Supplementary Information for:

A broadly conserved Gram-positive lipoprotein regulates cell elongation

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General Methods

Bacillus subtilis

All *B. subtilis* strains were derived from the prototrophic strain PY79 (1). Unless otherwise indicated, cells were grown in Luria-Bertani broth (LB) Miller (2) at 37 °C with aeration. Antibiotic concentrations used were: 100 µg/mL spectinomycin, 10 µg/mL kanamycin, 5 µg/mL chloramphenicol, 10 µg/mL tetracycline, 1 µg/mL erythromycin and 25 µg/mL lincomycin. Insertion-deletion mutations were generated by isothermal assembly (3) of PCR products followed by direct transformation into *B. subtilis*. Antibiotic cassettes were removed using temperature sensitive plasmids expressing Cre recombinase (4). Ectopic integration alleles were generated by direct transformation of digested plasmid templates.

Staphylococcus aureus

All *S. aureus* strains were derived from the NCTC8325 derivative HG003 (5). Cells were grown in Tryptic Soy Broth (TSB) at 37 °C with aeration. Antibiotic concentrations were: 200 µg/mL spectinomycin, 50 µg/mL kanamycin and 50 µg/mL neomycin, 10 µg/mL chloramphenicol, 5 µg/mL tetracycline, 10 µg/mL erythromycin. Insertion-deletion mutants and ectopic integration events were generated by recombineering (6) using a PCR product amplified from a plasmid template. Ectopic integrations were at neutral chromosomal sites between convergently transcribed genes with less than 250 base pairs between the stop codons, no annotated small RNAs, and with transcription termination sites as predicted by RNA-seq (7). These were: between SAOUHSC_0309 and SAOUHSC_0310, between SAOUHSC_02626 and SAOUHSC_02627, and between SAOUHSC_02923 and SAOUHSC_02924. The integration constructs contained 1000 bp of flanking DNA and synthetic bidirectional transcriptional terminators flanking the integrated DNA. All genomic modifications were transduced to a clean background using phi85. Antibiotic cassettes were flanked by *loxP* sites and excised using a temperature sensitive plasmid expressing Cre recombinase (pAB440). All transformations were conducted by electroporation.

Bacillus anthracis

All *B. anthracis* strains were derived from the plasmid-free non-pathogenic Sterne derivative *B. anthracis* 9131 called BaR1 (8). Cells were grown in Brain Heart Infusion broth (BHI) at 37 °C with aeration. Antibiotic concentrations used were: 300 µg/mL spectinomycin and 25 µg/mL kanamycin. Deletions and ectopic integrations were generated by allelic exchange (8). All transformation was conducted by electroporation. Ectopic integration was at a neutral chromosomal locus between BAIDGICL_00395 and BAIDGICL_00396 (locus00395_6).

All experiments were performed as follows unless otherwise noted: A freshly streaked colony was grown in 3 mL media at 37 °C to mid-log phase. This pre-culture was back-diluted into 25 mL media in 250 mL baffled flasks to an OD600 = 0.025 and grown to OD ~0.2-0.5 at 37 °C with agitation. Cells were harvested and analyzed by fluorescence and phase-contrast microscopy, labeled with Bocillin-FL and/or lysed and resolved by SDS-PAGE and detected on a fluorescence imager or by immunoblot.

TnSeeker

The TnSeeker viewer application was developed as part of this work to facilitate fast and easy visualization of TnSeq datasets. It functions as a fully standalone web tool and only requires a user's browser to run. The application is built using HTML and CSS to render components, with JavaScript providing interactivity. Several external JavaScript libraries are included to provide enhanced functionality: Coloris provides the color picker, D3 handles SVG manipulation operations, SortableJS enables list item rearrangement, Tom Select provides enhanced searchable option lists, and Cash-DOM simplifies document manipulation.

Users can select from a list of pre-loaded model organism GenBank genomes or upload their own GenBank file to load in a bacterial genome of their choice. Once a genome has been loaded, they can add text files representing the number of transposon read insertions detected at every site in the genome. The application parses these files into a memory-efficient indexed representation that enables rapid interactive exploration

of insertion patterns across the full chromosome at multiple scales. Users can quickly browse mega base long regions, inspect individual genes, and dynamically adjust display settings such as colors, tracks, and ordering to better compare datasets. It also parses genomes to link genes to UniProt entries to present additional metadata during browsing so that users can more quickly interpret results and assess any potential biological significance.

The viewer was designed to remain lightweight and responsive even when working with large bacterial genomes and multiple insertion datasets, making it practical for routine use without requiring any server-side infrastructure or specialized software installation. By combining genome loading, insertion file parsing, and interactive visualization into a single browser-based interface, the tool provides an accessible and efficient way to examine Tn-Seq results and generate clear, publication-ready views directly from input files. The use of SVG as the rendering engine enables the viewer to export resolution-independent images that remain sharp at any scale and are well suited for figures, presentations, and publication.

Strain Construction:
Insertion-deletion mutants in *B. subtilis* were constructed by transforming 3-piece isothermal assemblies containing 1000bp upstream homology, 1000bp downstream homology, and an antibiotic resistance cassette. They were generated using the following oligos: oAB27/28 (universal oligos for antibiotic cassettes), *pbpA* (oJM24/25 & oJM30/31), *pbpH* (oAB1238/1239 & oAB1240/1241), *clcR* (oAB1108/1109 & oAB1110/1111), *yaaL* (oAB1426/1427 & oAB1428/1429), *mntA* (oAB1422/1423 & oAB1424/1425). The deletion of Δ*clcR*(ECD) in *B. subtilis* was made by allelic exchange using the pminiMAD based plasmid pAB493.

Plasmid Construction:
All plasmids were sequence verified using nanopore sequencing.

pAB53 [yhdG--Pspank-*pbpA* (erm) (amp)]
pAB53 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB97 & oAB98 and pCB57 [yhdG—Pspank (erm) (amp)] using HindIII & SpeI.

pAB397 [0309 0310 integration (tet) (amp)]
pAB397 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB886 & oAB887, a PCR product amplified from the loxP flanked SaTetR cassette cassette using oAB27 & oAB28, a PCR product amplified from HG003 gDNA using oAB884 & oAB885, and pMR91 [pMAD3.1] digested using NcoI & BamHI.

pAB401 [02626-02627 integration (kan) (amp)]
pAB401 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB896 & oAB897, a PCR product amplified from the loxP flanked SaKanR cassette using oAB27 & oAB28, a PCR product amplified from HG003 gDNA using oAB898 & oAB899, and pMR91 [pMAD3.1] digested with NcoI & BamHI.

pAB402 [02626-02627::Pveg-GFP (kan) (amp)]
pAB402 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from GFP using oAB928/903 and pAB401 [02626-02627 integration (kan) (amp)] digested with BamHI & SpeI.

pAB405 [02923 02924 integration (spc) (amp)]

pAB405 was generated in a 4-piece isothermal assembly using a PCR product amplified from HG003 gDNA using oAB907 & oAB908, a PCR product amplified from the loxP flanked SaSpcR casette cassette using oAB27 & oAB28, a PCR product amplified from HG003 gDNA using oAB909 & oAB910, and pMR91 [pMAD3.1] digested using NcoI & BamHI.

pAB438 [yhdG-Phy-spank-gfp (spc) (amp)]
pAB438 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from GFP using oAB987 & oAB988 and pCB101 [yhdG::Pspank (spc) (amp)] digested with SpeI & PstI.

pAB440 [pACYC ts Ptet-cre (erm) (amp)]
pAB440 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from pPM300 [Ptet-cre] using oAB998 & oAB999 and a PCR product amplified from pACYC using oAB1000 & oAB1001.

pAB450 [0309 0310 integration Phy-spank-optRBS-gfp (tet) (amp)]
pAB450 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from pAB438 [yhdG::Physpank-gfp (spc) (amp)] using oAB888 & oAB889 and pAB397 [0309 0310 integration (tet)] digested using EcoRI & BamHI.

pAB461 [0309-0310 Phy-spank-gfp (tet) (amp) pACYC]
pAB461 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from pACYC using oAB1046 & oAB1047 and a PCR product amplified from pAB450 [0309 0310 integration Phy-spank-optRBS-gfp (tet)] using oAB1045 & oAB1048.

pAB485 [yhdG-Pspank-*pbpH* (erm) (amp)]
pAB485 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1106 & oAB1107 and pCB57 [yhdG—Pspank (erm) (amp)] using HindIII & SpeI.

pAB486 [yhdG-Pspank-*clcR* (erm) (amp)]
pAB486 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1112 & oAB1113 and pCB57 [yhdG—Pspank (erm) (amp)] using HindIII & SpeI.

pAB487 [(pMAD3.1)-Δ*clcR*::kan (amp)]
pAB487 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB1114 & oAB1115, a PCR product amplified from HG003 gDNA using oAB1116 & oAB1117, a PCR product amplified from the *S. aureus* KanR cassette using oAB27 & oAB28, and pMR91 [pMAD3.1] digested using EcoRI & BamHI.

pAB488 [(pMAD3.1)-Δ*pbp3*::kan (amp)]
pAB488 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB1118 & oAB1119, a PCR product amplified from HG003 gDNA using oAB1120 & oAB1121, a PCR product amplified from the *S. aureus* KanR cassette using oAB27 & oAB28, and pMR91 [pMAD3.1] digested using EcoRI & BamHI.

pAB493 [(miniMAD) Δ*clcR*(ECD)]

pAB493 was generated in aa 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1132 & oAB1133, a PCR product amplified from PY79 gDNA using oAB1134 & oAB1135, and pminiMAD digested with HindII & EcoRI.

pAB495 [yhdG-Pspank-*clcR*-His6 (erm) (amp)]
pAB495 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1112 & oAB1138 and pCB57 [yhdG—Pspank (erm) (amp)] using HindIII & SpeI.

pAB504 [yhdG-Pspank-*clcR*-R76E-His6 (erm) (amp)]
pAB504 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1112 & oAB1156, a PCR product amplified from PY79 gDNA using oAB1138 & oAB1155, and pCB57 [yhdG—Pspank (erm) (amp)] using HindIII & SpeI.

pAB513 [yvbJ-PxylA-*clcR* (kan) (amp)]
pAB513 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1188 & oAB1189 and pCB133 [yvbJ—Pxyl (kan) (amp)] using XhoI & BamHI.

pAB515 [yvbJ-PxylA-*clcR*-R76E (kan) (amp)]
pAB515 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1188 & oAB1156, a PCR product amplified from PY79 gDNA using oAB1189 & oAB1155, and pCB133 [yvbJ—Pxyl (kan) (amp)] using XhoI & BamHI.

pAB532 [02923-02924 (spc) (amp) pACYC]
pAB532 was generated in a 2-piece isothermal assembly using a PCR product amplified from pACYC using oAB1214 & oAB1215 and a PCR product amplified from pAB405 [02923-02924 (spc)] using oAB1220 & oAB1221.

pAB554 [(pMAD3.1)-*clcR*Δ69-391::kan (amp)]
pAB553 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB1271 & oAB1272, a PCR product amplified from HG003 gDNA using oAB1116 & oAB1117, a PCR product amplified from the *S. aureus* KanR cassette using oAB27 & oAB28, and pMR91 [pMAD3.1] digested using EcoRI & BamHI.

pAB557 [0309-0310 Phy-spank-*clcR* (tet) (amp)]
pAB557 was generated in a 3-piece isothermal assembly using a PCR product amplified from HG003 gDNA using oAB1136 & oAB1137, a PCR product amplified from pAB461 [0309-0310 Phy-spank-gfp (tet)] using oAB1297 & oAB1289, and a PCR product amplified from pAB461 using oAB1298 & oAB1288.

pAB558 [0309-0310 Phy-spank-*clcR*-His6 (tet) (amp)]
pAB558 was generated in a 3-piece isothermal assembly using a PCR product amplified from HG003 gDNA using oAB1136 & oAB1145, a PCR product amplified from pAB461 [0309-0310 Phy-spank-gfp (tet)] using oAB1297 & oAB1289, and a PCR product amplified from pAB461 using oAB1298 & oAB1288.

pAB565 [ycgO-PxylA-*pbpA*-ALFA (spec) (amp)]
pAB565 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1305 & oAB1306 and pCB96 [ycgO—Pxyl (spec) (amp)] using XhoI & HindIII.

pAB568 [ycgO-PxylA-*pbpA* (spec) (amp)]

pAB568 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1305 & oAB1142 and pCB96 [ycgO—Pxyl (spec) (amp)] using XhoI & HindIII.

pAB580 [02923-02924 *pbp3* (spc) (amp) pACYC]
pAB580 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB1324 & oAB1325 and pAB532 [02923-02924 (spc) pACYC] digested with BamHI & XhoI.

pAB584 [Δ*mreCD*::kan (amp) pACYC]
pAB584 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB1314 & oAB1315, a PCR product amplified from HG003 gDNA using oAB1328 & oAB1317, a PCR product amplified from the *S. aureus* KanR cassette using oAB27 & oAB28, and a PCR product amplified from pACYC backbone using oAB1214 & oAB1215.

pAB585 [Δ*rodA*::kan pACYC]
pAB585 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from HG003 gDNA using oAB1329 & oAB1330, a PCR product amplified from HG003 gDNA using oAB1331 & oAB1332, a PCR product amplified from the *S. aureus* KanR cassette using oAB27 & oAB28, and a PCR product amplified from pACYC backbone using oAB1214 & oAB1215.

pAB587 [yhdG-Pspank-*pbpB* (erm) (amp)]
pAB587 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1335 & oAB1336 and pCB57 [yhdG—Pspank (erm) (amp)] using HindIII & SpeI.

pAB588 [02923-02924 *pbp3*-ALFA (spc) (amp) pACYC]
pAB588 was generated via KLD (NEB) using a PCR product amplified from pAB580 [02923-02924 PBP3 (spc) (amp) pACYC] using oAB1337 & oAB1338.

pAB589 [0309-0310 Phy-spank-*clcR*-S76E (tet) pACYC]
pAB589 was generated via transformation of a PCR product amplified using Pfu Turbo (Agilent) from pAB557 [0309-0310 Phy-spank-clcR (tet) (amp] using oAB1339 & oAB1340.

pAB590 [0309-0310 Phy-spank-*clcR*-S76E-His6 (tet) pACYC]
pAB590 was generated via transformation of a PCR product amplified using Pfu Turbo (Agilent) from pAB558 [0309-0310 Phy-spank-clcR-His6 (tet) (amp] using oAB1339 & oAB1340.

pAB596 [locus003956::Pspank-*clcR1*(BAIDGICL347) (spec) (kan)]
pAB596 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from BaR1 gDNA using oAB1355 & oAB1356 and pCZ18 [pMAD3 locus003956] digested with NcoI & SpeI.

pAB597 [locus003956::Pspank-*clcR2*(BAIDGICL4892) (spec) (kan)]
pAB597 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from BaR1 gDNA using oAB1357 & oAB1358 and pCZ18 [pminiMAD3 locus003956] digested with NcoI & SpeI.

pAB598 [pminiMAD3 Δ*clcR1*(BAIDGICL347) (spec) (amp)]
pAB598 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from BaR1 gDNA using pAB1359 & oAB1360, a PCR product amplified from BaR1 gDNA using oAB1361 & oAB1362, and pCZ9 [pminiMAD3] digested with EcoRI & BamHI.

pAB599 [pminiMAD3 Δ*clcR2*(BAIDGICL4892) (spec) (amp)]
pAB598 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from BaR1 gDNA using oAB1363 & oAB1364, a PCR product amplified from BaR1 gDNA using oAB1365 & oAB1366, and pCZ9 [pminiMAD3] digested with EcoRI & BamHI.

pAB600 [locus003956::Pspank-*rodA* (spec) (kan)]
pAB600 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from BaR1 gDNA using oAB1367 & oAB1368 and pCZ18 [pMAD3 locus003956] digested with NcoI & SpeI.

pAB601 [pminiMAD3 Δ*rodA* (spec) (amp)]
pAB598 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from BaR1 gDNA using oAB1369 & oAB1370, a PCR product amplified from BaR1 gDNA using oAB1371 & oAB1372, and pCZ9 [pminiMAD3] digested with EcoRI & BamHI.

pAB602 [yvbJ::PxylA-*clcR*-Q203E (kan) (amp)]
pAB602 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1188 & oAB1374, a PCR product amplified from PY79 DNA using oAB1189 & oAB1373, and pCB133 [yvbJ::PxylA (kan) (amp)] digested with XhoI & BamHI.

pAB603 [yhdG::Pspank-*clcR*-Q203E (erm) (amp)]
pAB603 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1112 & oAB1374, a PCR product amplified from PY79 gDNA using oAB1113 & oAB1373, and pCB57 [yhdG::Pspank (erm) (amp)] digested with HindIII & SpeI.

pAB605 [yhdG::Pspank-*clcR*-Q203E-His6 (erm) (amp)]
pAB605 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1112 & oAB1374, a PCR product amplified from PY79 gDNA using oAB1138 & oAB1373, and pCB57 [yhdG::Pspank (erm) (amp)] digested with HindIII & SpeI.

pAB626 T25-*clcR* (kan)
pAB626 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1430 & oAB1431 and pKT25 [T25-MCS (kan)] digested with XbaI & EcoRI.

pAB627 T25-*pbpA* (kan)
pAB627 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1432 & oAB1433 and pKT25 [T25-MCS (kan)] digested with XbaI & EcoRI.

pAB628 T25-*pbpH* (kan)
pAB628 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1434 & oAB1435 and pKT25 [T25-MCS (kan)] digested with XbaI & EcoRI.

pAB629 *clcR*-T25 (kan)
pAB629 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1436 & oAB1437 and pKNT25 [MCS-T25 (kan)] digested with HindIII & BamHI.

pAB630 *pbpA*-T25 (kan)
pAB630 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1438 & oAB1439 and pKNT25 [MCS-T25 (kan)] digested with HindIII & BamHI.

pAB631 *pbpH*-T25 (kan)
pAB631 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1440 & oAB1441 and pKNT25 [MCS-T25 (kan)] digested with HindIII & BamHI.

pAB632 *clcR*-T18 (amp)
pAB632 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1442 & oAB1443 and pUT18 [MCS-T18 (amp)] digested with HindIII & BamHI.

pAB633 *pbpA*-T18 (amp)
pAB633 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1444 & oAB1445 and pUT18 [MCS-T18 (amp)] digested with HindIII & BamHI.

pAB634 *pbpH*-T18 (amp)
pAB634 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1446 & oAB1447 and pUT18 [MCS-T18 (amp)] digested with HindIII & BamHI.

pAB635 T18-*clcR* (amp)
pAB635 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1448 & oAB1449 and pUT18C [T18-MCS (amp)] digested with XbaI & EcoRI.

pAB636 T18-*pbpA* (amp)
pAB636 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1450 & oAB1451 and pUT18C [T18-MCS (amp)] digested with XbaI & EcoRI.

pAB637 T18-*pbpH* (amp)
pAB637 was generated in a 2-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1452 & oAB1453 and pUT18C [T18-MCS (amp)] digested with XbaI & EcoRI.

pAB645 [yvbJ::PxylA-yerH-R76E-Q203E (kan) (amp)]
pAB645 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from pAB602 [yvbJ::PxylA-*clcR*-Q203E (kan) (amp)] using oAB1188 & oAB1156, a PCR product amplified from pAB602 [yvbJ::PxylA-*clcR*-Q203E (kan) (amp)] using oAB1155 & oAB1189, and pCB133 [yvbJ::PxylA (kan) (amp)] digested with BamHI & XhoI.

pAB646 [yhdG::Pspank-yerH-R76E-Q203E-His6 (erm) (amp)]
pAB646 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from pAB605 [yhdG::Pspank-*clcR*-Q203E-His6 (erm) (amp)] using oAB1112 & oAB1156, a PCR product amplified from pAB605 [yhdG::Pspank-*clcR*-Q203E-His6 (erm) (amp)] using oAB1155 & oAB1138, and pCB57 [yhdG::Pspank (erm) (amp)] digested with HindIII & XhSpeIoI.

pAB647 *clcR*-R76E-T18
pAB647 was generated in a 3-piece isothermal assembly reaction using a PCR product amplified from PY79 gDNA using oAB1442 & oAB1156, a PCR product amplified from PY79 gDNA using oAB1443 & oAB1155, and pCH363 [MCS-T18] digested with HindiII & BamHI.

pAB653 [pMAD4 mbl-mNeonGreen (spec) (amp)]

pAB653 was generated in a 4-piece isothermal assembly reaction using a PCR product amplified from BaR1 gDNA using oAB1481 & oAB1482, a PCR product amplified from PY79 gDNA containing an mreB-mNeonGreen fusion using oAB1468 & oAB1479, a PCR product amplified from BaR1 gDNA using oAB1483 & oAB1484, and pCZ54 [pMAD4 pACYC origin] digested with BamHI & EcoRI.

Supplemental References

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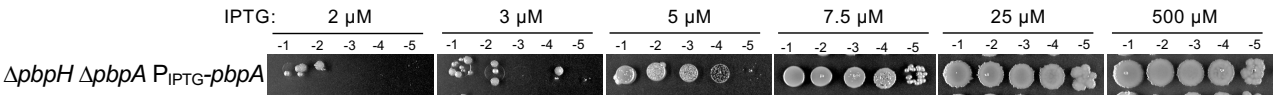

Figure S1. Reducing PBP2a expression in the absence of PBPH results in a sensitized background. Spot titers of the indicated strain harboring an IPTG-regulated allele of *pbpA*, encoding PBP2a, on LB agar supplemented with the indicated IPTG concentrations. 6 μM IPTG (not assayed here) was used as the condition for the Tn-seq.

Figure S2
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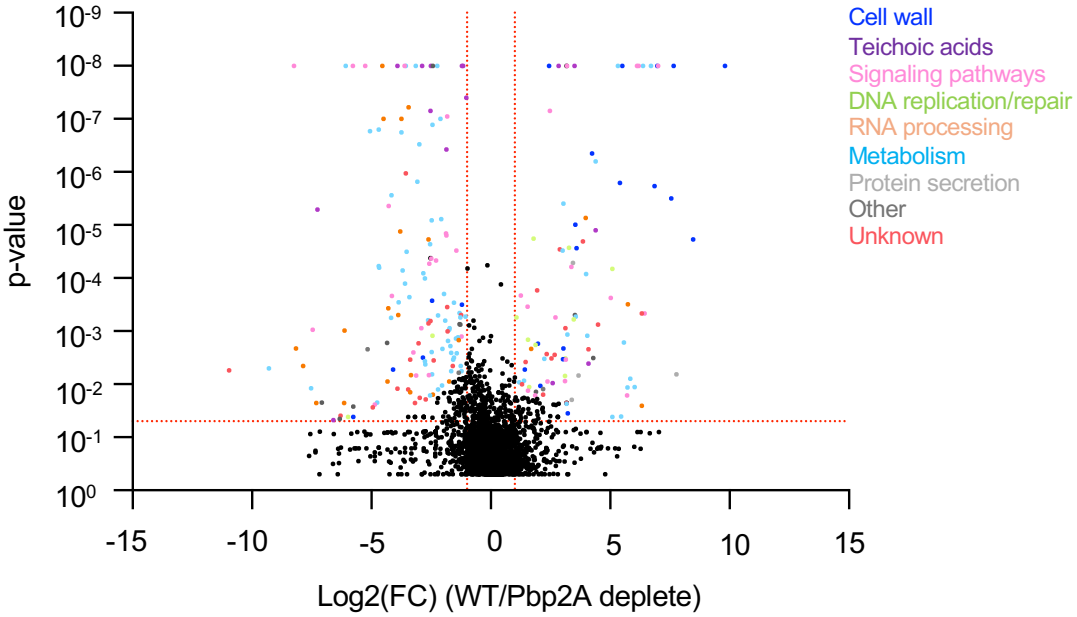

Figure S2. Results of the PBP2a depletion Tn-seq. Scatter plot showing the fold change (FC) in transposon insertions between wild-type and cells with low levels of PBP2a. Red lines indicate a fold change > 2 and a p-value < 0.05. Each circle represents a gene and are colored by their previously defined role in the indicated cellular process.

Figure S3
Brogan et al.

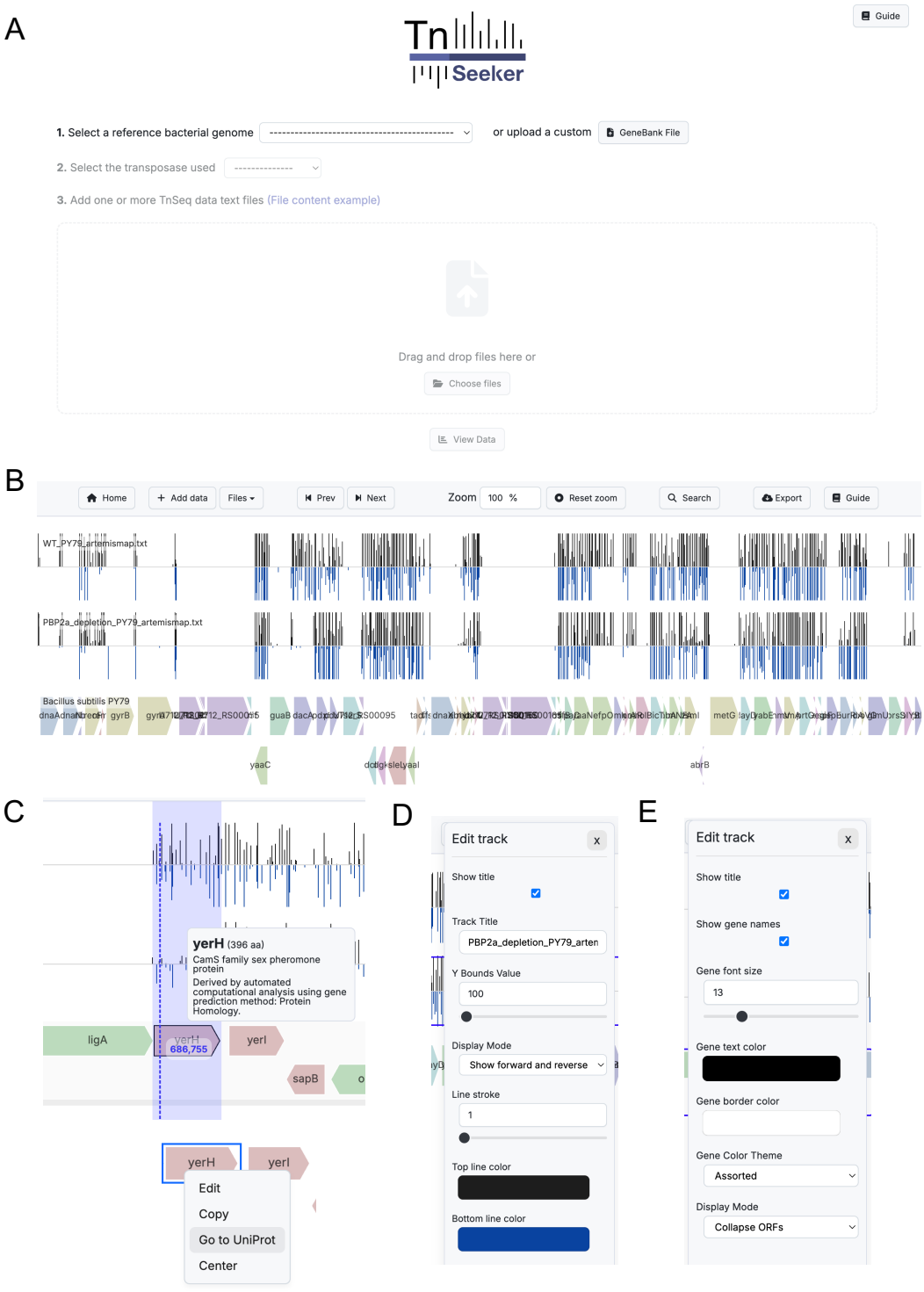

Figure S3. TnSeeker genome viewer. (A) Home page of TnSeeker. Users can select from a pre-assembled list of annotated bacterial genomes or upload their own. After selecting the type of transposon used, users upload a text file mapping genomic position to the number of reads mapped in the forward and reverse directions. **(B)** Tn-seq tracks plotted onto genomic position. Black lines indicate the number of reads mapped in the forward position and blue lines indicate the number of reads mapped in the reverse direction. Users can search for genes, zoom, add tracks, or export an SVG file of the screen. **(C)** Clicking an ORF highlights its genomic range across the tracks and displays annotation from the Genbank file and Uniprot. Users can also link out to the protein's entry on Uniprot. **(D)** Each Tn-seq library track can be edited to alter colors, name, y-axis range, line stroke, and toggle display mode to show forward and reverse insertions or combined. **(E)** The genome track can be edited to change gene color(s), name, and font sizes.

Figure S4
Brogan et al.

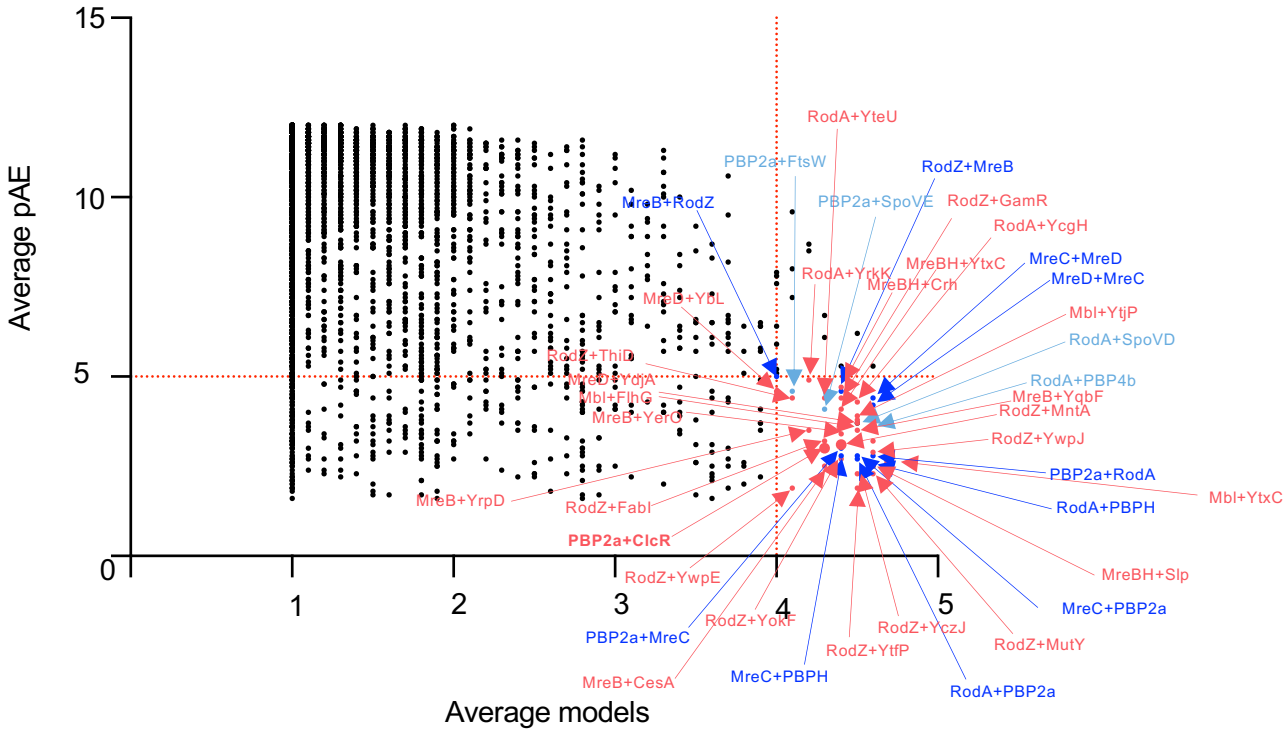

Figure S4. Detailed results of the Alphafold-Multimer screen. Scatter plot showing the results of the eight one-by-proteome AF-M screens. Only predictions with an average models score ≥ 1 are labeled. Red lines indicate an average models score ≥ 4 and average pAE ≤ 4 Å. Predictions are annotated with the bait protein written first followed by the prey. Known interactions between Rod complex components are annotated in dark blue, predicted interactions between non-cognate SEDS-bPBP pairs are annotated in light blue, and unknown interactions are annotated in red.

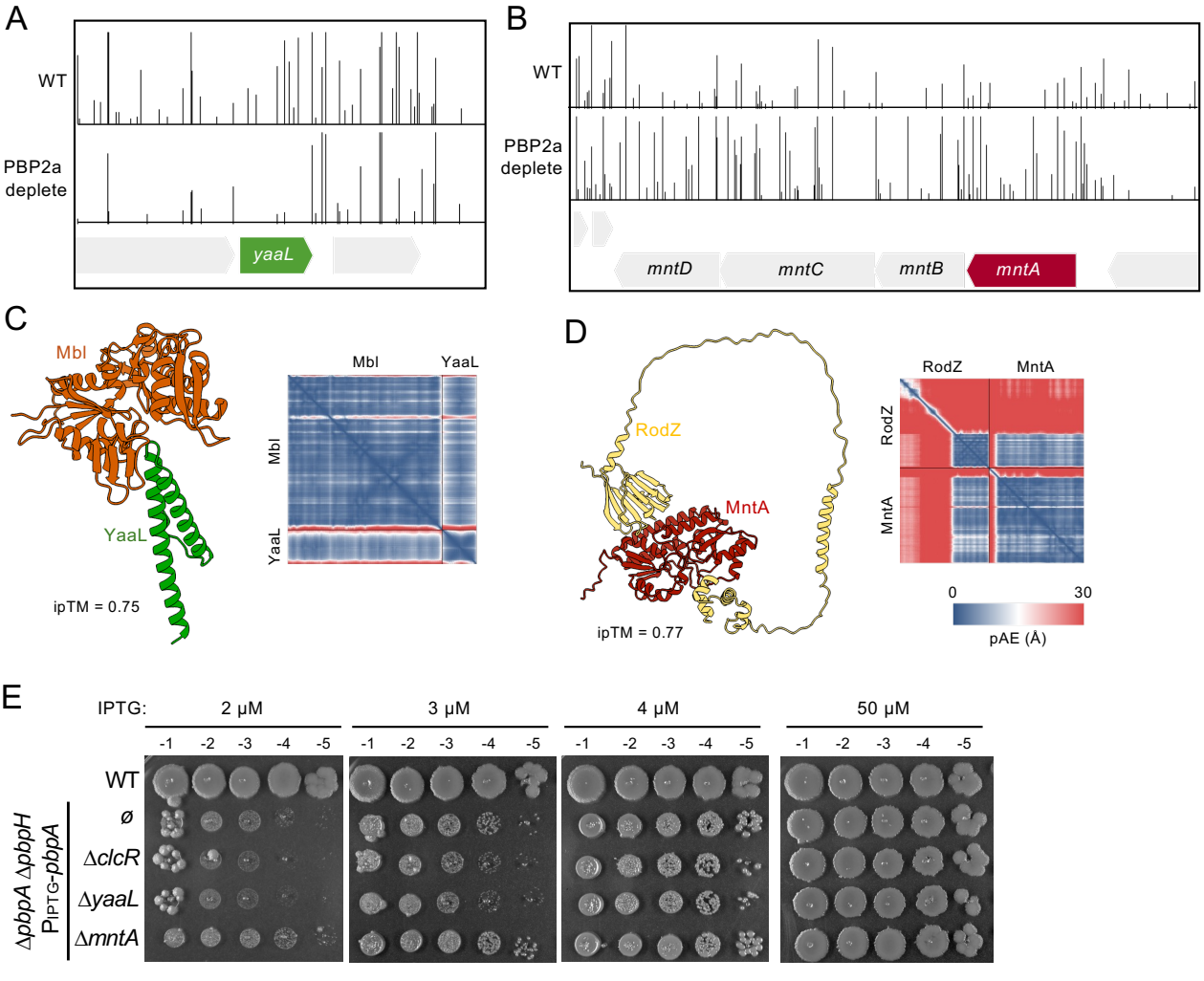

Figure S5. *clcR* (*yerH*), *yaaL*, and *mntA* are potential Rod complex interactors.
(A & B) Tn-seq profiles from wild-type *B. subtilis* and cells with low levels of PBP2a. Genomic positions that contain *yaaL* (A) and the *mnt* operon (B) are shown. **(C & D)** AlphaFold-Multimer prediction and pAE plot for the Mbl-YaaL interaction (C) and the RodZ-MntA interaction (D). **(E)** Spot titers of the indicated strains on LB agar supplemented with the listed IPTG concentrations. Deletion of *clcR* and *yaaL* subtly impair growth and deletion of *mntA* subtly improves growth of cell with low levels of PBP2a.

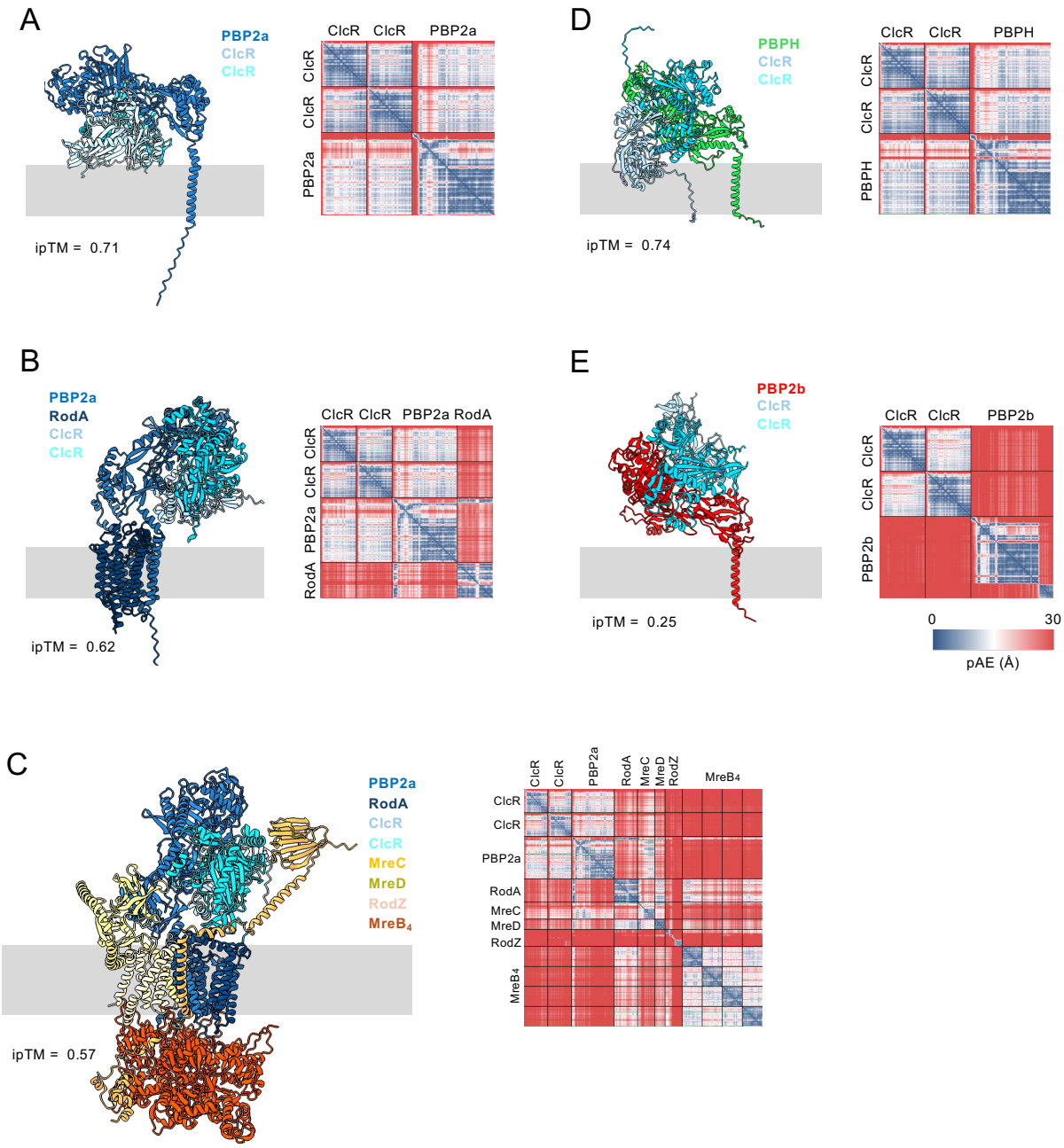

Figure S6. Alphafold3 predictions are consistent with a role for ClcR in the Rod complex. Alphafold3 predictions and pAE plots for **(A)** $ClcR_2$-PBP2a; **(B)** $ClcR_2$-PBP2a-RodA; **(C)** $ClcR_2$-PBP2a-RodA-MreC-MreD-RodZ-$MreB_4$ **(D)** $ClcR_2$-PBPH; **(E)** $ClcR_2$-PBP2b.

Figure S7
Brogan et al.

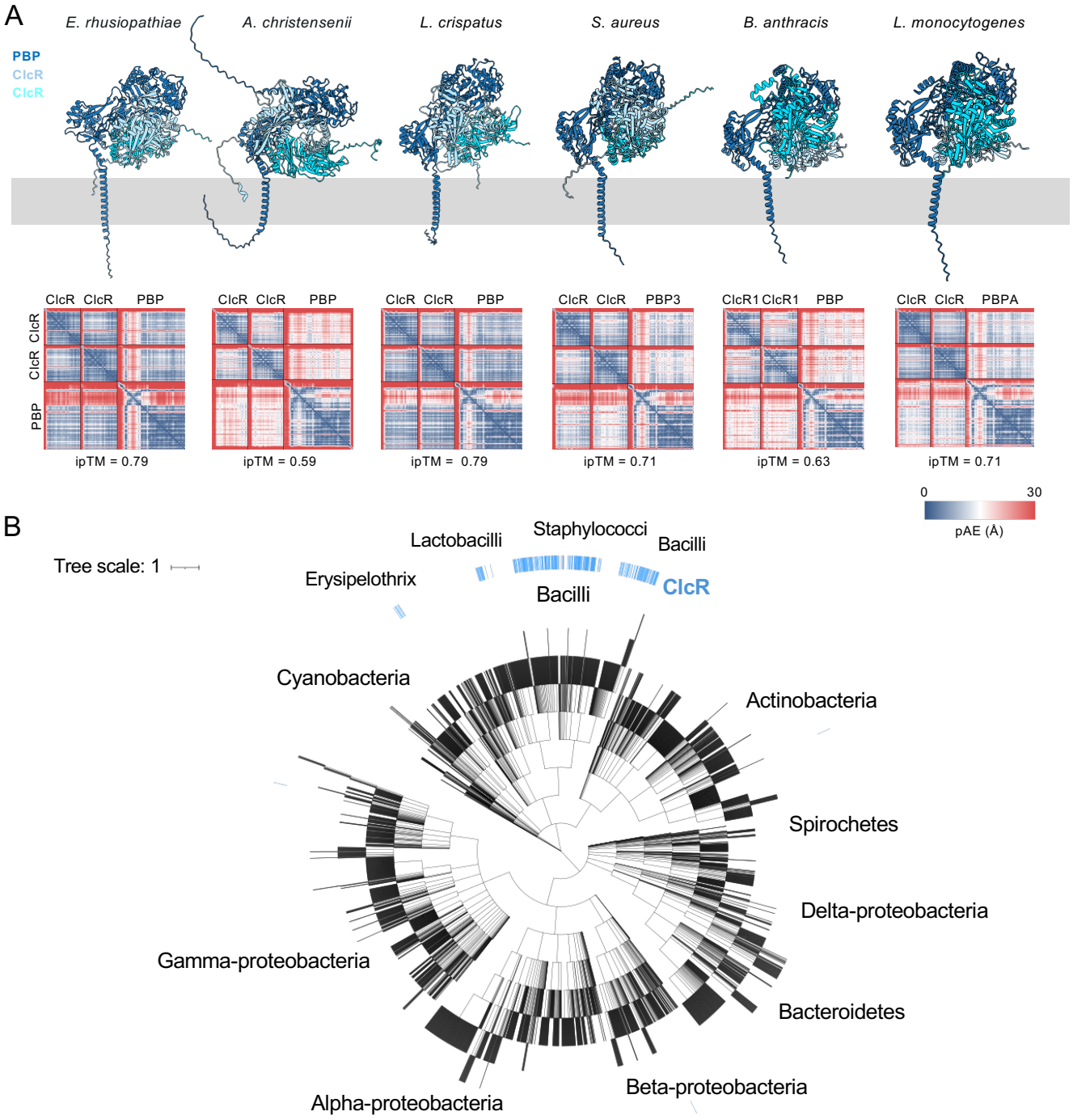

Figure S7. ClcR and PBP2a paralogs are predicted to interact across Gram-positive bacteria. (A) Alphafold3 models and pAE plots of $ClcR_2$-bPBP interactions in the indicated species. For species that lacked an annotated Rod complex bPBP, the closest homolog of *B. subtilis* PBP2a is shown. **(B)** Phylogenetic tree from 6,176 species (black lines) with assembled genomes available in the Prokaryotic Refseq genomes database. Species highlighted in blue encode at least one ClcR homolog as determined by blastp using *B. subtilis* ClcR as a query. The tree scale represents 1 substitution per site.

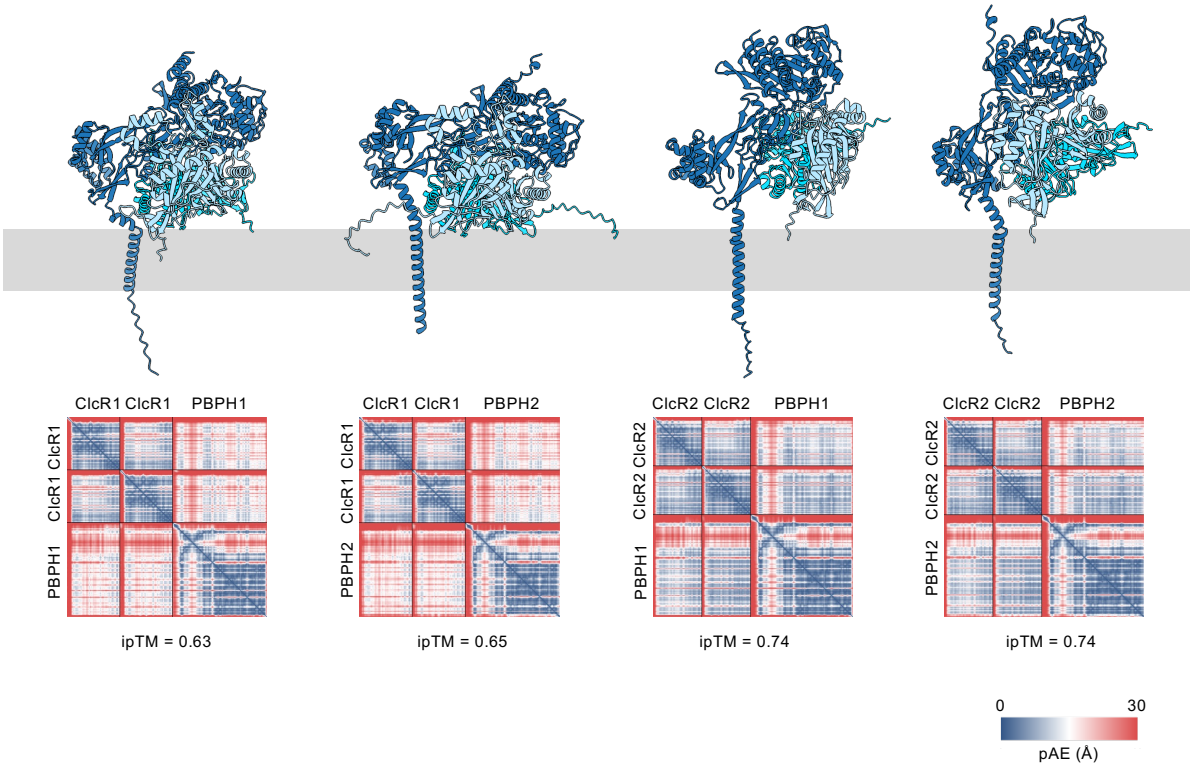

Figure S8. *B. anthracis* ClcR1 and ClcR2 are predicted to interact with two bPBP paralogs. Alphafold3 models and pAE plots for ClcR1, ClcR2, and the two most closely related bPBPs in *B. anthracis* to *B. subtilis* PBP2a and PBPH. The membrane is shown in grey.

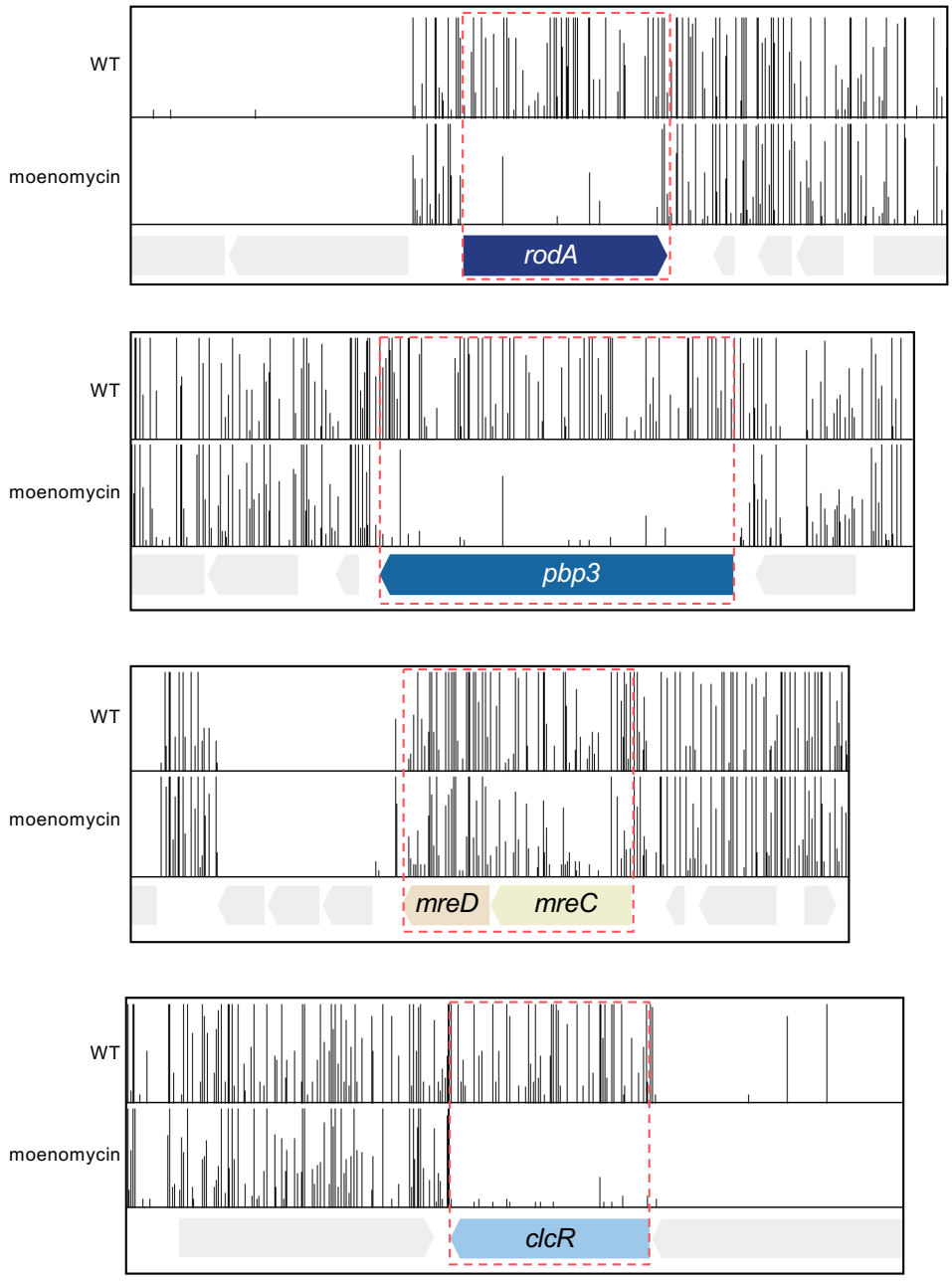

Figure S9. *S. aureus* genes encoding Rod complex components and ClcR become critical in the presence of moenomycin. Tn-seq profiles of four regions of the genome comparing transposon insertions in a wild-type *S. aureus* library in the absence and presence of 8 ng/mL moenomycin (36).

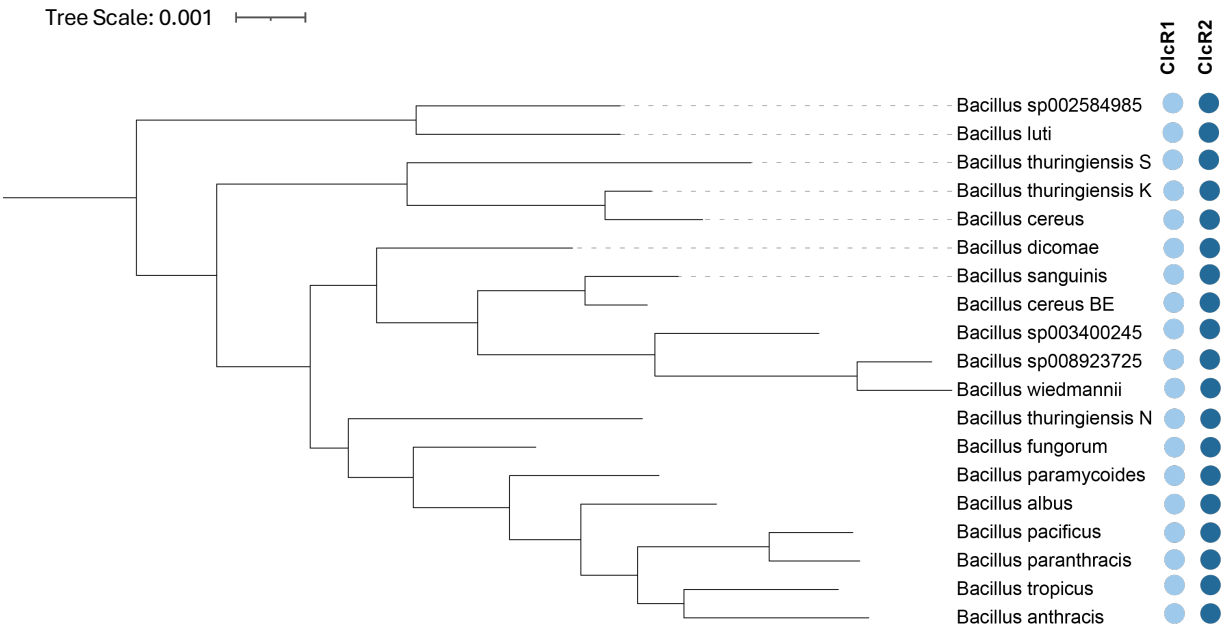

Figure S10. ClcR2 is conserved throughout Bacillus A. A phylogenetic tree displaying the 20 most closely related representative species in the Genome Taxonomy Database GTDB to *B. anthracis*. Those that encode ClcR1 and ClcR2 paralogs are highlighted in light and dark blue respectively.

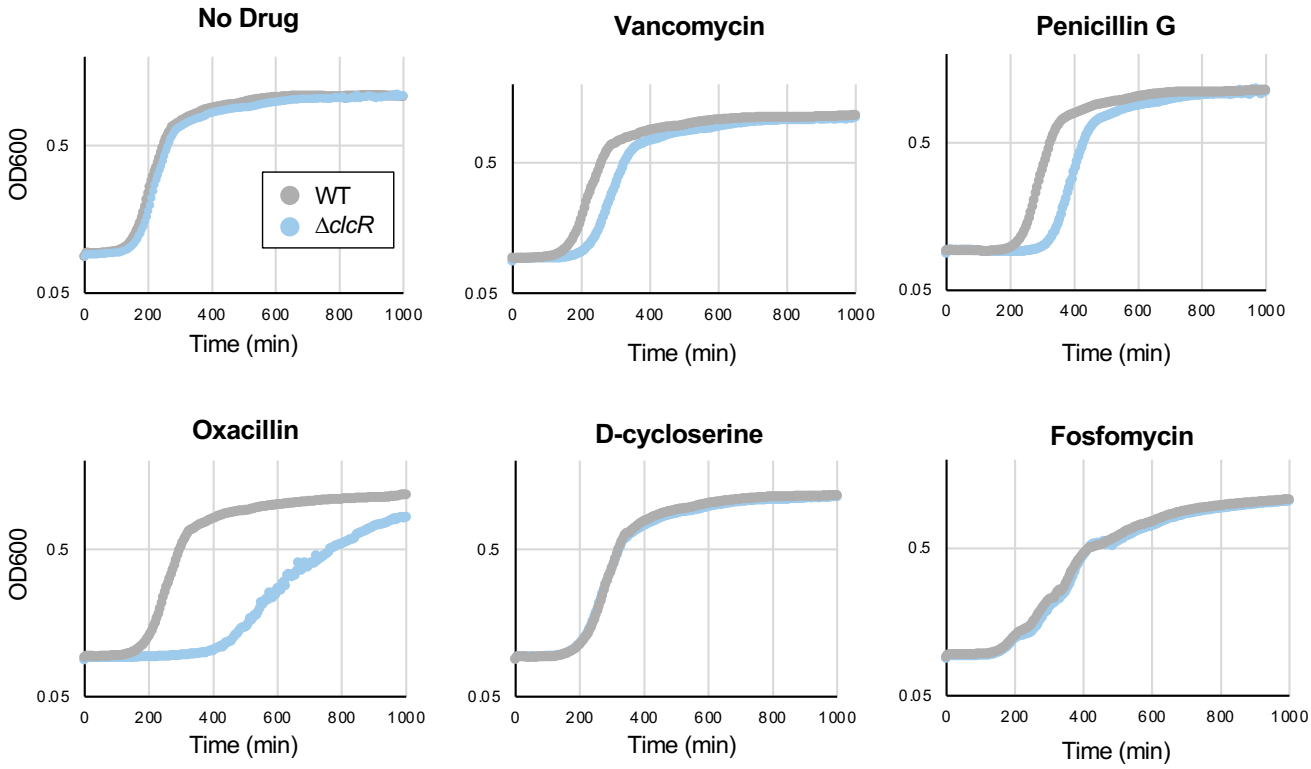

Figure S11. The B. subtilis Δ*clcR* mutant is sensitive to cell envelope targeting antibiotics. Representative growth curves of the indicated strains treated with 0.25-1X MIC of the indicated antibiotics. OD600 was monitored every 5 minutes while cells were grown in an Infinite M Plex plate reader (Tecan) at 37°C with 432 rpm shaking. All experiments in this figure were performed in biological triplicate and representative growth curves are shown.

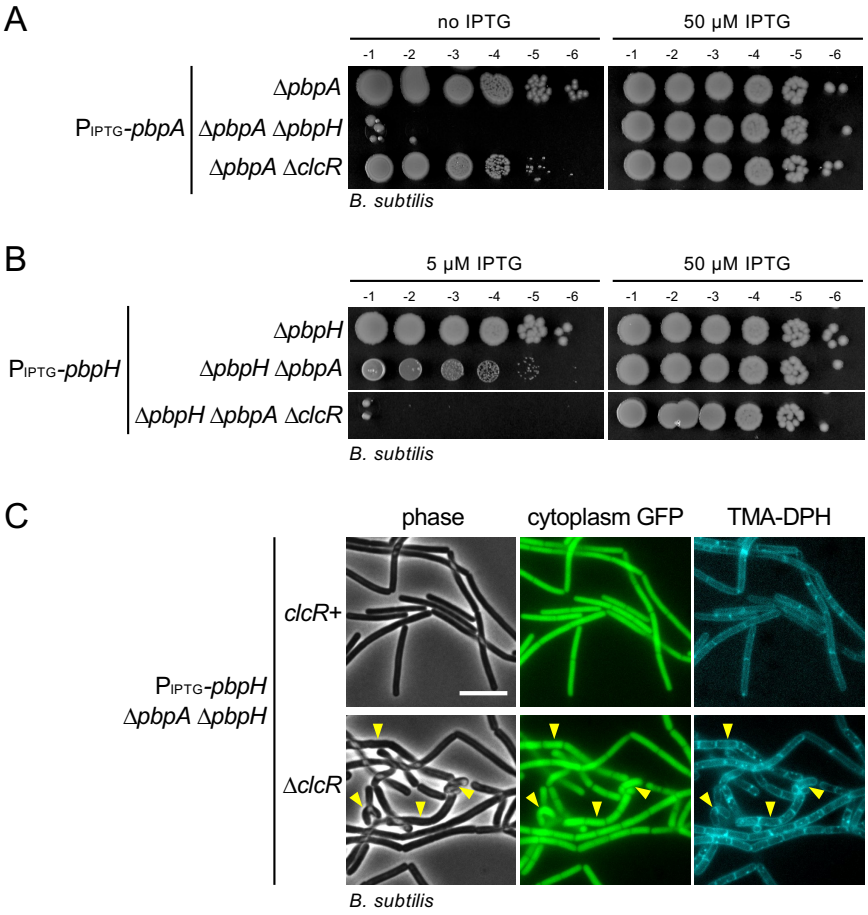

Figure S12. ClcR is required for proper PBPH function. (A&B) Spot titers of the indicated strains on LB agar supplemented with 0, 5, or 50 μM IPTG. **(C)** Fluorescent and phase-contrast micrographs of *B. subtilis* cells expressing *pbpH* as the sole Rod complex bPBP in the presence or absence of *clcR*. Yellow carets highlight fat and twisted cells. The cytoplasm is labeled with a constitutively expressed GFP and the membranes are labeled with 50 μM TMA-DPH. Scale bar indicates 5 μm.

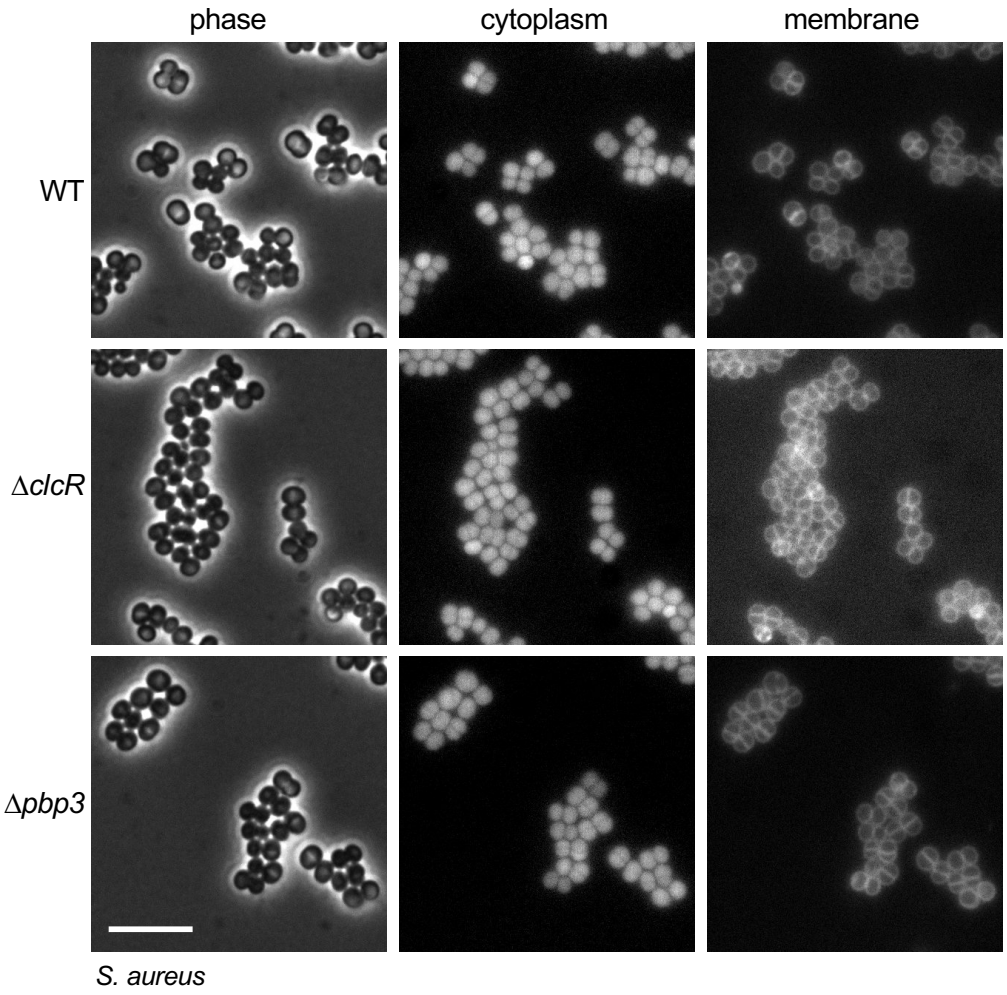

Figure S13. Images from *S. aureus* eccentricity analysis. Representative fluorescent and phase-contrast images of *S. aureus* cells used for the eccentricity analysis. The cytoplasm was labeled with constitutively expressed GFP and the membranes were labeled with 1 µg/mL Nile Red. Larger fields were used to quantify cell eccentricity as described in Figure 3E.

Figure S14
Brogan et al.

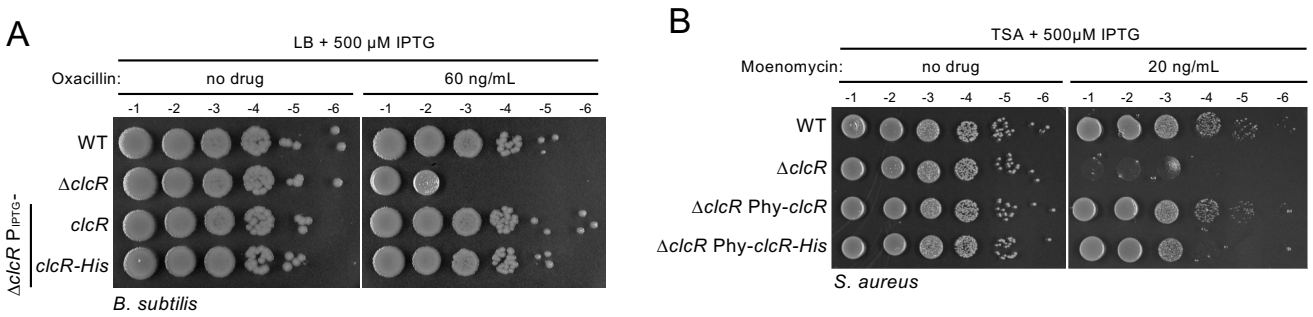

Figure S14. ClcR-His6 tagged alleles are functional. Spot titers of the indicated *B. subtilis* and *S. aureus* strains on **(A)** LB agar supplemented with 500 µM IPTG and 60 ng/mL oxacillin or no drug. **(B)** TSA supplemented with 500 µM IPTG and 20 ng/mL moenomycin or no drug. Both genes are fused at their C-terminus to $His_{6.}$

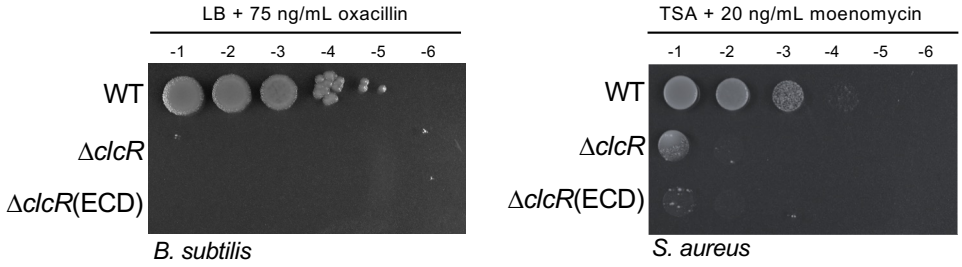

Figure S15. Deletion of the extracytoplasmic domain of *clcR* phenocopies Δ*clcR*. Serial dilutions of the indicated *B. subtilis* (left) and *S. aureus* (right) strains on LB or TSA supplemented with 75 ng/mL oxacillin or 20 ng/mL moenomycin. The *B. subtilis* Δ*clcR*(ECD) allele is an in-frame deletion generated by allelic exchange removing all amino acids between E28 and the stop codon. The *S. aureus* allele was designed based on a previous study (48) and deletes all residues between F69 and the stop codon, leaving a *lox72* scar.

Table S1. Combined AF-M and Tn-seq hits.

known interaction
known interaction with paralog
unknown
unknown with significant Tn-seq hit

Bait	Prey	avg_n_models	best_pae_avg	TnHit
Mbl	YaaL	4.7	2.6	yes
RodZ	MutY	4.6	2.3	no
RodA	PBPH	4.6	2.5	n/a
PBP2a	RodA	4.6	2.8	essential
RodZ	YwpJ	4.6	2.9	no
Mbl	YtxC	4.6	3.2	no
RodA	PBPI	4.6	3.6	essential
MreD	MreC	4.6	4.2	essential
MreC	MreD	4.6	4.4	essential
RodZ	YtfP	4.5	1.9	no
RodZ	YczJ	4.5	2.3	no
RodA	PBP2a	4.5	2.7	essential
MreC	PBP2a	4.5	2.8	essential
MreB	YqbF	4.5	3.5	no
RodA	SpoVD	4.5	3.7	no
Mbl	YlxH(FlhG)	4.5	3.7	no
MreD	YdjA(BsuRC)	4.5	3.8	no
Mbl	PepVL(YtjP)	4.5	3.9	no
RodA	YcgH	4.5	4.3	no
RodZ	YokF(NukF)	4.4	2.7	no
MreC	PBPH	4.4	2.8	n/a
PBP2a	MreC	4.4	3	essential
RodZ	MntA	4.4	3.1	yes
MreB	YerO	4.4	3.4	no
RodZ	GamR	4.4	4.4	no
RodZ	MreB	4.4	4.6	essential
MreB	Nap(CesA)	4.3	2.5	no
PBP2a	YerH(ClcR)	4.3	3	yes
RodZ	FabI	4.3	3.2	yes
PBP2a	SpoVE	4.3	4.1	no
RodA	YteU(RmgU)	4.3	4.4	no

MreB	YrpD	4.2	3.5	no
RodA	YrkK	4.2	4.9	no
RodZ	YwpE	4.1	1.9	no
RodZ	ThiD	4.1	4.4	no
PBP2a	FtsW	4.1	4.6	essential
MreD	YjbL	4	4.6	yes
MreB	RodZ	4	5	essential
MreBH	Slp	4.6	2.5	no
MreBH	Crh	4.4	4.7	no
MreBH	YtxC	4.4	4.1	no

Table S2. Strains used in this study.

Organism		Strain	Genotype	Reference	Figure
Bacillus subtilis	PY79	BAB2238	*yhdG::Pspank-pbpA (kan) pbpA::lox72 pbpH::cat*	This Study	Fig. 1, Fig. S1
Bacillus subtilis	PY79	BAB2347	*yhdG::Pspank-pbpA (kan) pbpA::lox72 pbpH::cat yerH::tet*	This Study	Fig. 1
Staphylococcus aureus	HG003	aTP3	wild-type	Pang et al	Fig. 2, Fig.3, Fig. 4
Staphylococcus aureus	HG003	aAB276	*clcR::lox72*	This Study	Fig. 2, Fig.3, Fig. S14, Fig. S15
Staphylococcus aureus	HG003	aAB277	*pbp3::lox72*	This Study	Fig. 2, Fig.3, Fig. 4
Staphylococcus aureus	HG003	aAB279	*clcR::lox72 pbp3::kan*	This Study	Fig. 2
Bacillus anthracis	BaR1	BAB2440	wild-type	Ramirez-Guadiana et al	Fig. 2, Fig.3
Bacillus anthracis	BaR1	BAB2491	*3956::Pspank-clcR1 (kan) ΔclcR1*	This Study	Fig. 2, Fig.3
Bacillus anthracis	BaR1	BAB2594	*3956::Pspank-clcR2 (kan) ΔclcR2*	This Study	Fig. 2, Fig.3
Bacillus anthracis	BaR1	BAB2501	*3956::Pspank-clcR1 (kan) ΔclcR1 ΔclcR2*	This Study	Fig. 3
Bacillus subtilis	PY79	BAB1	wild-type	Youngman et al	Fig. 3, Fig. 4, Fig. S5, Fig. S11, Fig. S14, Fig, S15
Bacillus subtilis	PY79	BAB2119	*pbpA::cat*	This Study	Fig. 3
Bacillus subtilis	PY79	BAB222	*yhdG::Pspank-pbpA (erm) pbpA::cat*	This Study	Fig. 3
Bacillus subtilis	PY79	BAB2120	*clcR::tet*	This Study	Fig. 3, Fig. 4, Fig. S11, Fig. S14, Fig. S15
Bacillus subtilis	PY79	BAB2266	*yhdG::Pspank-clcR (erm) clcR::tet*	This Study	Fig. 3, Fig. S14
Bacillus subtilis	PY79	BAB2338	*yhdG::Pspank-pbpA (erm) clcR::tet*	This Study	Fig. 3
Bacillus subtilis	PY79	BAB2349	*yhdG::Pspank-pbpB (erm) clcR::tet*	This Study	Fig. 3
Bacillus subtilis	PY79	BAB1505	*sacA::Pveg-GFP (phleo)*	Brogan et al	Fig. 3

Bacillus subtilis	PY79	BAB2098	*yhdG::Pspank-pbpA (erm) pbpA::cat pbpH::spc sacA::Pveg-gfp (Phleo)*	This Study	Fig. 3, Fig. S5
Bacillus subtilis	PY79	BAB2100	*yhdG::Pspank-pbpA (erm) pbpA::cat clcR::tet pbpH::spc sacA::Pveg-gfp (Phleo)*	This Study	Fig. 3, Fig. S5
Staphylococcus aureus	HG003	aAB428	*mreCD::kan*	This Study	Fig. 3
Staphylococcus aureus	HG003	aAB430	*clcR::lox72 mreCD::kan*	This Study	Fig. 3
Staphylococcus aureus	HG003	aAB452	*rodA::kan*	This Study	Fig. 3
Staphylococcus aureus	HG003	aAB30	*02626-02627::Pveg-GFP (kan)*	This Study	Fig. 3, Fig. S13
Staphylococcus aureus	HG003	aAB278	*clcR::lox72 02626-02627::Pveg-GFP (kan)*	This Study	Fig. 3, Fig. S13
Staphylococcus aureus	HG003	aAB280	*PBP3::lox72 02626-02627::Pveg-GFP (kan)*	This Study	Fig. 3, Fig. S13
Bacillus anthracis	BaR1	BAB2497	*3956::Pspank-rodA (kan) ΔrodA*	This Study	Fig. 3
Bacillus subtilis	PY79	BAB2269	*clcR::tet yvbJ::PxylA-clcR (kan)*	This Study	Fig. 4, Fig. 5
Bacillus subtilis	PY79	BAB2270	*clcR::tet yvbJ::PxylA-clcR-R76E (kan)*	This Study	Fig. 4, Fig. 5
Bacillus subtilis	PY79	BAB2111	*yhdG::Pspank-clcR-His6 (erm)*	This Study	Fig. 4
Bacillus subtilis	PY79	BAB2169	*yhdG::Pspank-clcR-R76E-His6 (erm)*	This Study	Fig. 4
Staphylococcus aureus	HG003	aAB389	*clcR::lox72 0309-0310::Phy-spank-clcR (tet)*	This Study	Fig. 4, Fig. S14
Staphylococcus aureus	HG003	aAB450	*clcR::lox72 0309-0310::Phy-spank-clcR-S76E (tet)*	This Study	Fig. 4
Staphylococcus aureus	HG003	aAB390	*clcR::lox72 0309-0310::Phy-spank-clcR-His6 (tet)*	This Study	Fig. 4, Fig. S14
Staphylococcus aureus	HG003	aAB451	*clcR::lox72 0309-0310::Phy-spank-clcR-S76E-His6 (tet)*	This Study	Fig. 4
Bacillus subtilis	PY79	BAB2428	*clcR::tet yhdG::Pspank-clcR-Q203E (erm)*	This Study	Fig. 5
Bacillus subtilis	PY79	BAB2267	*yhdG::Pspank-clcR-His6 (erm) clcR::tet*	This Study	Fig. 5, Fig. S14
Bacillus subtilis	PY79	BAB2445	*clcR::tet yhdG-Pspank-clcR-Q203E-His6 (erm)*	This Study	Fig. 5
Bacillus subtilis	PY79	BAB2511	*yhdG::Pspank-clcR-R76E-Q203E-His6 (erm)*	This Study	Fig. 5
Bacillus subtilis	PY79	BAB2516	*yvbJ::PxylA-clcR-Q203E (kan) clcR::tet*	This Study	Fig. 5
Bacillus subtilis	PY79	BAB2512	*clcR::tet yvbJ::PxylA-clcR-R76E-Q203E (kan)*	This Study	Fig. 5
Bacillus subtilis	PY79	BAB2455	*yhdG::Pspank-pbpA (erm) pbpA::cat clcR::tet pbpH::spc*	This Study	Fig. 5

			sacA::Pveg-gfp (Phleo) yvbJ::PxylA-clcR-Q203E (kan)		
Bacillus subtilis	PY79	BAB2498	*yhdG::Pspank-pbpA (erm) pbpA::cat clcR::tet pbpH::spc sacA::Pveg-gfp (Phleo) yvbJ::PxylA-clcR (kan)*	This Study	Fig. 5
Bacillus anthracis	BaR1	BAB2523	*mbl-mNeonGreen*	This Study	Fig. 5
Bacillus anthracis	BaR1	BAB2527	*3956::Pspank-rodA (kan) ΔrodA mbl-mNeonGreen*	This Study	Fig. 5
Bacillus anthracis	BaR1	BAB2539	*3956::Pspank-yerH(347) (kan) ΔyerH(347) ΔyerH(4892) mbl-mNeonGreen*	This Study	Fig. 5
Bacillus subtilis	PY79	BAB2504	*yhdG::Pspank-pbpA (erm) pbpA::cat pbpH::spc sacA::Pveg-gfp (Phleo) mntA::tet*	This Study	Fig. S5
Bacillus subtilis	PY79	BAB2505	*yhdG::Pspank-pbpA (erm) pbpA::cat pbpH::spc sacA::Pveg-gfp (Phleo) yaaL::tet*	This Study	Fig. S5
Bacillus subtilis	PY79	BAB222	*yhdG::Pspank-pbpA (erm) pbpA::cat*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB241	*yhdG::Pspank-pbpA (erm) pbpA::cat pbpH::spc*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB2078	*yhdG::Pspank-pbpA (erm) pbpA::cat clcR::tet*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB2076	*yhdG::Pspank-pbpH (erm) pbpH::spc*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB2092	*yhdG::Pspank-pbpH (erm) pbpH::spc pbpA::cat*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB2094	*yhdG::Pspank-pbpH (erm) pbpH::spc clcR::tet pbpA::cat*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB2099	*yhdG::Pspank-pbpH (erm) pbpH::spc pbpA::cat sacA::Pveg-gfp (Phleo)*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB2101	*yhdG::Pspank-pbpH (erm) pbpH::spc clcR::tet pbpA::cat sacA::Pveg-gfp (Phleo)*	This Study	Fig. S12
Bacillus subtilis	PY79	BAB2314	*pbpA::cat ycgO-PxylA-pbpA-ALFA (spec)*	This Study	Fig. S14
Bacillus subtilis	PY79	BAB2315	*pbpA::cat ycgO::PxylA-pbpA (spec)*	This Study	Fig. S14
Staphylococcus aureus	HG003	aAB432	*PBP3::lox72 02923-02924::PBP3 (spc)*	This Study	Fig. S14
Staphylococcus aureus	HG003	aAB442	*PBP3::lox72 02923-02924::PBP3-ALFA (spc)*	This Study	Fig. S14
Bacillus subtilis	PY79	BAB2136	*ΔclcR(29-396)*	This Study	Fig. S15
Staphylococcus aureus	HG003	aAB395	*clcRΔ69-391 (lox72)*	This Study	Fig. S15

Table S3. Plasmids used in this study.

Plasmid	Description	Reference
pCB57	yhdG::Pspank (erm) (amp)]	lab stock
pCB96	ycgO::Pxyl (spec) (amp)	lab stock
pCB101	yhdG::Phy-spank (spc) (amp)	lab stock
pCB133	yvbJ::PxylA (kan) (amp)	lab stock
pKT25	T25-MCS (kan)	Karimova et al (2018)
pKNT25	MCS-T25 (kan)	Karimova et al (2018)
pUT18	MCS-T18 (amp)	Karimova et al (2018)
pUT18C	T18-MCS (amp)	Karimova et al (2018)
pWX496	PPA-cre (erm) (amp)	Meeske et al (2016)
pCZ9	pMAD3 (spc) (amp), for *B.anthracis* allelic replacement	Zhang et al (2026)
pCZ18	pMAD3 locus003956::Pspank (kan) (spc) (amp)	Zhang et al (2026)
pCZ54	pMAD4 (pACYC origin) (spc) (amp), for *B.anthracis* allelic replacement	Zhang et al (2026)
pEB352	T25-TolB (kan)	Battesti & Bouveret (2008)
pEB356	T18-Pal (amp)	Battesti & Bouveret (2008)
pPM260	expressing *S. aureus* recombineering machinery	Banh et al (2023)
pminiMAD	for *B. subtilis* allelic replacement	Patrick & Kearns (2008)
pMR91	pMAD3.1, for *S. aureus* allelic replacement	Bartlett et al (2024)
pAB53	yhdG::Pspank-*pbpA* (erm) (amp)	This Study
pAB397	0309-0310-integration (tet) (amp)	This Study
pAB401	02626-02627 integration (kan) (amp)	This Study
pAB402	02626-02627::Pveg-GFP (kan) (amp)	This Study
pAB405	02923-02924 integration (spc) (amp)	This Study
pAB438	yhdG-Phy-spank-gfp (spc) (amp)	This Study
pAB440	pACYC ts Ptet-cre (erm) (amp)	This Study
pAB450	0309 0310 integration Phy-spank-optRBS-gfp (tet) (amp)	This Study
pAB461	0309-0310::Phy-spank-gfp (tet) (amp) pACYC	This Study
pAB485	yhdG::Pspank-*pbpH* (erm) (amp)	This Study
pAB486	yhdG::Pspank-*clcR* (erm) (amp)	This Study
pAB487	(pMAD3.1)-Δ*clcR*::kan (amp)	This Study
pAB488	(pMAD3.1)-Δ*pbp3*::kan (amp)	This Study
pAB493	pminiMAD) Δ*clcR*(ECD)	This Study
pAB495	yhdG::Pspank-*clcR*-His6 (erm) (amp)	This Study
pAB504	yhdG::Pspank-*clcR*-R76E-His6 (erm) (amp)	This Study
pAB513	yvbJ::PxylA-*clcR* (kan) (amp)	This Study
pAB515	yvbJ::PxylA-*clcR*-R76E (kan) (amp)	This Study

pAB532	02923-02924 (spc) pACYC	This Study
pAB554	(pMAD3.1)-*clcR*Δ69-391::kan (amp)	This Study
pAB557	0309-0310::Phy-spank-*clcR* (tet) (amp)	This Study
pAB558	0309-0310::Phy-spank-*clcR*-His6 (tet) (amp)	This Study
pAB565	ycgO::PxylA-*pbpA*-ALFA (spec) (amp)	This Study
pAB568	ycgO::PxylA-*pbpA* (spec) (amp)	This Study
pAB580	02923-02924::*pbp3* (spc) (amp) pACYC	This Study
pAB584	Δ*mreCD*::kan (amp) pACYC	This Study
pAB585	Δ*rodA*::kan pACYC	This Study
pAB587	yhdG::Pspank-*pbpB* (erm) (amp)	This Study
pAB588	02923-02924::*pbp3*-ALFA (spc) (amp) pACYC	This Study
pAB589	0309-0310::Phy-spank-*clcR*-S76E (tet) pACYC	This Study
pAB590	0309-0310::Phy-spank-*clcR*-S76E-His6 (tet) pACYC	This Study
pAB596	locus003956::Pspank-*clcR1*(BAIDGICL347) (spec) (kan)	This Study
pAB597	locus003956::Pspank-*clcR2*(BAIDGICL4892) (spec) (kan)	This Study
pAB598	pminiMAD3 Δ*clcR1*(BAIDGICL347) (spec) (amp)	This Study
pAB599	pminiMAD3 Δ*clcR2*(BAIDGICL4892) (spec) (amp)	This Study
pAB600	locus003956::Pspank-*rodA* (spec) (kan)	This Study
pAB601	pminiMAD3 Δ*rodA* (spec) (amp)	This Study
pAB602	yvbJ::PxylA-*clcR*-Q203E (kan) (amp)	This Study
pAB603	yhdG::Pspank-*clcR*-Q203E (erm) (amp)	This Study
pAB605	yhdG::Pspank-*clcR*-Q203E-His6 (erm) (amp)	This Study
pAB626	T25-*clcR* (kan)	This Study
pAB627	T25-*pbpA* (kan)	This Study
pAB628	T25-*pbpH* (kan)	This Study
pAB629	*clcR*-T25 (kan)	This Study
pAB630	*pbpA*-T25 (kan)	This Study
pAB631	*pbpH*-T25 (kan)	This Study
pAB632	*clcR*-T18 (amp)	This Study
pAB633	*pbpA*-T18 (amp)	This Study
pAB634	*pbpH*-T18 (amp)	This Study
pAB635	T18-*clcR* (amp)	This Study
pAB636	T18-*pbpA* (amp)	This Study
pAB637	T18-*pbpH* (amp)	This Study
pAB645	yvbJ::PxylA-yerH-R76E-Q203E (kan) (amp)	This Study
pAB646	yhdG::Pspank-yerH-R76E-Q203E-His6 (erm) (amp)	This Study
pAB647	*clcR*-R76E-T18	This Study
pAB653	pMAD4 mbl-mNeonGreen (spec) (amp)	This Study

Table S4. Oligonucleotides used in this study.

Oligo	Sequence
oAB27	TTCTGCTCCCTCGCTCAG
oAB28	CAGGGAGCACTGGTCAAC
oAB97	GTGAGCGGATAACAATTAAGCTTtaaatagaaaaggtgatgttatgaggagaaataaacc
oAB98	GCATGCgaGCTAGCatCTGCAGttACTAGTttagttatcagaagacgttgtgttttctgc
oAB884	atataaaatatactcaaaatattatcaaggttatgacgtacaaacgacg
oAB885	AACGGTAGTTGACCAGTGCTCCCTGAAAAAAAAACCCCGCTTTCGCGGGGccttttatcaagaggaaagccacttac
oAB886	AACGGTACTGAGCGAGGGAGCAGAAGAATTCatAAGCTTgtCTCGAGatACTAGTatGGATCCAAAAAAAAACCCCGCCGAAGCGGGGTTTTTTTTggcattcactcaatatgtgacgg
oAB887	TCACGTTCGCTCGCGTATCGGTGATggctgcgtttttagcaatcattttaac
oAB888	TTCGGCGGGGTTTTTTTTTGGATCCcGACTCTCTAGCTTGAGGCATC
oAB889	ACTGAGCGAGGGAGCAGAAGAATTCTCACTGCCCGCTTTCCAG
oAB896	tataaaatatactcaaaatattatcgtgtatgtcttaatttattttagtatgatagtcac
oAB897	AACGGTAGTTGACCAGTGCTCCCTGAAAAAAAAACCCCGCTTTCGCGGGGacgtaattgggttttgtttttgaaaatatagc
oAB898	AACGGTACTGAGCGAGGGAGCAGAAGAATTCatAAGCTTgtCTCGAGatACTAGTatGGATCCAAAAAAAAACCCCGCCGAAGCGGGGTTTTTTTTtttcatgtcaaatatcatcttgcatgaaatc
oAB899	ACGTTCGCTCGCGTATCGGTGATggatacataaaatatataaaggtaggttaagacattg
oAB903	CatAAGCTTgtCTCGAGatACTAGTttatttgtatagttcatccatgccatgtg
oAB907	atataaaatatactcaaaatattatgctgctatcgttattttattaactgcattattac
oAB908	AACGGTAGTTGACCAGTGCTCCCTGAAAAAAAAACCCCGCTTTCGCGGGGtctggatgttaatcagatgggaaatg
oAB909	AACGGTACTGAGCGAGGGAGCAGAAGAATTCatAAGCTTgtCTCGAGatACTAGTatGGATCCAAAAAAAAACCCCGCCGAAGCGGGGTTTTTTTTtgtggaaatgatttttttattttatattaagataatcgc
oAB910	TCACGTTCGCTCGCGTATCGGTGATgcatatacagggcgtccttatatc
oAB928	TTCGGCGGGGTTTTTTTTTGGATCCCttgaaaacctgcataggagagctatg
oAB987	ATTAAGCTTgtCCCGGGtaACTAGTTacataaggaggaactactatgagtaaaggag
oAB988	CTTGCATGCgaGCTAGCatCTGCAGttatttgtatagttcatccatgccatgtg
oAB998	GTGGACGGACTGGTTACAAGTGGAAatcacggaaaaaggttatgctgcttttaag
oAB999	GCCGTCAATTGTCTGATTCGTTACCGAATTCAGTCATAGCTGTACCCCACG
oAB1000	aagcagcataacctttttccgtgatTTCCACTTGTAACCAGTCCGTCC
oAB1001	GTGGGGTACAGCTATGACTGAATTCGGTAACGAATCAGACAATTGACGGC
oAB1045	gagaccacaacggtttccctCTAGcaaggttatgacgtacaaacgacg
oAB1046	tcgtttgtacgtcataaccttgCTAGagggaaaccgttgtggtc
oAB1047	taaaatgattgctaaaaacgcagccGGCCGGCtgagcaataactag
oAB1048	tgctagttattgctcaGCCGGCCggctgcgtttttagcaatcattttaac
oAB1106	TTGTGAGCGGATAACAATTAAGCTTtttgagaggggtaaccagaagtgactg
oAB1107	CgaGCTAGCatCTGCAGttACTAGTttatttttttactgtgtttttttttcgagctctgcg

oAB1108	gagcttcaggcgaagcgcg
oAB1109	GGTACTGAGCGAGGGAGCAGAAgtctttttcaatagaaaacactcctcttatttctttag
oAB1110	AACGGTAGTTGACCAGTGCTCCCTGatttatgactgatagaaaaccctttgtgcc
oAB1111	gctgtaatcggcgtgccattctc
oAB1112	TTGTGAGCGGATAACAATTAAGCTTaataagaggagtgttttctattgaaaaagacgttg
oAB1113	CgaGCTAGCatCTGCAGttACTAGTtcagtcataaatgtgaacagtcggc
oAB1114	aaaatattatCCATGGtatGAATTCggacgaacaggtgtagtcacacc
oAB1115	AACGGTACTGAGCGAGGGAGCAGAAgtacgcttcatcgacatacccctc
oAB1116	ACGGTAGTTGACCAGTGCTCCCTGgaaccgcaagttcatatttacagtaattaattg
oAB1117	TCGCTCGCGTATCGGTGATGGATCCccaaggtgtcattatgttaattgcgatg
oAB1118	aaaatattatCCATGGtatGAATTCggtgtatgtgagtcttgccaagattaatataaag
oAB1119	AACGGTACTGAGCGAGGGAGCAGAActttttaacaaaactacctacctctattcaaagtc
oAB1120	AACGGTAGTTGACCAGTGCTCCCTGaaagacaaataaaatttaacctgacgattgtgtag
oAB1121	TCGCTCGCGTATCGGTGATGGATCCgtctatgttcatttagtcctccactatgc
oAB1132	ctatgaccatgattacgccaagcttggataaggttgtcgtcaaaaaagcg
oAB1133	cacaagggttttctatcactccttctccccccccgaaac
oAB1134	tcgggggggagaaggagtgatagaaaacccttgtgccatattggc
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oAB1137	gaGCTAGCatCTGCAGttACTAGTttaattactgtaaatatgaacttgcggttctttgtc
oAB1138	tCTGCAGttACTAGTtcaATGATGGTGATGATGATGgtcataaatgtgaacagtcggctc
oAB1142	ATTCGCCAGGGGGATCCatCTCGAGttagttatcagaagacgttgtgttttctgc
oAB1145	gaGCTAGCatCTGCAGttACTAGTttaATGATGGTGATGATGATGattactgtaaatatgaacttgcggttctttgtc
oAB1155	gaacagctgaatacaGAActggacattgacg
oAB1156	cgtcaatgtccagTTCtgtattcagctgttc
oAB1188	atgAAGCTTgaGCTAGCatCTCGAGaataagaggagtgttttctattgaaaaagacgttg
oAB1189	cacctcaaatggttcgctggGATCCtcagtcataaatgtgaacagtcggc
oAB1214	CTAGagggaaaccgttgtggtc
oAB1215	GGCCGGCtgagcaataactagc
oAB1220	ggagaccacaacggtttccctCTAGgctgctatcgttatttttattaactgcattattac
oAB1221	tatgctagttattgctcaGCCGGCCgcatatacagggcgtccttatatc
oAB1238	ggtgaagaaaacagcggaacacag
oAB1239	AACGGTACTGAGCGAGGGAGCAGAAatttcagtcacttctggttacccctc
oAB1240	ACGGTAGTTGACCAGTGCTCCCTGagtaaaaaataaaaaacagggtgcacaactaaaaga
oAB1241	ccaggattgcttgacaatttcagattctg
oAB1271	aaaatattatCCATGGtatGAATTCcctgaagttgtacgtagtattccagaacg
oAB1272	AACGGTACTGAGCGAGGGAGCAGAAaaaagtcctcttgcctggctttc
oAB1288	AACTAGTtaCCCGGGacAAGC
oAB1289	CTGCAGatGCTAGCtcGCATGC

oAB1297	ctaggctcatgagcccgaagtg
oAB1298	cgccacttcgggctcatgag
oAB1305	gcatctgtatttgaatgAAGCTTtaaatagaaaaggtgatgttatgaggagaaataaacc
oAB1306	GCCAGGGGGATCCatCTCGAGttaTTCAGTTAACCGTCTCCGAAGCTCCTCCTCGA GGCGGGAgttatcagaagacgttgtgttttctgc
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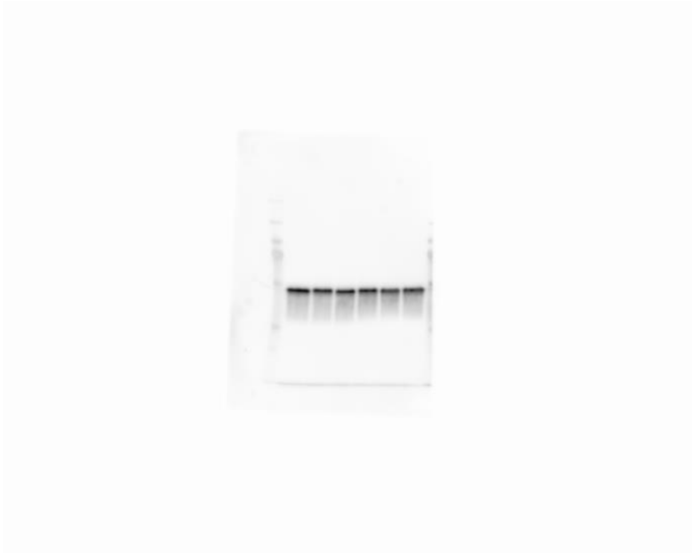
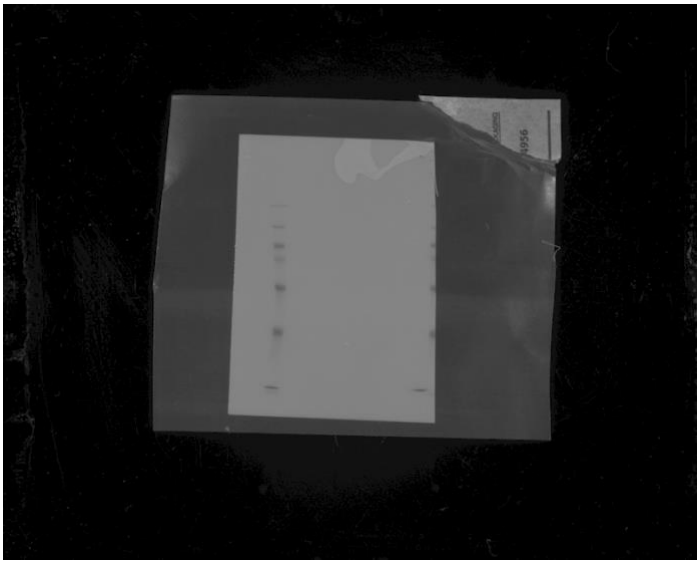
Uncropped immunoblots/SDS-PAGE gels

Figure 4E:
Anti-His





Anti-SigA

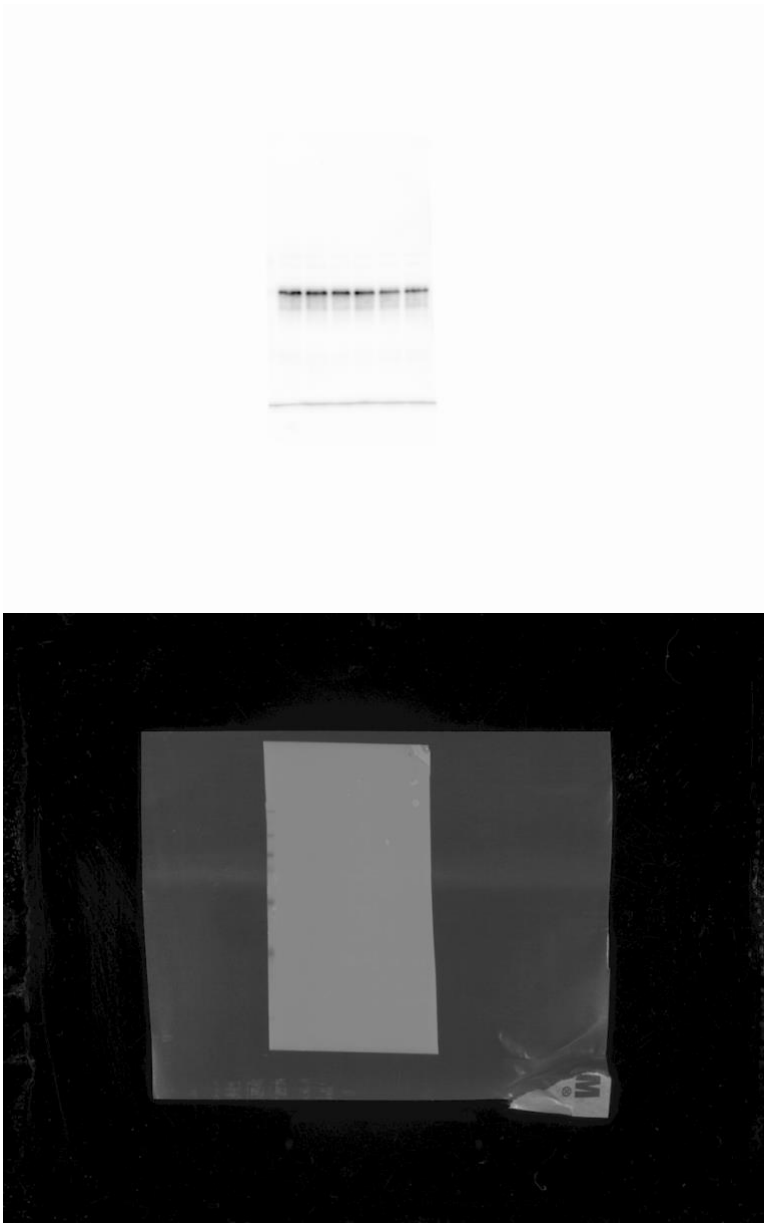
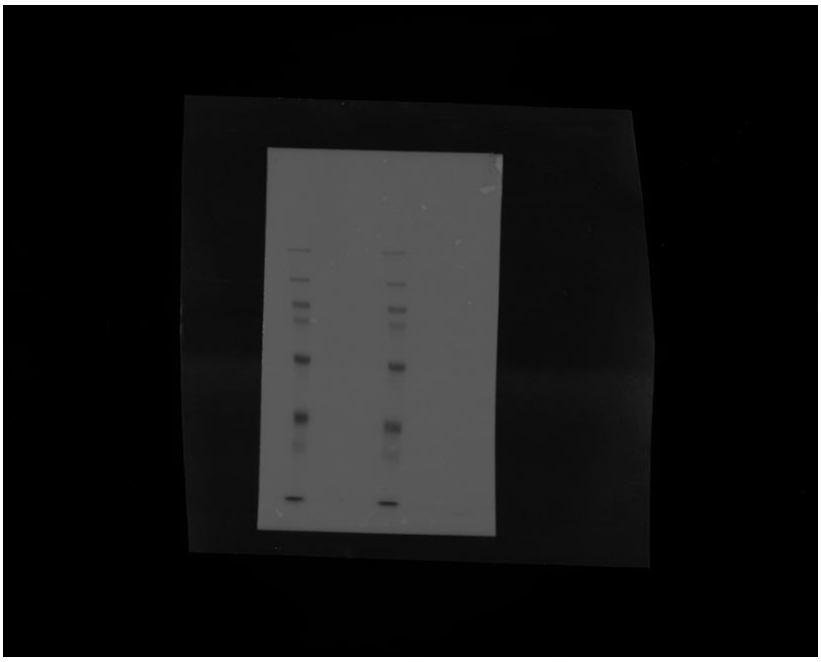


Figure 4G:
Anti-His



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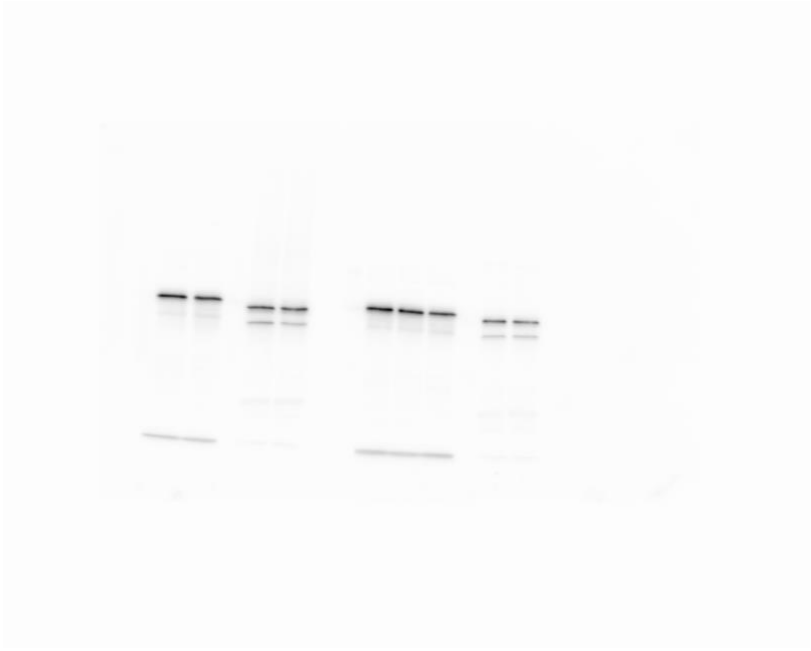


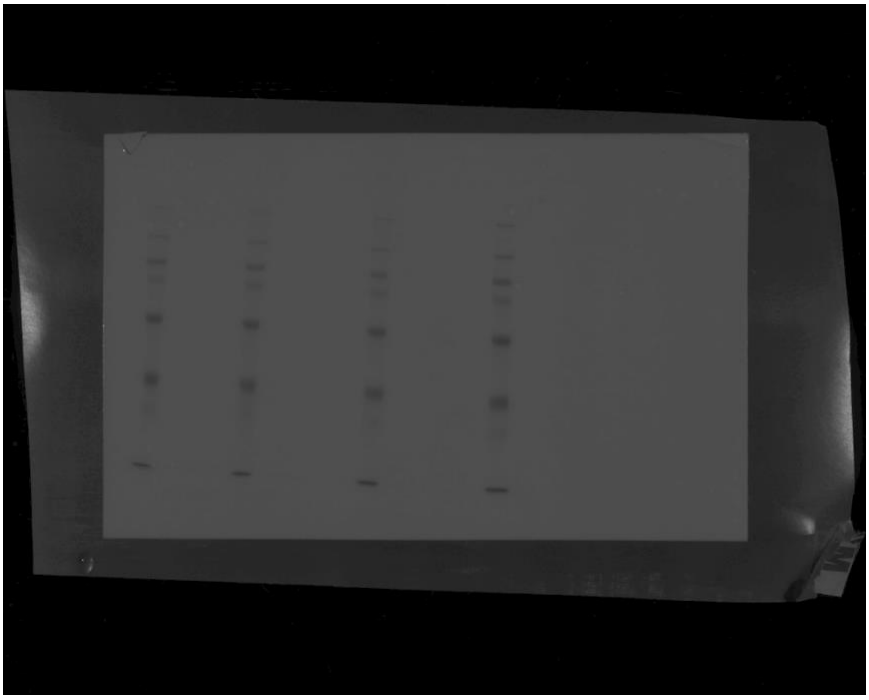
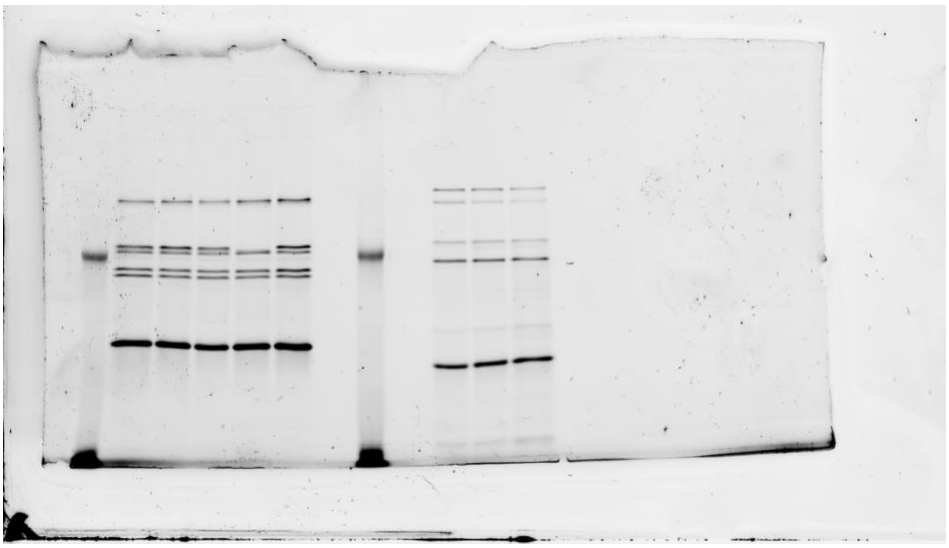
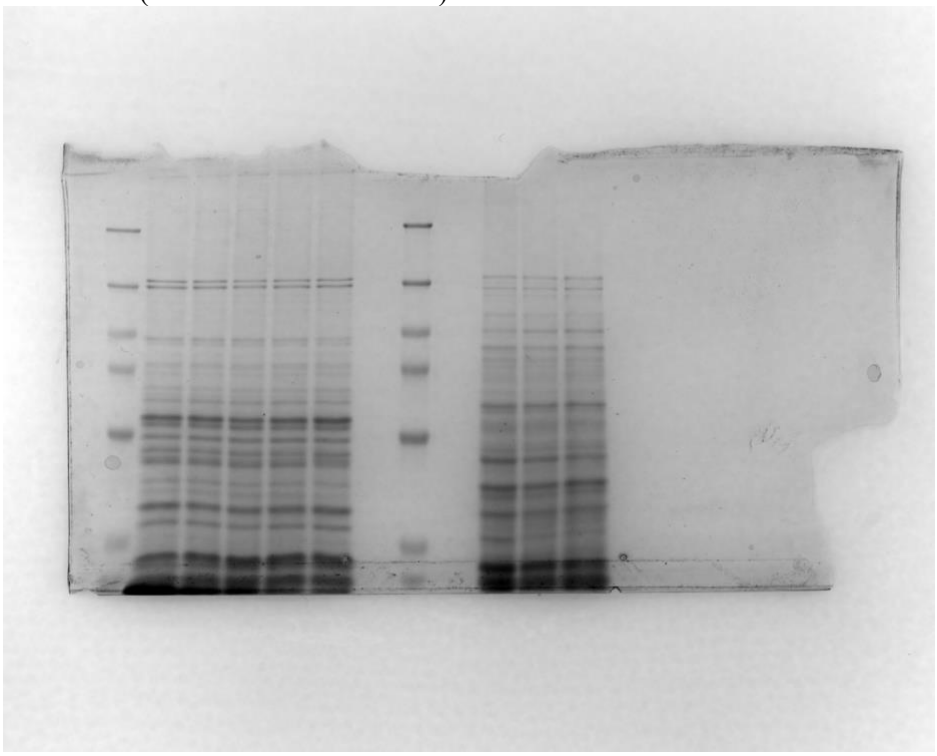


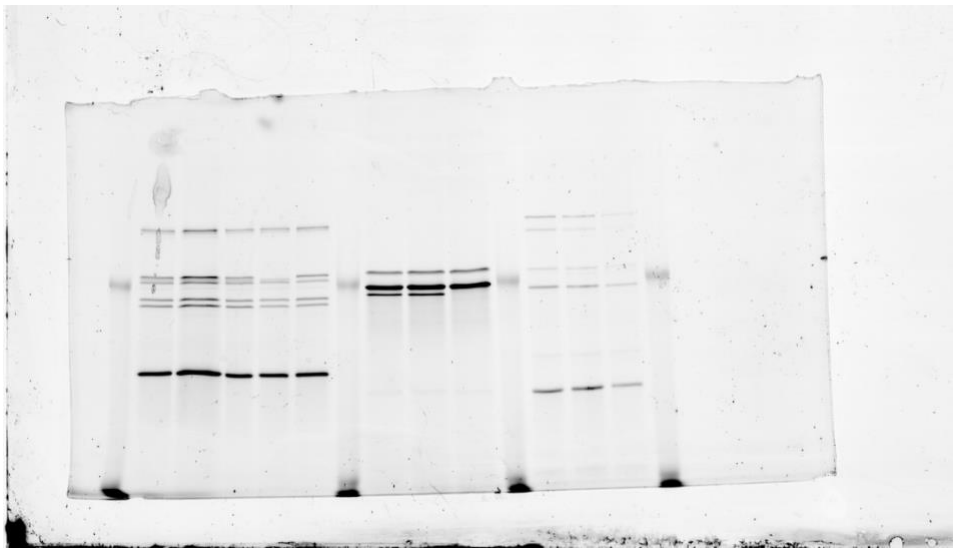
Figure 5A:
Bocillin-FL (B. subtilis. / B. anthracis)



Coomassie (B. subtilis. / B. anthracis)



Bocillin-FL (S. aureus)



Coomassie (S. aureus)

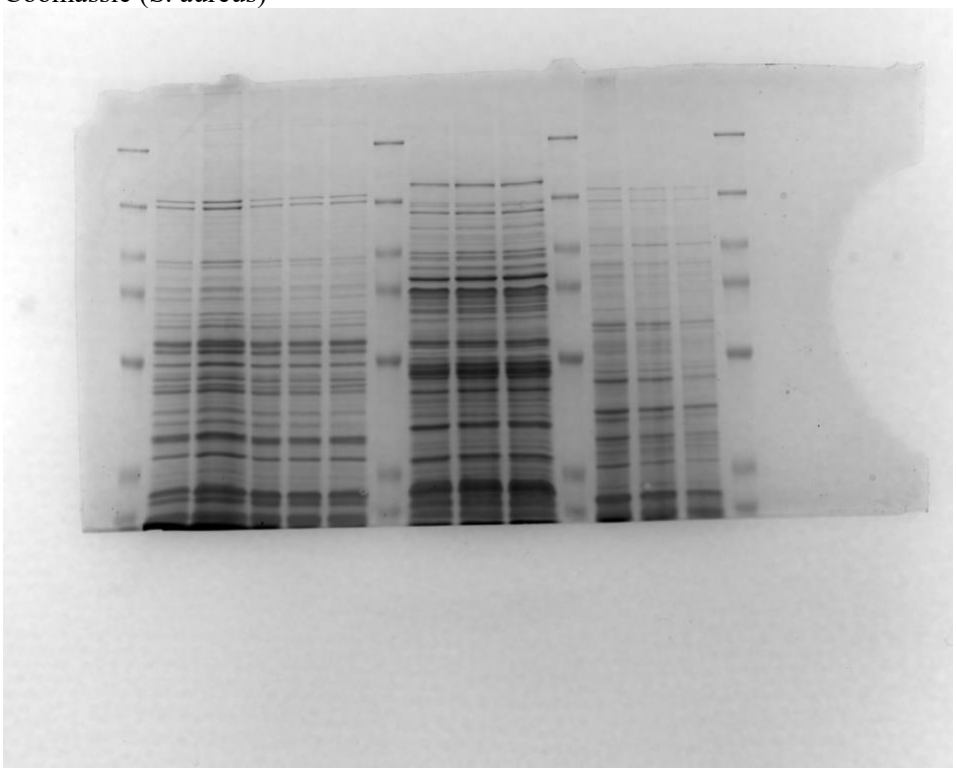
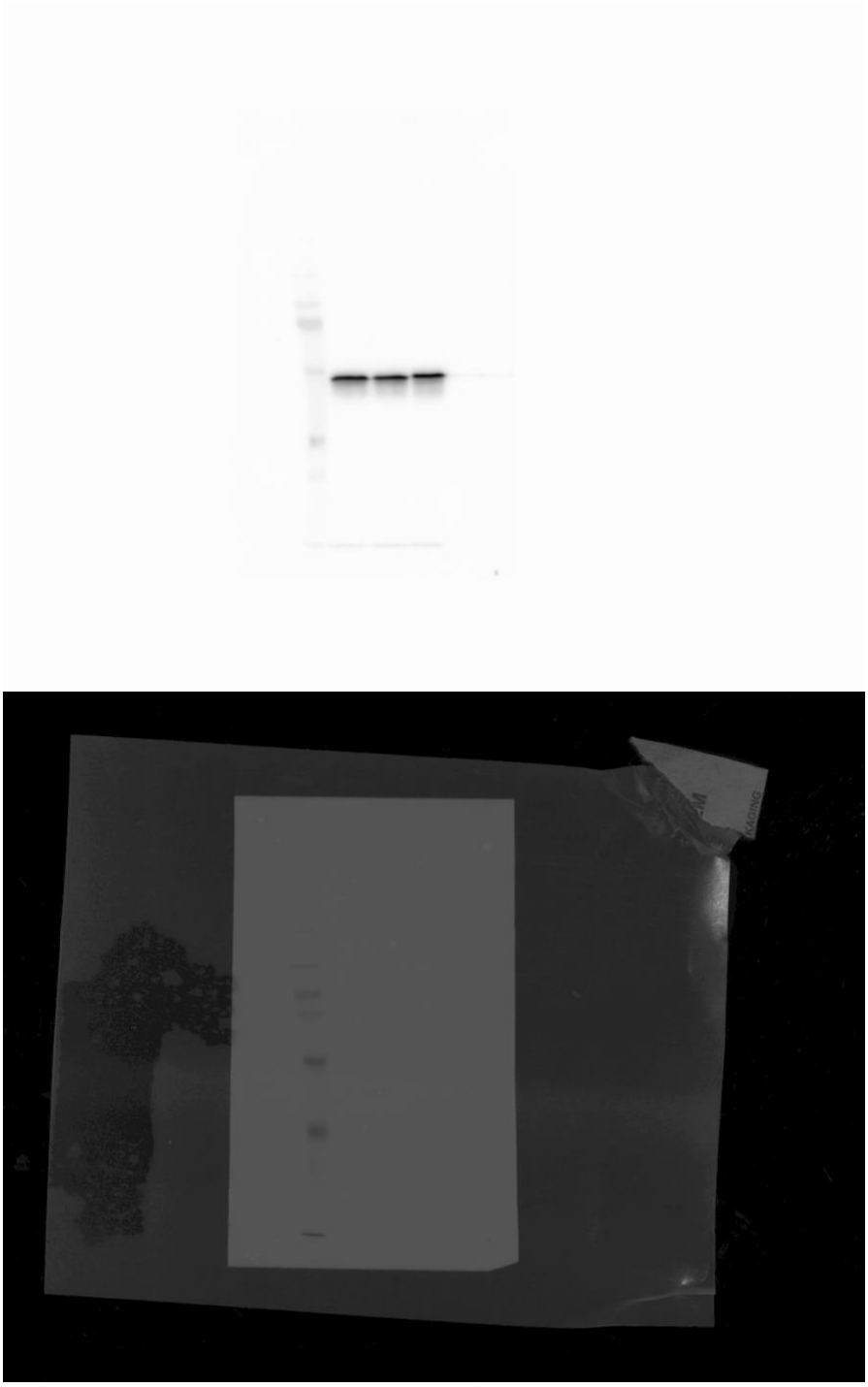


Figure 5E:
Anti-His



Anti-SigA