTTAACTTTTCAATAAACCAACGATT |
| oAT176 | TAAGGAGGAAAAATAATGAAGAAAAAGAGAAAAGGCTGTTTC |
| oAT177 | GTTACTGGAGGGATCTTATTCATGAGCCTTGGATTCC |
| oAT178 | TAAGGAGGAAAAATAATGAAAAAAGTGTTATTGATAATCTTGTT |

**Table S3. Oligonucleotide primers used in this study (cont.)**

| Primer | Sequence (5'-3') |
|---|---|
| oAT179 | GTTACTGGAGGGATCTTACTGCGCATTTTTTTCCTTAAG |
| oAT180 | TAAGGAGGAAAAATAATGAGTGAAAAGTCAAGAGAAGAAGAGA |
| oAT181 | GTTACTGGAGGGATCTTAGTTTAATTTGCTGTTGACATGTTCAG |
| oAT182 | ATCATTCAGGCAAGCAAGTCT |
| oAT183 | TCCTGCCTTTCCTCCCTCAAGTTTTTCCTCCTTGTTGATAATCC |
| oAT184 | GAGAGCACAGATACGGCGTAAACAAACTAAAATTATGTGATACTTCA |
| oAT185 | AATCTAAAGTATAGTGAAATGAAATAAAACATG |
| oAT186 | TTTACGTCTTTATGGGAGCAG |
| oJP202 | GCTGTCGGCAAGATGGCGAA |
| oJP203 | CCGCTGCGACTGGTAGAAG |
| oBR05 | CCAGTCGTGCTCGCTTCGCTACTTGGAGCC |
| oCS121 | GCTTTCTTGAGGCAATTCACTTGGTGC |
| oSK27 | CGTAGCCGTCTTTACCTGTGAAGT |
| oP1348 | TCTTCTTGCAGCCTTGAAAGAGGTGGCAGT |
| oP1349 | AGAGCAAACTAGGAAACTAGCCGCAGGTTG |
| oTT657 | CGCCCCAAGTTCATCACCAATGACATCAAC |
| oTT1406 | CCTTGGGCATACAAGATTGCAATATCACTTTGAAGTGGC |
| oTT1407 | GCCACTTCAAAGTGATATTGCAATCTTGTATGCCCAAGG |
| oTT1408 | TGTCAAAGTCGCAAGTACAGGTTCTTGGATAGGTAATAAGGAATCTAGCACGTACTGGTA |
| oTT1409 | CAGTACGTGCTAGATTCCTTATTACCTATCCAAGAACCTGTACTTGCGACTTTGACAATT |
| oTT1410 | TAGTAGACCTTGCCTTCTGTGACATCAACTTCATCATAGCCTTTTACTTTTTCTAAATCT |
| oTT1411 | TTTAGAAAAAGTAAAAGGCTATGATGAAGTTGATGTCACAGAAGGCAAGGTCTACTATGC |

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