Supplementary Information

Two broadly conserved families of polyprenyl-phosphate transporters

Ian J. Roney and David Z. Rudner*

Affiliations: Department of Microbiology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115

*Correspondence to: rudner@hms.harvard.edu

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Supplemental Fig. 2 Multiple sequence alignments of DedA (UptA) and PopT homologs that provide MX2401 resistance. (a) Multiple sequence alignment of DedA (UptA) homologs that provide MX2401 resistance. Two highly conserved arginines are boxed in black and two membrane re-entrant helices that are commonly found in membrane embedded transporters[13] are boxed in red and blue. **(b)** Multiple sequence alignment of DUF368 (PopT) homologs that provide MX2401 resistance. Homologous re-entrant helices that are commonly found in membrane embedded transporters[13] are boxed in red and blue.

Supplemental Fig. 3 DUF368 is broadly conserved in bacteria and archaea. Dendrograms highlighting the distribution of DUF368 domains in a broad range of bacterial **(a)** and archaeal **(b)** species. All members of the pfam04018 (DUF368) were mapped onto representative phylogenetic trees.

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Strain constructions

***B. subtilis* deletion mutants**

Most *B. subtilis* deletion mutants were made by isothermal assembly[54] followed by direct transformation. The assembly reactions contained three PCR products: two PCR products containing ~1500 base pairs upstream and downstream of the gene to be deleted, and a third PCR product containing an antibiotic resistance cassette. Antibiotic resistance cassettes with surrounding lox66/lox71 sites were amplified from pWX465(cat), pWX466(spec), pWX467(erm), pWX469(tet) and pWX470(kan) using the primers oJM028 and oJM029. The flanking regions for the respective deletions were amplified using PY79 genomic DNA as template and the following primer sets: *bceAB*(oIR626-629); *yngC* (oIR483-486); *ykoX*(oIR487-490); *ybfM*(oIR491-494); *yhjE*(oIR549-552); *yqeD* (oIR553-556); *ytxB*(oIR557-560); *uppS*(oIR447/oIR467,oIR468/oIR450); *ispH*(oIR733-736); *uppP*(oIR419-422); mraY(oIR344-347); murG(oIR388-391). The *bcrC* deletion was from the BKE collection and was backcrossed twice into PY79 and PCR confirmed.

Construction of the Δ6 mutant BIR712 [Δ*yngC*, Δ*ykoX*, Δ*ybfM*, Δ*yhjE*, Δ*yqeD*, Δ*ytxB*]

A Δ3 strain, BIR656 [*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ybfM::tet*] was made by successive transformations of isothermal assembly products to delete yngC, ykoX and ybfM with lox66/71 flanked erm, kan and tet resistance cassettes respectively. The antibiotic resistance cassettes were then looped out from the Δ3 strain using pDR244 [48]. The remaining three deletions of yhjE::kan, yqeD::erm and ytxB::tet were made by successive rounds of isothermal assembly and transformation to create BIR712 (*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-YFP (cat), yngC::lox72, ykoX::lox72, ybfM::lox72, yhjE::kan, yqeD::erm, ytxB::tet)*.

Construction of the P*yngC*(ΔP_{sigM}) mutant by allelic exchange

pIR361 [Pmad3.1-PyngC(ΔPsigM)(erm)(amp)] was passaged through a recA+ *E. coli* strain (AB1157) and transformed into PY79. A transformant obtained at 37˚C was grown overnight at 22˚C and then serial dilutions plated on LB agar at 37˚C. Single colonies were streaked onto LB and LB+MLS to identify strains that had looped out the integrated plasmid. The yngC promoter was PCR amplified from MLS(S) strains and sequenced confirm the promoter deletion.

Construction of *yngC* point mutations

Point mutations in yngC-his10 were made by isothermal assembly and direct transformation into *B. subtilis*. Two DNA fragments were amplified using the genomic DNA of BIR1271 [ycgO-Phyperspank-yngC-his10-spec-ycgO] as a template using oligos flanking the upstream and downstream homology arms (oIR999 and oIR1000) and mutation specific primers (R112A = oIR987/oIR988, R118A = oIR989/oIR990, R112A,R118A = oIR991/oIR992). The two resulting amplification products were purified and added to the isothermal assembly reaction followed by direct transformation into BIR648. All mutants were confirmed by sequencing.

Construction of deletion mutants of *uptA*(*Sa*), *popT*(*Sa*) and SAOUHSC_0091 in *S. aureus*

BIR683 [Δ02816::spec], BIR688 [Δ00846::kan], BIR1476[ΔSAOUHSC_00901] were made by allelic replacement of coding regions with antibiotic resistance cassettes (or a short scar in the case of SAOUHSC_0091) using a loop-in-loop-out approach as has been previously described [55] with minor modifications. Briefly, pMad based plasmids (pIR237[pMAD3.1-02816::spec(erm)(amp)], pIR238 [pMAD3.1-00846::kan(erm)(amp)] or pIR445[pMAD3.1-00901(erm)(amp)]) were electroporated into ATP001 (RN4220 WT) and selected on TSB agar + erm at 30C. Single colonies were inoculated in TSB + erm for 24 hours at 30C with two 1/100 back-dilutions. Serial dilutions were made of the cultures and aliquots were plated on TSA + erm at 42˚C to lose unintegrated plasmids. Colonies were inspected for the expression of mScarlet which is constitutively expressed from the plasmid. Pink colonies, indicating plasmid integration, were selected and inoculated in TSB and grown without antibiotics at 30 ˚C for 24 hours with two 1/100 back-dilutions. Serial dilutions were made of the cultures and aliquots were plated on TSA at 30 ˚C. White colonies were streaked on TSA, TSA + erm and (TSA or TSA + spec or kan) to confirm the integration of the deletion cassette and loss of plasmid. The deletion mutants were confirmed by PCR with primers flanking the target gene.

Construction of complementation strains

BIR691 [attB::Ptet-02816 (cat)] and BIR695 [attB::Ptet-00846 (cat)] were made by electroporating pIR239 [attB::Ptet-02816 (cat)(amp)] or pIR240 [attB::Ptet-00846 (cat)(amp)] into a L54a integrase expressing strain RN4220 (pTP044) and selecting on TSA + Cm at 42 ˚C to lose the integrase expressing plasmid. PIR239 and pIR240 were integrated into the attB (L54a) site within the *geh* gene. The correct integration was confirmed by PCR.

BIR715 [Δ02816::spec, Δ00846::kan] was constructed by phage transduction of the 846::kan deletion into BIR683[2816::spec] using phage 80alpha. PCR was performed to confirm the transduction with PCR primers flanking the deleted genes.

BIR1279 [[Δ02816::spec, Δ00846::kan, attB::Ptet-02816 (cat)], BIR1280 [[Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat)] were constructed by successive rounds of phage transduction of the 2816::spec and 846::kan markers respectively. The gene deletions were confirmed by PCR.

BIR1478 [Δ00901::scar, Δ02816::spec, Δ00846::kan, attB::Ptet-02816 (cat)], BIR1481 [Δ00901::scar, Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat)] were constructed by allelic exchange of pIR445 which had been electroporated into BIR1279 or BIR1281. The strains were grown in aTc the entire time and the deletion was confirmed by PCR.

Plasmid Constructions

pIR233 [ycgO::Phyperspank-bcrC (spec) (amp)]

pIR233 was generated in a two-piece ligation with PCR product containing the bcrC gene (amplified from PY79 gDNA with oIR374 and oIR375) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR234 [ycgO::Phyperspank-uppP (spec) (amp)]

pIR234 was generated in a two-piece ligation with PCR product containing the uppP gene (amplified from PY79 gDNA with oIR376 and oIR377) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR224 [ycgO::Phyperspank-yngC (spec) (amp)]

pIR224 was generated in a two-piece ligation with PCR product containing the yngC gene (amplified from PY79 gDNA with oIR496 and oIR497) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR226 [ycgO:: Phyperspank-ykoX (spec) (amp)]

pIR226 was generated in a two-piece isothermal assembly reaction with PCR product containing the ykoX gene (amplified from PY79 gDNA with oIR499 and oIR500) and pCB090 [ycgO::Phyperspank(spec)] digested with HIndIII and SpeI.

pIR239 [attB::Ptet-02816 (cat) (amp)]

pIR239 was generated in a two-piece ligation with PCR product containing the SAOUHSC_02816 gene (amplified from RN4220 gDNA with oIR534 and oIR535) and pTB005[attB::Ptet-02816] digested with KpnI and EcoRI.

pIR240 [attB::Ptet-00846 (cat) (amp)]

pIR240 was generated in a two-piece isothermal assembly reaction with PCR product containing the SAOUHSC_00846 gene (amplified from RN4220 gDNA with oIR536 and oIR537) and pTB005[attB::Ptet-02816] digested with KpnI and EcoRI.

pIR237 [Pmad3.1-2816::spec (amp)]

pIR237 was generated in a four-piece isothermal assembly reaction with PCR products flanking the SAOUHSC_02816 gene (amplified from RN4220 gDNA with oIR522/oIR523 and oIR526/oIR527), a PCR product encoding the spectinomycin resistance cassette (amplified from pWX466 with oIR524/oIR525) and pMR091[Pmad3.1 (amp)] digested with BamHI and EcoRI.

pIR238 [Pmad3.1-846::kan (amp)]

pIR237 was generated in a four-piece isothermal assembly reaction with PCR products flanking the SAOUHSC_00846 gene (amplified from RN4220 gDNA with oIR528/oIR529 and oIR532/oIR533), a PCR product encoding the kanamycin resistance cassette (amplified from pWX470 with oIR530/oIR531) and pMR091[Pmad3.1 (amp)] digested with BamHI and EcoRI.

pIR400 [ycgO::Phyperspank-popT(Sp) (spec) (amp)]

pIR226 was generated in a two-piece isothermal assembly reaction with PCR product containing the SPD_0872 gene (amplified from S.pneumoniae D39 gDNA with oIR1132 and oIR1133) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR388 [ycgO::Phyperspank-popT(Vc) (spec) (amp)]

pIR226 was generated in a two-piece isothermal assembly reaction with PCR product containing the MS6_A0029 gene of V. cholerae C6706 (amplified from gBlock Vc.popT with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR399 [ycgO::Phyperspank-popT(Bc) (spec) (amp)]

pIR226 was generated in a two-piece isothermal assembly reaction with PCR product containing the BC5158 gene of B. cereus atcc14579 (amplified from gDNA with oIR1130 and oIR1131) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR397 [ycgO::Phyperspank-uptA(Pa) (spec) (amp)]

pIR397 was generated in a two-piece isothermal assembly reaction with PCR product containing the PA4029 gene of P.aeruginosa PAO1 (amplified from gBlock Pa.uptA with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR393 [ycgO::Phyperspank-popT(Bb) (spec) (amp)]

pIR393 was generated in a two-piece isothermal assembly reaction with PCR product containing the BBUBOL26_RS01045 gene of B. burgdorferi (amplified from gBlock Bb.popT with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR405 [ycgO::Phyperspank-uptA(Bc) (spec) (amp)]

pIR405 was generated in a two-piece isothermal assembly reaction with PCR product containing the BC5040 gene of B. cereus atcc14579 (amplified from gDNA with oIR1148 and oIR1149) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR411 [ycgO::Phyperspank-popT(Ef) (spec) (amp)]

pIR411 was generated in a two-piece isothermal assembly reaction with PCR product containing the gene of E. faecium 13.SD.W.09 (amplified from gBlock Ef.popT with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR401 [ycgO::Phyperspank-popT(Cg) (spec) (amp)]

pIR401 was generated in a two-piece isothermal assembly reaction with PCR product containing the CGP_RS06655 gene of C. glutamicum MB001 (amplified from gDNA with oIR1134 and oIR1135) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR211 [ycgO::Pspank-uppS (spec) (amp)]

pIR211 was generated in a two-piece isothermal assembly reaction with PCR product containing the uppS gene (amplified from PY79 gDNA with oIR439 and oIR440) and pCB084 [ycgO::Pspank(spec)] digested with HindIII and SpeI.

pIR278 [ycgO::Pspank-ispH (spec) (amp)]

pIR278 was generated in a two-piece isothermal assembly reaction with PCR product containing the ispH gene (amplified from PY79 gDNA with oIR737 and oIR738) and pCB084 [ycgO::Pspank(spec)] digested with HindIII and SpeI.

pIR235 [ycgO::Phyperspank-yghB (spec) (amp)]

pIR235 was generated in a two-piece ligation with PCR product containing the yghB gene (amplified from MG1655 gDNA with oIR518 and oIR519) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI. The resulting plasmid was sequence confirmed.

pIR236 [ycgO::Phyperspank-yqjA (spec) (amp)]

pIR236 was generated in a two-piece ligation with PCR product containing the yqjA gene (amplified from MG1655 gDNA with oIR520 and oIR521) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR403[ycgO::Phyperspank-dedA (spec) (amp)]

pIR403 was generated in a two-piece isothermal assembly reaction with PCR product containing the dedA gene (amplified from MG1655 gDNA with oIR1144 and oIR1145) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR406 [ycgO::Phyperspank-yabI (spec) (amp)]

pIR406 was generated in a two-piece isothermal assembly reaction with PCR product containing the yabI gene (amplified from MG1655 gDNA with oIR1180 and oIR1181) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR407 [ycgO::Phyperspank-yohD (spec) (amp)]

pIR407 was generated in a two-piece isothermal assembly reaction with PCR product containing the yohD gene (amplified from MG1655 gDNA with oIR1182 and oIR1183) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR408 [ycgO::Phyperspank-ydjX (spec) (amp)]

pIR408 was generated in a two-piece isothermal assembly reaction with PCR product containing the ydjX gene (amplified from MG1655 gDNA with oIR1184 and oIR1185) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR409 [ycgO::Phyperspank-ydjZ (spec) (amp)]

pIR408 was generated in a two-piece isothermal assembly reaction with PCR product containing the ydjZ gene (amplified from MG1655 gDNA with oIR1186 and oIR1187) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR410 [ycgO::Phyperspank-yqaA (spec) (amp)]

pIR408 was generated in a two-piece isothermal assembly reaction with PCR product containing the yqaA gene (amplified from MG1655 gDNA with oIR1188 and oIR1189) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR385 [amyE::PyngC-yfp (cat) (amp)]

pIR385 was generated in a three-piece isothermal assembly reaction with PCR product containing the yfp gene with the yngC promoter in the primer overhang (amplified from pAM155 with oIR1105 and oIR1106) and pDG364 [amyE::cat] (PCR linearized with oIR1107 and oIR1108).

pIR361 [Pmad3.1-PyngC(ΔPsigM) (erm) (amp)]

pIR361 was generated in a three-piece isothermal assembly reaction with PCR products flanking the sigM promoter of yngC (oIR1031/oIR1032 and oIR1033/oIR1034) and pMR091 digested with BamHI and EcoRI.

pIR392 [ycgO::Phyperspank-uptA(Ab) (spec) (amp)]

pIR392 was generated in a two-piece isothermal assembly reaction with PCR product containing the AB17975 gene of *A. baumanni* (amplified from gBlock Ab.uptA with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR352 [ycgO::Phyperspank-yngC-his10 (spec) (amp)]

pIR352 was generated in a two-piece ligation with PCR product containing the yngC gene (amplified from PY79 gDNA with oIR496 and oIR986) and pIR301 [ycgO::Phyperspank-MCS-his10(spec)] digested with HIndIII and SpeI.

pIR451 [ycgO::Phyperspank-dedA(BS18575)(spec)(amp)]

pIR451 was generated in a two-piece isothermal assembly reaction with PCR product containing the B18575 gene of *B. simplex* (amplified from gBlock BS18575 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR455 [ycgO::Phyperspank-dedA(BS19690)(spec)(amp)]

pIR455 was generated in a two-piece isothermal assembly reaction with PCR product containing the B19690 gene of *B. simplex* (amplified from gBlock BS19690 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR456 [ycgO::Phyperspank-dedA(BS19110)(spec)(amp)]

pIR456 was generated in a two-piece isothermal assembly reaction with PCR product containing the B19110 gene of *B. simplex* (amplified from gBlock BS19110 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR457 [ycgO::Phyperspank-dedA(BS15480)(spec)(amp)]

pIR457 was generated in a two-piece isothermal assembly reaction with PCR product containing the B15480 gene of *B. simplex* (amplified from gBlock BS15480 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR458 [ycgO::Phyperspank-dedA(BS9915)(spec)(amp)]

pIR458 was generated in a two-piece isothermal assembly reaction with PCR product containing the BS9915 gene of *B. simplex* (amplified from gBlock BS9915 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR459 [ycgO::Phyperspank-dedA(BS7270)(spec)(amp)]

pIR459 was generated in a two-piece isothermal assembly reaction with PCR product containing the BS7270 gene of *B. simplex* (amplified from gBlock BS7270 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR460 [ycgO::Phyperspank-dedA(BS6870)(spec)(amp)]

pIR460 was generated in a two-piece isothermal assembly reaction with PCR product containing the BS6870 gene of *B. simplex* (amplified from gBlock BS6870 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR461 [ycgO::Phyperspank-dedA(BS6295)(spec)(amp)]

pIR461 was generated in a two-piece isothermal assembly reaction with PCR product containing the BS6295 gene of *B. simplex* (amplified from gBlock BS6295 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR449 [ycgO::Phyperspank-dedA(PC16690)(spec)(amp)]

pIR449 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC16690 gene of *P. cellulosyliticus* (amplified from gBlock PC16690 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR462 [ycgO::Phyperspank-dedA(PC28990)(spec)(amp)]

pIR462 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC28990 gene of *P. cellulosyliticus* (amplified from gBlock PC28990 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR463 [ycgO::Phyperspank-dedA(PC28030)(spec)(amp)]

pIR463 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC28030 gene of *P. cellulosyliticus* (amplified from gBlock PC28030 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR464 [ycgO::Phyperspank-dedA(PC16380)(spec)(amp)]

pIR464 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC16380 gene of *P. cellulosyliticus* (amplified from gBlock PC16380 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR465 [ycgO::Phyperspank-dedA(PC15755)(spec)(amp)]

pIR465 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC15755 gene of *P. cellulosyliticus* (amplified from gBlock PC15755 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR466 [ycgO::Phyperspank-dedA(PC7440)(spec)(amp)]

pIR466 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC7440 gene of *P. cellulosyliticus* (amplified from gBlock PC7440 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR467 [ycgO::Phyperspank-dedA(PC5445)(spec)(amp)]

pIR467 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC5445 gene of *P. cellulosyliticus* (amplified from gBlock PC5445 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR468 [ycgO::Phyperspank-dedA(PC4905)(spec)(amp)]

pIR468 was generated in a two-piece isothermal assembly reaction with PCR product containing the PC4905 gene of *P. cellulosyliticus* (amplified from gBlock PC4905 with oIR1128 and oIR1129) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR448 [ycgO::Phyperspank-SAOUHSC_00901(spec)(amp)]

pIR468 was generated in a two-piece isothermal assembly reaction with PCR product containing the SAOUHSC_00901 gene of *S. aureus* (amplified from RN4220 genomic DNA with oIR1367 and oIR1368) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR232 [ycgO::Phyperspank-SAOUHSC_00846(spec)(amp)]

pIR232 was generated in a two-piece isothermal assembly reaction with PCR product containing the SAOUHSC_00846 gene of *S. aureus* (amplified from RN4220 genomic DNA with oIR506 and oIR507) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR230 [ycgO::Phyperspank-SAOUHSC_02816(spec)(amp)]

pIR230 was generated in a two-piece ligation reaction with PCR product containing the SAOUHSC_02816 gene of *S. aureus* (amplified from RN4220 genomic DNA with oIR504 and oIR505) and pCB090 [ycgO::Phyperspank(spec)] digested with HindIII and SpeI.

pIR445 [pMad3.1-SAOUHSC_00901::scar]

pIR445 was generated in a three-piece isothermal assembly reaction with PCR products flanking the SAOUHSC_00901 gene (amplified from RN4220 gDNA with oIR1348/oIR1349 and oIR1350/oIR1351) and pMR091[Pmad3.1 (amp)] digested with BamHI and EcoRI.

pIR441 [Plow-uppS(ermR)(amp)]

pIR441 was generated in a two-piece ligation reaction with PCR product containing uppS (SAOUHSC_01237) gene of *S. aureus* (amplified from RN4220 gDNA with oIR1326 and oIR1327 and pLow digested with BamHI and EcoRI.

pIR442 [Plow-murAA(ermR)(amp)]

pIR442 was generated in a two-piece ligation reaction with PCR product containing murAA (SAOUHSC_02337) gene of *S. aureus* (amplified from RN4220 gDNA with oIR1332 and oIR1333 and pLow digested with BamHI and EcoRI.

All plasmids were sequence confirmed. All gene fusions to the Phyperspank promoter contained a synthetic optimized ribosome binding. All gene fusions to the Pspank promoter contained the native ribosome binding site.

Supplementary Table 1 Minimum inhibitory concentrations of *B. subtilis* (WT/Δ2) and *S. aureus* (WT/Δ2).

Antibiotic	***S. aureus* fold change (WT/Δ2)**	***B. subtilis* fold change (WT/Δ2)**
MX2401	256	8
Tunicamycin	128-512	1
Daptomycin	1	1
Vancomycin	1-2	1
Teicoplanin	4	1
Bacitracin	8	1-2
Fosfomycin	1	1
Penicillin G	2	1
Ampicillin	2	2
D-cycloserine	2	1
Tetracycline	2	1
Chloramphenicol	2	1
Nalidixic acid	1	1

Supplementary Table 2a Homologies between UptA from *B. subtilis* and *S. aureus* and the 8 *E. coli* DedA paralogs.

DedA member that provides MX2401 resistance	*E.coli* DedA paralogs	E-Value
YngC (UptA^{Bs}) (*Bacillus subtilis* PY79)	DedA (UptA^{Ec})	9e-13
	YabI	8e-12
	YohD	3e-11
	YqjA	9e-08
	YghB	2e-06
	YdjZ	--------
	YdjX	--------
	YqaA	--------
SAOUHSC_002816 (UptA^{Sa}) (*Staphylococcus aureus* NCTC8325)	DedA (UptA^{Ec})	1e-13
	YqjA	3e-12
	YghB	1e-08
	YdjZ	6e-06
	YabI	3e-06
	YohD	1e-06
	YqaA	0.002
	YdjX	------

Supplementary Table 2b Homologies between the DedA family members in *B. thailendensis, B. glumae,* and *K. pneumoniae* and the 8 *E. coli* DedA paralogs.

DedA member that provides colistin resistance	*E.coli* DedA paralogs	E-Value
DbcA (*Burkholderia thailendensis E264*)	DedA ($UptA^{Ec}$)	2e-71
	YqjA	5e-34
	YghB	4e-28
	YabI	2e-12
	YohD	1e-10
	YdjZ	1e-06
	YdjX	--------
	YqaA	--------
DbcA (*Burkholderia glumae*)	DedA ($UptA^{Ec}$)	6e-72
	YqjA	2e-37
	YghB	5e-30
	YohD	5e-12
	YabI	2e-12
	YdjZ	7e-07
	YdjX	4e-05
	YqaA	--------
DkcA (*Klebsiella pneumoniae* ST258)	DedA ($UptA^{Ec}$)	4e-145
	YqjA	2e-28
	YghB	2e-24
	YabI	2e-15
	YohD	3e-14
	YdjZ	4e-09
	YdjX	---------
	YqaA	----------
NMB1052 (*Neisseria meningitidis* NMB)	DedA ($UptA^{Ec}$)	6e-107
	YqjA	1e-29
	YghB	3e-27
	YabI	4e-15
	YohD	1e-12
	YdjZ	4e-07
	YqaA	2e-04
	YdjX	--------

Supplementary Table 3 Uniprot IDs for the proteins included in gene neighborhood analyses

Neighborhood analysis	Organism	Uniprot ID
Figure 3A	*B. smithii*	A0A0H4PDF0
	B. simplex	A0A127DB63
	C. baratii	A0A0A7FUF6
	C. drakei	A0A2U8DT63
	B. nakamurai	A0A150F9V4
	B. megaterium	D5DCB1
	P. aeruginosa	Q9HX16
Figure 3A	*H. salinarum*	Q9HQP1
	H. volcanii	A0A558GE34
	S. rubrum	A0A2I8VIS1
	H. walsybi	U1PMD6
	H. borinquense	A0A6G9MFC0
	H. inordinatus	A0A1I2PR27
Extended data fig. 5a	*L. pentosiphilus*	A0A1Z5IL31
	L. paracollinoides	A0A1B2IX91
	L. suebicus	A0A0R1W5I3
	L. casei	K6Q4L5
	P. sp. Soil766	A0A0Q9PMV8
	P. whitsoniae	A0A430JDZ5
	P. polymyxa	E3E4F9
	S. scp. 75	A0A3D9NMI5
Extended data fig. 5b	*B. glycinfermentans*	A0A1C3SHT6
	B.shackletonii	A0A0Q3WW54
	P. putida	R9V9H3
	P. indica	A0A1G9ATL5
	P. kribbensis	A0A4Y8VBJ8
	P. pectinilyticus	A0A1C1A1K2
	P. ginsengarvi	A0A3B0CM75
	P. ferrarius	A0A1V4HKY6
Extended data fig. 5d	*S. nanhensis*	A0A1E7LPD7
	S. carminius	A0A2M8M1J9
	S. filamentosus	D6AIM5
	S. spTverLS-915	A0A1C4J7J3
	S. himastatinicus	D9WFT4
	P. odorifer	A0A1R0ZHQ8
	P. cellulosilyticus	A0A2V2YUK8
	P. macerans	A0A090ZGU8
Extended data fig. 6a	*H. volcanii*	D4GYH6
Extended data fig. 6c	*H. desulfuricum*	A0A343TMB0
	H. persicus	A0A1H3MBV2
	H. salinus	A0A368ND00
	H. spMBLA0076	A0A6A8GHZ9
	H. limi	A0A1I6GGR4
	H. aquaticum	A0A1I3B2G6
	H. elongans	M0HQX5
	H. spCB1230	A0A7D5C923

Supplementary Table 4 Strains used in this study

Strain	***Background***	**Genotype**	**Source**	**Figure**
ATP001	*S. aureus* RN4220	*wild-type*	[1]	2cde,4a, ED4bc,ED7c, ED9abc, ED10a
BDR2660	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo)*	Unpublished	4a,ED7c
BIR003	*B. subtilis PY79*	*wild-type*	[2]	2a,ED2h, ED7ab, ED8ab, ED10b
BIR019	*B. subtilis PY79*	*Δ(sigM-yhdL-yhdK)::erm*	This study	ED2h
BIR0334	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat)*	This study	2bf,4bc,ED2b, ED3a
BIR0634	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ycgO::Pspank-uppS (spec), uppS::tet*	This study	ED3a
BIR0644	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm*	This study	2b,ED2h, ED3ac
BIR0645	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ykoX::kan*	This study	2b,ED3c
BIR0646	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ybfM::tet*	This study	ED3c
BIR0648	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan*	This study	2bf,4bc,ED2b
BIR0650	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ycgO::Phyperspank-yngC (spec)*	This study	2b
BIR0672	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::tet*	This study	ED3d
BIR0683	*S. aureus* RN4220	*Δ02816::spec*	This study	2cd
BIR0688	*S. aureus* RN4220	*Δ00846::kan*	This study	2cd
BIR0691	*S. aureus* RN4220	*attB::Ptet-02816 (cat)*	This study	2c
BIR0695	*S. aureus* RN4220	*attB::Ptet-00846 (cat)*	This study	2c

BIR0712	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::lox72, ykoX::lox72, ybfM::lox72, yhjE::kan, yqeD::erm, ytxB::tet*	This study	2b
BIR0715	*S. aureus* RN4220	*Δ02816::spec, Δ00846::kan*	This study	2cde, 4abc,ED7c, ED9abc, ED10a
BIR0769	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), bceAB::kan*	This study	ED9e
BIR0852	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ycgO::Pspank-ispH (spec), ispH::kan*	This study	ED3a
BIR1113	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yngC (spec)*	This study	2f,4b,ED2b
BIR1114	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-ykoX (spec)*	This study	2f
BIR1116	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-02816 (spec)*	This study	2f
BIR1117	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-00846 (spec)*	This study	2f
BIR1118	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yghB(Ec) (spec)*	This study	ED9d
BIR1119	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yqjA(Ec) (spec)*	This study	ED9d
BIR1120	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yngC-his10 (spec)*	This study	ED2bc

BIR1132	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan,ycgO::Phyperspank-yngC(R112A)-his10 (spec)*	This study	ED2bc
BIR1133	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yngC(R118A)-his10 (spec)*	This study	ED2bc
BIR1134	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yngC(R112A,R118A)-his10 (spec)*	This study	ED2bc
BIR1172	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan*	This study	ED8ab,ED9ac, ED10b
BIR1186	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-yngC(spec)*	This study	4a,ED7c, ED8ab
BIR1187	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-00846 (spec)*	This study	4a,ED7c, ED8ab
BIR1191	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-02816 (spec)*	This study	ED8c
BIR1193	*B. subtilis PY79*	*sacA::Pveg-mCherry (phleo), ykoX::kan, yngC::erm*	This study	4a,ED8cd, ED9b
BIR1200	*B. subtilis PY79*	*yngC::tet*	This study	ED8ab
BIR1211	*B. subtilis PY79*	*(PsigM-Loopout)-yngC*	This study	ED2h
BIR1218	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyper-uptA(A.baumanii) (spec)*	This study	4b
BIR1219	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-popT(B.burgdorferi) (spec)*	This study	4c
BIR1227	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-popT(C.glutamicum) (spec)*	This study	4c
BIR1232	*B. subtilis PY79*	*amyE::PyngC-optRBS-(ATG)-yfp (cat), sacA::Pveg-mTag-BFP(phleo)*	This study	ED2fg

BIR1233	*B. subtilis PY79*	*amyE::PyngC-optRBS-(ATG)-yfp (cat), Δmlk::erm, sacA::Pveg-mTag-BFP(phleo)*	This study	ED2fg
BIR1252	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ycgO::Pspank-uppS(spec), uppS::tet, yngC::kan*	This study	ED3a
BIR1259	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ycgO::Pspank-ispH (spec), ispH::kan, yngC::tet*	This study	ED3a
BIR1262	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), bceAB::kan, bcrC::erm*	This study	ED9e
BIR1263	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), bceAB::kan, uppP::erm*	This study	ED9e
BIR1266	*B. subtilis PY7*	*bceAB::kan, yngC::tet*	This study	ED9e
BIR1267	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-dedA(E.coli) (spec)*	This study	4b, ED8c
BIR1269	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-uptA(B.cereus) (spec)*	This study	4b
BIR1270	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-popT(E.faecium) (spec)*	This study	4c
BIR1273	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yabI(E.coli) (spec)*	This study	ED9d
BIR1274	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yohD(E.coli) (spec)*	This study	ED9d
BIR1275	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-ydjX(E.coli) (spec)*	This study	ED9d

BIR1276	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-ydjZ(E.coli) (spec)*	This study	ED9d
BIR1277	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yngC::erm, ykoX::kan, ycgO::Phyperspank-yqaA(E.coli) (spec)*	This study	ED9d
BIR1279	*S. aureus* RN4220	*attB::Ptet-02816 (cat), Δ02816::spec, Δ00846::kan*	This study	2cd
BIR1280	*S. aureus* RN4220	*attB::Ptet-00846 (cat), Δ02816::spec, Δ00846::kan*	This study	2cd
BIR1300	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), bceAB::kan, ycgO::Phyperspank-bcrC (spec)*	This study	ED9e
BIR1301	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), bceAB::kan ycgO::Phyperspank-uppP (spec)*	This study	ED9e
BIR1302	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), bceAB::kan ycgO::Phyperspank-yngC (spec)*	This study	ED9e
BIR1305	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-popT(S.pneumoniae) (spec)*	This study	4c, ED8d
BIR1306	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-popT(V.cholerae) (spec)*	This study	4c, ED8d
BIR1307	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-popT(B.cereus) (spec)*	This study	4c, ED8d
BIR1308	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank - uptA(P.aeruginosa) (spec)*	This study	4b, ED8c
BIR1309	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank-dedA(E.coli) (spec)*	This study	4b

BIR1310	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yqeD::erm*	This study	ED3c
BIR1311	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), yhjE::kan*	This study	ED3c
BIR1312	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), amyE::Pamj-yfp (cat), ytxB::tet*	This study	ED3c
BIR1429	*B. subtilis PY79*	*sacA::Pveg-mCherry, yngC::erm, ycgO::Pspank-rodA-his10(spec), rodA::kan*	This study	ED3a
BIR1430	*B. subtilis PY79*	*sacA::Pveg-mCherry(phleo), yngC::erm, ycgO::Pspank-mraY, mraY::tet*	This study	D3a
BIR1431	*B. subtilis PY79*	*sacA::Pveg-mCherry (phleo), yngC::erm, ycgO::Pspank-murG(spec), murG::tet*	This study	D3a
BIR1433	*B. subtilis PY79*	*sacA::Pveg-mCherry, ycgO::Pspank-rodA-his10(spec), rodA::kan*	This study	D3a
BIR1434	*B. subtilis PY79*	*sacA::Pveg-mCherry(phleo), ycgO::Pspank-mraY, mraY::tet*	This study	D3a
BIR1435	*B. subtilis PY79*	*sacA::Pveg-mCherry (phleo), ycgO::Pspank-murG(spec), murG::tet*	This study	D3a
BIR1454	*B. subtilis PY79*	*sacA::Pveg-mCherry (phleo), ycgO::Phyperspank-yngC(spec),yngC::erm, ykoX::kan*	This study	ED9a
BIR1455	*B. subtilis PY79*	*sacA::Pveg-mCherry (phleo), ycgO::Phyperspank-SAOUHSC_00846(spec), yngC::erm, ykoX::kan*	This study	ED9a
BIR1458	*B. subtilis PY79*	*sacA::Pveg-gfp (phleo), ycgO::Phyperspank-yngC(spec),yngC::erm, ykoX::kan*	This study	ED9bc
BIR1459	*B. subtilis PY79*	*sacA::Pveg-gfp (phleo), ycgO::Phyperspank-SAOUHSC_00846(spec), yngC::erm, ykoX::kan*	This study	ED9bc
BIR1467	*S. aureus* RN4220	*pLow-empty(erm)*	This study	ED4d
BIR1468	*S. aureus* RN4220	*pLow-uppS(ermR)*	This study	ED4d
BIR1469	*S. aureus* RN4220	*pLow-murA(ermR)*	This study	ED4d

BIR1473	*S. aureus* RN4220	*Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat), pLow-empty(ermR)*	This study	ED4d
BIR1474	*S. aureus* RN4220	*Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat), pLow-uppS(ermR)*	This study	ED4d
BIR1475	*S. aureus* RN4220	*Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat), pLow-murA(ermR)*	This study	ED4d
BIR1476	*S. aureus* RN4220	*Δ00901::scar*	This study	2d
BIR1478	*S. aureus* RN4220	*Δ00901::scar, Δ02816::spec, Δ00846::kan, attB::Ptet-02816 (cat)*	This study	2d
BIR1481	*S. aureus* RN4220	*Δ00901::scar, Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat)*	This study	2d
BIR1489	*S. aureus* RN4220	*00901::scar, Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat), pLow-empty(ermR)*	This study	2g, ED4d
BIR1490	*S. aureus* RN4220	*00901::scar, Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat), pLow-uppS(ermR)*	This study	2g, ED4d
BIR1491	*S. aureus* RN4220	*00901::scar, Δ02816::spec, Δ00846::kan, attB::Ptet-00846 (cat), pLow-murA(ermR)*	This study	2g, ED4d
BIR1492	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -SAOUHSC_00901 (spec)*	This study	2g
BIR1493	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC16690) (spec)*	This study	ED5g
BIR1495	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(BS18575) (spec)*	This study	ED5f
BIR1500	*B. subtilis PY79*	*ycgO::Pspank-uppS(spec), uppS:tet*	This study	ED7ab
BIR1504	*B. subtilis PY79*	*ycgO::Phyperspank-uppS(spec)*	This study	ED7a
BIR1505	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan,*	This study	ED5f

		ycgO::Phyperspank -dedA(BS19690) (spec)		
BIR1506	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(BS19110) (spec)*	This study	ED5f
BIR1507	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(BS15480) (spec)*	This study	ED5f
BIR1508	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(BS9915) (spec)*	This study	ED5f
BIR1509	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(BS7270) (spec)*	This study	ED5f
BIR1510	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(BS6870) (spec)*	This study	ED5f
BIR1511	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(BS6295) (spec)*	This study	ED5f
BIR1512	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC28030) (spec)*	This study	ED5g
BIR1513	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC16380) (spec)*	This study	ED5g
BIR1514	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC15755) (spec)*	This study	ED5g
BIR1515	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC7440) (spec)*	This study	ED5g

BIR1516	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC5445) (spec)*	This study	ED5g
BIR1517	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC4905) (spec)*	This study	ED5g
BIR1518	*B. subtilis PY79*	*sacA::Pveg-mTagBFP (phleo), yngC::erm, ykoX::kan, ycgO::Phyperspank -dedA(PC28990) (spec)*	This study	ED5g

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1. Pang, T., Wang, X., Lim, H. C., Bernhardt, T. G. & Rudner, D. Z. The nucleoid occlusion factor Noc controls DNA replication initiation in Staphylococcus aureus. *Plos Genet* 13, e1006908 (2017).

2. Youngman, P., Perkins, J. B. & Losick, R. Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in Bacillus subtilis or expression of the transposon-borne erm gene. *Plasmid* 12, 1–9 (1984).

Supplementary Table 5 Plasmids used in this study

Plasmid	Description	Source
pAM155	amyE::Pamj-yfp(cat)(amp)	[1]
pDR244	Ppa-cre, ori(ts) (spec) (amp)	[2]
pIR211	ycgO::Pspank-uppS(spec)(amp)	This study
pIR224	ycgO::Phyperspank-yngC (spec) (amp)	This study
pIR226	ycgO::Phyperspank-ykoX (spec) (amp)	This study
pIR233	ycgO::Phyperspank-bcrC (spec) (amp)	This study
pIR234	ycgO::Phyperspank-uppP (spec) (amp)	This study
pIR235	ycgO::Phyperspank-yghB(spec)(amp)	This study
pIR236	ycgO::Phyperspank-yqjA(spec)(amp)	This study
pIR237	Pmad3.1-2816::spec (erm)(amp)	This study
pIR238	Pmad3.1-846::kan (erm)(amp)	This study
pIR239	attB::Ptet-02816 (cat) (amp)	This study
pIR240	attB::Ptet-00846 (cat) (amp)	This study
pIR242	Himar1C9 IR-specPpen-IR terminators (erm)(amp)	[3]
pIR278	ycgO::Pspank-ispH (spec)(amp)	This study
pIR352	ycgO::Phyperspank-yngC-his10 (spec)(amp)	This study
pIR361	Pmad3.1-PyngC(ΔPsigM) (erm)(amp)	This study
pIR385	amyE::PyngC-yfp (cat)(amp)	This study
pIR388	ycgO::Phyperspank-popT(Vc) (spec)(amp)	This study
pIR392	ycgO::Phyperspank-uptA(Ab)(spec)(amp)	This study
pIR393	ycgO::Phyperspank-popT(Bb) (spec)(amp)	This study
pIR397	ycgO::Phyperspank-uptA(Pa) (spec)(amp)	This study
pIR399	ycgO::Phyperspank-popT(Bc) (spec)(amp)	This study
pIR400	ycgO::Phyperspank-popT(Sp)(spec)(amp)	This study
pIR401	ycgO::Phyperspank-popT(Cg) (spec)(amp)	This study
pIR403	ycgO::Phyperspank-dedA(Ec) (spec)(amp)	This study
pIR405	ycgO::Phyperspank-uptA(Bc) (spec)(amp)	This study
pIR406	ycgO::Phyperspank-yabI(Ec) (spec)(amp)	This study
pIR407	ycgO::Phyperspank-yohD(Ec) (spec)(amp)	This study
pIR408	ycgO::Phyperspank-ydjX(Ec) (spec)(amp)	This study
pIR409	ycgO::Phyperspank-ydjZ(Ec) (spec)(amp)	This study
pIR410	ycgO::Phyperspank-yqaA(Ec) (spec)(amp)	This study
pIR411	ycgO::Phyperspank-popT(Ef) (spec)(amp)	This study
pIR451	ycgO::Phyperspank-dedA(BS18575)(spec)(amp)	This study
pIR455	ycgO::Phyperspank-dedA(BS19690)(spec)(amp)	This study
pIR456	ycgO::Phyperspank-dedA(BS19110)(spec)(amp)	This study
pIR457	ycgO::Phyperspank-dedA(BS15480)(spec)(amp)	This study
pIR458	ycgO::Phyperspank-dedA(BS9915)(spec)(amp)	This study
pIR459	ycgO::Phyperspank-dedA(BS7270)(spec)(amp)	This study
pIR460	ycgO::Phyperspank-dedA(BS6870)(spec)(amp)	This study
pIR461	ycgO::Phyperspank-dedA(BS6295)(spec)(amp)	This study

pIR449	ycgO::Phyperspank-dedA(PC16690)(spec)(amp)	This study
pIR462	ycgO::Phyperspank-dedA(PC28990)(spec)(amp)	This study
pIR463	ycgO::Phyperspank-dedA(PC28030)(spec)(amp)	This study
pIR464	ycgO::Phyperspank-dedA(PC16380)(spec)(amp)	This study
pIR465	ycgO::Phyperspank-dedA(PC15755)(spec)(amp)	This study
pIR466	ycgO::Phyperspank-dedA(PC7440)(spec)(amp)	This study
pIR467	ycgO::Phyperspank-dedA(PC5445)(spec)(amp)	This study
pIR468	ycgO::Phyperspank-dedA(PC4905)(spec)(amp)	This study
pIR448	ycgO::Phyperspank-SAOUHSC_00901(spec)(amp)	This study
pIR232	ycgO::Phyperspank-SAOUHSC_00846(spec)(amp)	This study
pIR230	ycgO::Phyperspank-SAOUHSC_02816(spec)(amp)	This study
pIR445	pMAD3.1-SAOUHSC_00901::scar (erm)(amp)	This study
pIR441	pLow-Pspac-uppS(erm)(amp)	This study
pIR442	Plow-Pspac-murA(erm)(amp)	This study
pLow	Pspac-MCS, LacI (erm)(amp)	[4]

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Supplementary Table 7 gBlocks used in this study

gBlock	Sequence
Vc.popT	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGAATTACTTATCCACTTATTTAAAGGGTCTG GCAATGGGTGCGGCCGATGTGGTCCCAGGGGTATCCGGCGGGACGATAGCTTTTATTACGGGAATATACGACA CTTTACTTGAGAGCATTCGCCGTATAAACCCAAGTCTGCTGAAAGTGTGGAAAGCGCAAGGGTTAGCGGGTGT TTTTCGGCATATCAATGGGCTGTTTCTCATCACGTTGTTCGGCGGGATCTTTACTTCTATTGCTACTTTAGCCAAG TTAATTAGCTGGCTCTTAGTTACCCATCCGATTCCGATTTGGAGTTTCTTCTTTGGTCTGATACTGGTTTCCGTAT GGCACATGTTGCGTCAAATCGAACAGAAGAAGCTCTCCCGGCTCCTGTGGTTAATAGCTGGTGCAATTTTCGCT TACGGTATAACTGTGTTAAAACCATTGCATTTGGAACCAACCTACATAAACGTTCTGATTTCCGGCGCAATAGCA ATATGTGCAATGATTTTACCAGGAATTAGTGGTTCCTTCATTCTTTTACTGATAGGTATGTACGCCCCAGTTTTGG GGGCCGTTAAGACTTTCCAGCTCGACATACTCCTGATCTTTCTCACTGGGTGTGTAATTGGCCTGCTCAGTTTTTC TCATATCTTAAGCTGGCTCCTCCGCAGATATCGTGACGTTACACTCACTTTCTTAACGGGTTTGATGTTGGGAAC TCTGCCAAAGATCTGGCCGTGGAAAGAAACGCTGTCTTGGCGGGTAAATTCAAGCGGAGAACAAGTGCCTCTT CTTCAACGCAATCTCAGTCCATTCGAATTTGAGACTCTGACTAGCCAACCTTCCCAGTGGTTGCTTGCGTTAGTC CTTATGCTTGCAGCAGTGGCGCTTGTGCTTGGTTTGGAGAAATATGCTGAAAAATAAACTAGTaaCTGCAGatGC TAGCtcGCATGC
Pa.uptA	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGGATTTCAATCCTATCGACTTGATATTGCACT TAGACACCTATCTTGCCATGCTTGTTAGTAATTATGGCGTCTGGATATATGCGATATTGTTTCTGGTTATTTTTTG CGAGACGGGTCTCGTGGTGACACCGTTCCTGCCTGGCGATTCTTTGCTCTTTATAGCGGGCGCTATATGTGCTA CGGGAGGCATGGACCCTTGGCTTCTGGGCGGACTTTTAATGGTTGCGGCCATAACCGGAGATAGCACTAATTA CGTCATCGGACGGACATTGGGGAAAAGACTTTTTTCTAATCCTGATAGCAAAGTTTTTAGACGTGATTATTTAGA CCGCACCCATGAGTTTTATGAGCGGCATGGAGGGAAGACCGTTACCTTAGCCCGCTTCCTCCCAATAGTTAGAA CATTTGCGCCTTTTGTCGCCGGAATGGCCAAGATGCACTACCCAAGATTCGTAATGTTTTCTGTAGTGGGCACC GTGGCTTGGGTCGGTGGCTTGGTCACTTTAGGGTACTTTTTCGGGAATGTGCCTTTCATCAAGAAGAACTTATC CCTTCTGGTGATAGGTATTATCCTTTTGTCTTTGCTCCCAATGATACTGGGATTCATTCGGCATCGCCTGCAAGCC AGTGCAGCAAAGCAAGCCAAGGCTCAGAGCGATTAAACTAGTaaCTGCAGatGCTAGCtcGCATGC
Bb.popT	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGTTAAATATATACATTAAAGGCATACTGCTT GGGATTGCCAACATAATACCAGGGGTCTCTGGAGGGACCCTGGCATTGATCCTTAAAATATATTACAAGATCAT TAACTCTATTAGTGAGATATTGAAGCTTACAGAGATCAAGAAGAACCTGATGTTCCTCACTATATTGGCTACTGG AATGTTGACCAGCATTCTCCTTACGGCGAAAATCTTCAAGACTTACGCTTTTGACAATGGCATTATAGAGGCGTT GCTCATCGTATTTTTTATAGGTCTTGCGTTCGGCAATATTCTGACGTTAAAAACCGAGATCAGCATCAAGGAAAT TAACAGCAACACCAAAATTCTTAATAACCTGCTTTTTTTCATTGGTATGTCTATCATAGTCTTATTCTTAATCTTGA AGGAATCAAACATCCAGTTGCAGAGCACCATCCCGAAGGATAAGAACAGTATCAAGTACTATCTTTTACTCATA AGTAGTGGGACAATATCCGGGGCAAGTATGATACTGCCTGGAATCTCCGGGTCCGCGATGTTATTGCTTCTTGG ATTCTATAAAGAGATCATATTGATTGTATCTGAGTTCAATATCATATTGATTACTATATTCGCCGCGGCAGCGAC GATGGGTATTATAACGTCAATCTTAATAATTAAAAAAATCATCGACAAACATTTGAACAACTTCATCTATCTTAG CAAGGGGTTAATCTTCGGCTCAATTCTTCAAATGATCCTTATCGTCCTTAAACTCAATTTCAAAATCGGATTTACG TCCTTCACGTCTTTAGGCACGAGTTTCATACTTGGCATTTTTATAAATAAAAAACTTGCGGAGAAGTATAAATAA ACTAGTaaCTGCAGatGCTAGCtcGCATGC
Ef.popT	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGGAGAAGCAGCGGAATGAAAGTATATTATC ACGGTTCCTGAAAGGAGTTGTTATCGCTCTTGGGTTTATACTCCCAGGCGTGTCCGGTGGGGTTCTTGCTGCGA TACTCGGCATATACGAACGGATGCTCCGTTTTATGGCACACTTGACCAAAAACTTTAAGGAAAATCTGCTGTTTT TTTTACCGGTTGGTATAGGAGGGATAGTCGGTATAGGCTTATTATCTAAACCTCTCGAGTGGCTGCTCCAGAAT TATCAAATAATAGTATTGTGGGGCTTCGCTGGGGCTATCATTGGGACTCTCCCGGCGCTGGCTAAAGAAAGTAC GCTGAAGTCAAAAAGAACAACCGGAGACCTTTTATGGTTCTTCGGTAGTATGGTCATAGGTTTCCTCTTTCTGTA CTTCATGGGAGACATCCTGGGCACTATCCCAGCAAATTTCATCGGTTTTGTTATAGCAGGAGGACTGATCGCTC TGGGCGTTTTAGTCCCTGGATTATCACCAAGCAATCTGTTACTTGTTCTTGGTTTATATGACCCTATGTTGAAAG GATTCAAATCATTCGATATAATGGGGGTGTTTCTGCCTATCGCCATCGGTGGACTCGCGGTCATGTTGCTTTTCT CAAAGTTAATGGAATGGCTGTTATCACATTACCATAGCAAAGTGTACCACTTTATACTCGGCATAGTAATCGCTA GTACAATACTTATTGTTATACCACCGGTCGCGAATTATTCCGGGTTTACCTGGGTGACTGCGGTCATAGGAATA ATCCTCTTCGTCCTCGGAGCCTGGCTCGGCTTATGGATGTCAAAGCTGGAAGAAAAGTATAAATAAACTAGTaa CTGCAGatGCTAGCtcGCATGC

Ab.uptA	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGGAACTCTTAGATTTTATACTCCACGTAGAT CAGCATCTGGCGGAATTTATAACGAACTACGGTACTTGGATTTACGGGATTCTGTTCTTGATTATCTTTGTGGAA ACTGGTCTCGTCGTAATGCCATTCCTTCCAGGCGATTCTCTGTTGTTCGCTGCTGGTGCACTTGCCGCGTCTACA GGTACTATAGATCCGTGGCTCTTAGGAATCCTCCTGTTTATAGCCGCCGTTCTCGGAGACACCGTGAACTATCAT ATAGGGCGTTTCATAGGCCCGCGCGTCTTTGAGATGAACTCACGGTTTATAAATAAGCAGCACTTAATAAAGAC GCATGAGTTCTTCGCGCGGCATGGTGGCAAAACAATTATTTTCGCTCGTTTCATACCTTTCGCCCGTACTTTCGC GCCATTCGTCGCGGGAGCGGGGAGTATGAACTATAAATTCTTCCTTACTTATAACATAATCGGCGCTTTTTGCTG GGTTGCCAGCTTTGTCACGCTCGGCTATTTATTTGGGGGCCACCCGCTGGTCAAGGACAATTTTACTCACCTTAT ATTCGGCATTATTATTATTTCAATTCTTCCTGGAGTGATAGGGTTTATCAGACAAAAACTCAAAAATCGGAAGGC GAACGTATAAACTAGTaaCTGCAGatGCTAGCtcGCATGC
BS18575	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGGAGAATTGGATCACAGAATTTATGAACGA TTTCGGCTATATGGGCATCTTTCTTTTAATTGCCCTGGAAAATATCTTTCCACCTATACCGAGCGAGGTCATATTA ACTTTTGGTGGCTTCATGACAACGACTGCCAACATGACTATAATGGGGGTGGTCATCGCCTCCACAATAGGCTC CGTGGCCGGTGCCGTAGTTTTATATGGGATTGGATTACTGTTGGACGTAAAGGTGATCGAGAAGTTCGTGGAA AGATGGGGACATATATTGCGGCTGACCGTCTCAGACGTCCACAAAGCTAATAGCTGGTTCGATAAATACGGGG CCTGGACAGTGTTTTTTTGCAGACTTGTGCCTCTGATACGCTCTCTGATCTCAATACCTGCGGGTATGTCACACA TGAGCTTTTGGCTCTTCCTCCTGTACACGACCTCTGGAACTCTGATTTGGAATATAATTCTCGTAAACATAGGGG CGGCGGTCGGCAGCTCATGGGAAGATATTGTTGGTTATATGGACATATATAGCAATATAGTGTATGCAATACTT GCGCTTCTTTTCATCACATTTGTCGTGTTGATCTTTAGACGCAATAAGGGAAAAGTATAAACTAGTaaCTGCAGat GCTAGCtcGCATGC
BS19690	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGAACGATAAACTCACCTTGGTGATGTCCTAC GTCCAGTCTGGGTCCGTTTTTGCCCCGTTAATATTTATTACGTTCCACCTCCTCCGTCAGTTCCTTTTCATTCCGGT TATCGTGGTGTGTATGTCCGGGGGCGTCTTGTTCGGCGCTGCCTTTGGCACCATTTATTCCGTCATTGGCCTCAC CTTGTCATCAATGGCATTCTATTTCGTGATCGGTAAATTCCCGAAAACGAAAGACAGACTGTTGCGCTTGAAAA ACAAGTTGTTCGGAAACCGTAAGTTAAACTCCCGCCAGATTGCGGTGCTTCGTCTGATCCCATTCATTCATTTCT ATCTTCTGAGTCTCTGTCTTTTAGAATCAAATAAAGGACTTAAGAACTACTTCTACCTTAGTTTCATGACGAATCT CCCGGTCGCGTTTGTTTATACGGTATTCGGTCATTACATCAGTGATTTCTCCCCGACTCTTATAGTTATTATCCTC CTGTCTCTCACACTCCTTTTGTATCTGTTGCGGGAAAAGCAGACTGTAGTCCCTTGGAAAGAATTTTTCAGAAGT GACGACTGAACTAGTaaCTGCAGatGCTAGCtcGCATGC
BS19110	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGGATATAGTGAAGGACTTGATTTCCAATTAT GGCTACTTTGCGATCTACGGACTCCTTGCCATCGGAATTATAGGTCTGCCTGTACCGGACGAGTTTATGATGAC GTTCGTTGGTTATTTATCATCCATCTCAGTGTTAAACGTGCAAGGGGCATTTTTAGTGTCTTTCCTCGGGAGTAT CTCAGGAATGCTCATATCATACTTCATTGGAAAAAAGGTGGGTAAGCCATTCCTCAGAAAACATGGGAAGTGG ATCAAAATGACACCTGCCAGACTGGAGAAGCTTGAGAAATGGTTTAATAAGTACGGTCCATGGACGATCATAA TAGCTTACTTCATACCTGGAGTCCGTCATTTTGCTTCTTACATCAGCGGAATGAACGGCATGGGCAAGAGAAAG TACTTCATCTTTGCCGGTGCAGGAGCCTTTTCATGGTGCCTTGTTTTTACAACATTCGGATACTTTATTGGAGTTC TTACATGAACTAGTaaCTGCAGatGCTAGCtcGCATGC

BS15480	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGAACATCGACACGTTGCTGCATTTCATAGAC AGTTACGGCTACCTCATAATCTTCTTATTCTTATTTTTCGGCATTGTCGGAGTACCTGCGCCGGAGGAGTCCCTTC TGTTTCTCATTGGGGTGTTAGTAGTCCAAGGTAAGCTCAGCATGGGGTTAGCCATGTTATGTGCTATACTTGGA GCTTTTATTGGAATGCTCGCAGCGTATGCCTGTGGTAAGTACGTCGGCTATCCTTTCATCAACAAATATGGGCG CTTCATTGGTATTACGTCTGAACGTTGGGAAAAAGCAAAAAAGAATTACACAGACAACGCTGAAAAGACATTG GTCTTAGGCTTCTACATACCTGGGATCCGGCAGATATCTCCGTACTTTGCGGGGATATCTAGCATACCATTCCGC AAATACTTCCTCTTTAGTCTGCTGGGGACTATACTTTGGACAGTTCCATTTATAGTCGCCGGATATTATGTAGGC GATGCTTTTAACGTCAACCCAAAATACGTGCCGTACCTGGGCGTTGTATTCTTAGTCATATTTGTTTTGTACGTG ACTATTAAATACTTCAAGAAACGGAAGCAGATGAGTAAATCCTAAACTAGTaaCTGCAGatGCTAGCtcGCATGC
BS9915	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGTCTAAAAACGGGGGAGGTCCGTTGAAAAA GGTTTTGACGATCATCGGGTTGCTCGTAATCGCAATCTTTATCTACTCAAACGCGGAGCTGTTTACACTCGTTTG GAAAAGCGATCTTGATTCTGTGATAGGAATATTAAAAGAAAATCTCCTGTTAACGTTCATAGTTACATTTGTCTT AATGTTCGTTCAAAACAGTTTCACGATCATACCATTGATCTTATTGTTAACGATAAACGTTACTATCTTTGGTTTC ATCTACGGGTATCTGTGGAGTTGGTTCACCTCCGTCGTGGCTAGTGGTTTCATTTTTTATGCTGTTCGGAATTGG TTCCAAGAGCTGTTACTGAAGAAGATAGGCGAAAAGTGGCAAGAGACTGTAGTGGAGCATGGGTTCATGTAC GTGTTTACGGGCCGCATTTTTCCATTGATTCCTACGAGCTTAATCAACCTTGCCGCCGGAGTGTCCACTGTGACC TTTAAGGACTTCCTGCTTGCTACTGCTTTAGGAAACTTAATTTATTTCTTTTTTTTGTCACTTATCCCTTATGGACT CCTGTCAGTAGAAATGAATCAGTATACTTTAGTAGCCTTAGCCCTCCTTACCCTGCTGTTCTTCATAATCTACAAG CGCGCGAAGAAAAAGAAAAAATTGACGTTTTTTAGAAAAAACCGCTAGACTAGTaaCTGCAGatGCTAGCtcGC ATGC
BS7270	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGAATTTCATTTCAGAAGCACTGTTGAGCTCA GGCCCATTTAGTATCGCGGTATCACTCGTGTTCAACATTCTGATAAGTGTTTTAGGCTTCATCCCTAGCGTCTTTA TAACGGCTGCGAACATTACAGTTTTTGGATTCGAGAAGGGCCTGATCCTCAGTTACATGGGCGAGATAGCAGG TGCAGTCGTGTCCTTCTGGTTATACCGTAAGGGCTTTCAGACGTTCAAACCAAAGTTCATGAAAAATCGTTGGG TTATGAAATTGCAGAAAAGCCAAGGATTCCATGCTTTCTGGATGATCCTGATGCTTCGCCTCCTTCCGTTTATTCC GTCTGGTGTTATAAATCTCACTGCGGCTCTGAGCAAGACTGGAATGATGATATTCTTTCTCGCCACGTCAATCGG CAAACTCCCGGCATTGCTTGTTGAAGCGTACTCTGTCACCCAAGTCTTGAAAGCCTCCGATGATGTTCGGATAGT CTTGGTCCTTCTTATCCTGGTGATCGCTGTCTTTTACTATTTCAACAGAAATAAAAAGAAAGGCATGTAAACTAG TaaCTGCAGatGCTAGCtcGCATGC
BS6870	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGAGTCAGCTTATGATGACCATACTTAATTGG TTTGCGGAAATGGGATACATGGGAATTTTATTAGGGCTCATGGTTGAGATATTACCATCAGAATTGGTACTTGG GTACGGAGGATACCTTGTGGGATTGGGGAAGATGAATTTCTGGGGAGCCGTTTTAGCAGGCGTTGCCGGTGG AACCATGGCTCAACTGTTCTTGTACTGGGCTGGATATTATGGTGGGCGGCCATTTCTGCTGAAATATGGGAAAT ATATTCTTATTAAGCAAAACCATATAGTAAACGCGGAAGAATGGTTTCAACGCTATGGAGTAGGCGTGATCTTC ACAGCAAGATTCATCCCTGTCGTTCGCCACGCCATTTCTATACCAGCCGGAATCGCGCGGATGTCCGTATGGAA GTTCATCTTCTACACAGTCGCTGCAGTGATCCCATGGACGATCTTATTTCTTACTCTGGGTCGGATATTGGGTGA AAACTGGCAACAAATTCGTGAAATCACTGAACCATATTTAATTCCTGCGGCAGGGTTTAGTTTTATACTGATAAT GCTGTACATATTGTGGAAAAAGAAATCAACCCCACCTATCGTCACAGTAAAGAAGATGGGTCGGTTCCCTGATA AATAGACTAGTaaCTGCAGatGCTAGCtcGCATGC

BS6295	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGGATCTTGACACAATTAGAGAGTGGTTTACT CTCGACCATATCTCCGAGTTAATTCAGCAATATCGCTCTTTTGGACCGATACCGGGAATATTATTGCCAATGTTG GAAGCATTCTTACCTTTTCTTCCACTCGTCGTTTTCGTTCTCGCAAACGCTACAGCTTTTGGGCTCTGGTGGGGGT TCTTGTTTTCATGGATTGGCGCGGTGGCTGGTTCATTCCTTATTTTTTTGGTGATTCGCCGTTACGGCCAAATGC GTTTCTTTCGTTTCCTTCAGAAGCACAAGCAAGTTCAGCGCCTCATGGTTTGGGTCGAGACCCACGGTTTTGGCC CGCTTTTTATTCTTCTCTGTTTCCCATTCACTCCAAGTGCAATTGTAAATATTGTCGCTGGGCTGTCAAAAGTTAG CCCTTTGCAGTACGGGCTCGCTGTGGTCGGCGGTAAGGCGGTTATGATTTTCACCATTTCATTTGTCGGCTACG ATTTGGTCTCCCTGATTCATAAGCCGGTACGCACCATTATAATAGGGATTGTCATATTTATTTTATGGTATGTAG GGAAACGTCTGGAAGTTAGATTGAACAAAAGCATGAAGCGCGAAGAAGGTCAGTGAACTAGTaaCTGCAGatG CTAGCtcGCATGC
PC16690	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGCATCAGCTTATAGACTGGTTCAGTCGGACC GCAATACATTTGATCGACACTTTAGGCCTCACTGGCATTTTCATCGGTATGATCCTTGAGTCAGCCTGTATCCCG ATCCCTAGTGAAGTAATTCTCCTCGGTGGTGGCGCAGCCTCTGCTTTAGGCTCCATGACCTATTGGGGAGTTGTT GCAGCCGGAGTATTCGGCAACCTCGTCGGTAGCATAATCGCATACTATATTGGTGCGCTTGGTGGGAGACGGC TTCTCGAACAATACGGCAAGTATATACTGTTCAAACAAAGTCATTATGAACAAGCGCAGAGATGGTTCGACAGA CACGGGGAACTCACTGTCCTTATAGCTCGTAACCTGCCATTTGTTCGGACGTTTATCTCATTACCTGCGGGTATT GCATCCATGCGCGTGACTAAATTCATACTTTTTACTTTTATAGGTTGTATCCCATGGAATATGGCACTCGCGTTC GCCGGATATAAGTTGGGCCAGTCTGACCAGATAGAGCGCATTTTGCATCCTCTGAGTTACGGGATTGCAGGGA TCATTTTGGTAGCAGGGCTCTTCTGGTTATTGAAAAAGCGGAGAGGCATACACTAAACTAGTaaCTGCAGatGCT AGCtcGCATGC
PC28990	AATTGTGAGCGGATAACAATTAAGCTTacataaggaggaactactATGAACAAACGCAAGCTGATTGTACGTGTGGT CGGGTACATAGCCTTTTTACTTTGTTTGTTCTATCTGATTCGGTCAAGTGGCTACACGGTCGCTGACCTTACGCC AGACACTATCCGGGAGATAGCTCACGACGATATCATTCTCATCATGTTAATTATGCTCGTTATCATGACCTTGCA AAACATATTTACATTCATACCTCTTATTTTAGTGATTACAGCCAACATCACCTTATTTGGTTTCGTTAAAGGATAC TTATACGGATGCCTTTGTTCAGTGATCGGTTCCACCATTGTTTTCCTCTCCATAAGATATTTATTCCATGATGTATT CGCGGGCAGTCCTAAGATGCAGCAATTTCAAGAAAAAATAGAGAAAAATGGTTTTACTTACGTCTTATTCGGGA GAATCCTGCCGTTAATGCCTACAAATCTTATTAACATAACATCAGGCCTCTCATCAATCCGTACCCTCCATTTCGT TACGGCCACGACAATCGGGAATATGGTCTATGGTTTGGTACTTAGCAGTCTCTCTTTTGGTTTAATCAGCGCCTC CGCGCATAATAGAGTCTATATAATTGCAACGGTAATAGTTGTACTTGCTATCGTATGGTTATATAGATTGAATAA GCGTCGCCACAAAAAGGCAGTGGAAGTCGAAATGAAATAAACTAGTaaCTGCAGatGCTAGCtcGCATGC
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Supplementary Table 6 oligonucleotides used in this study

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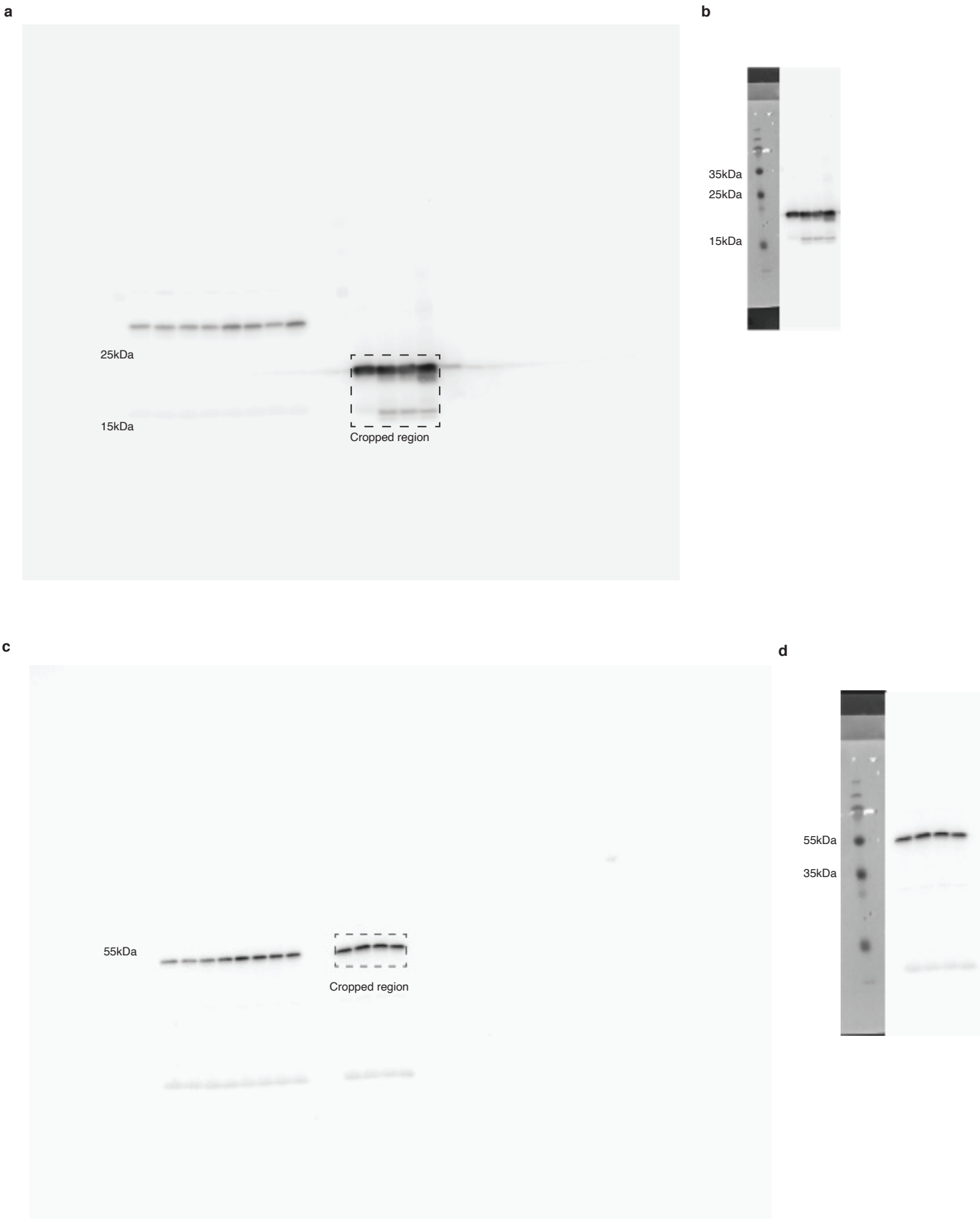
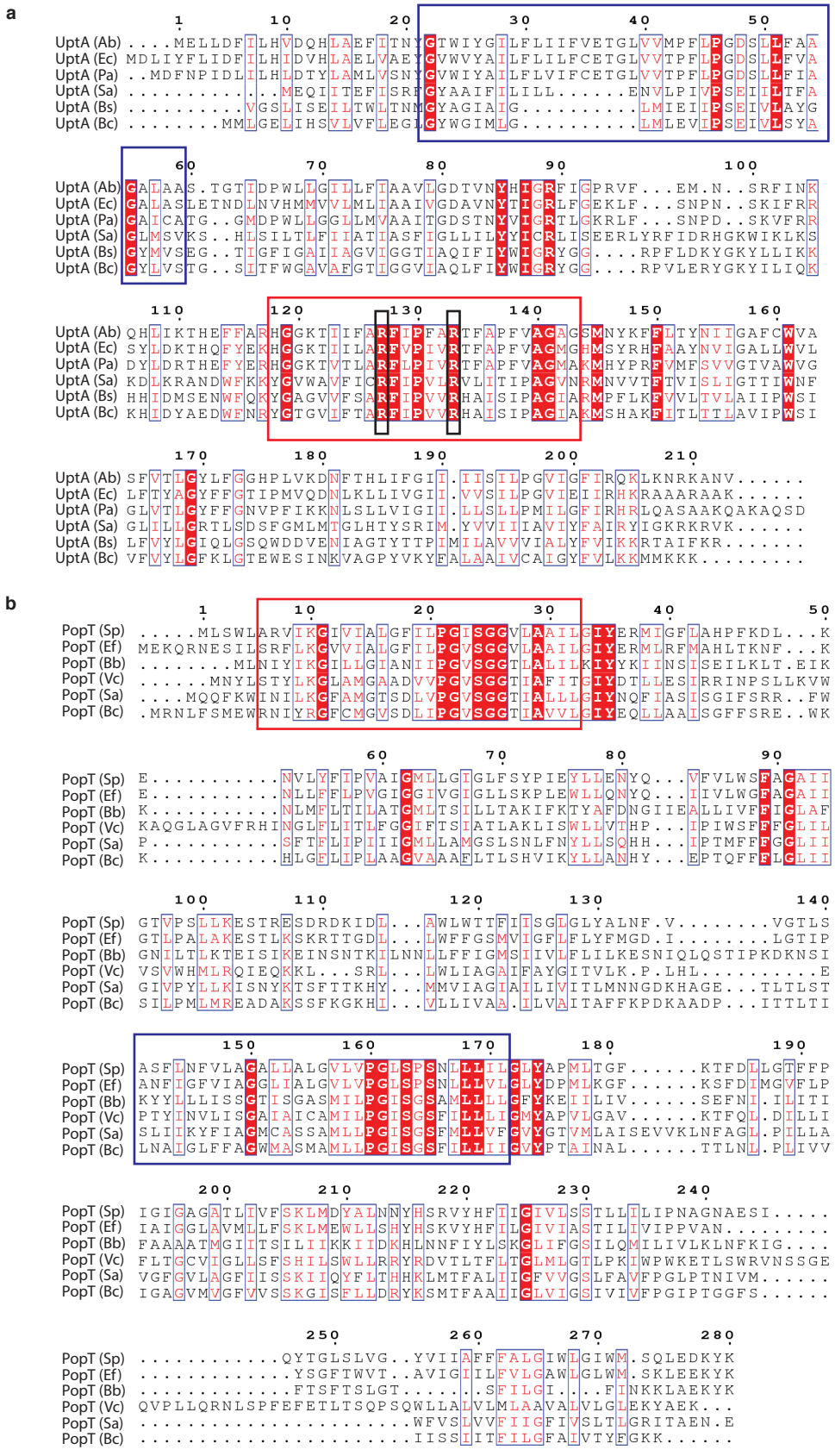
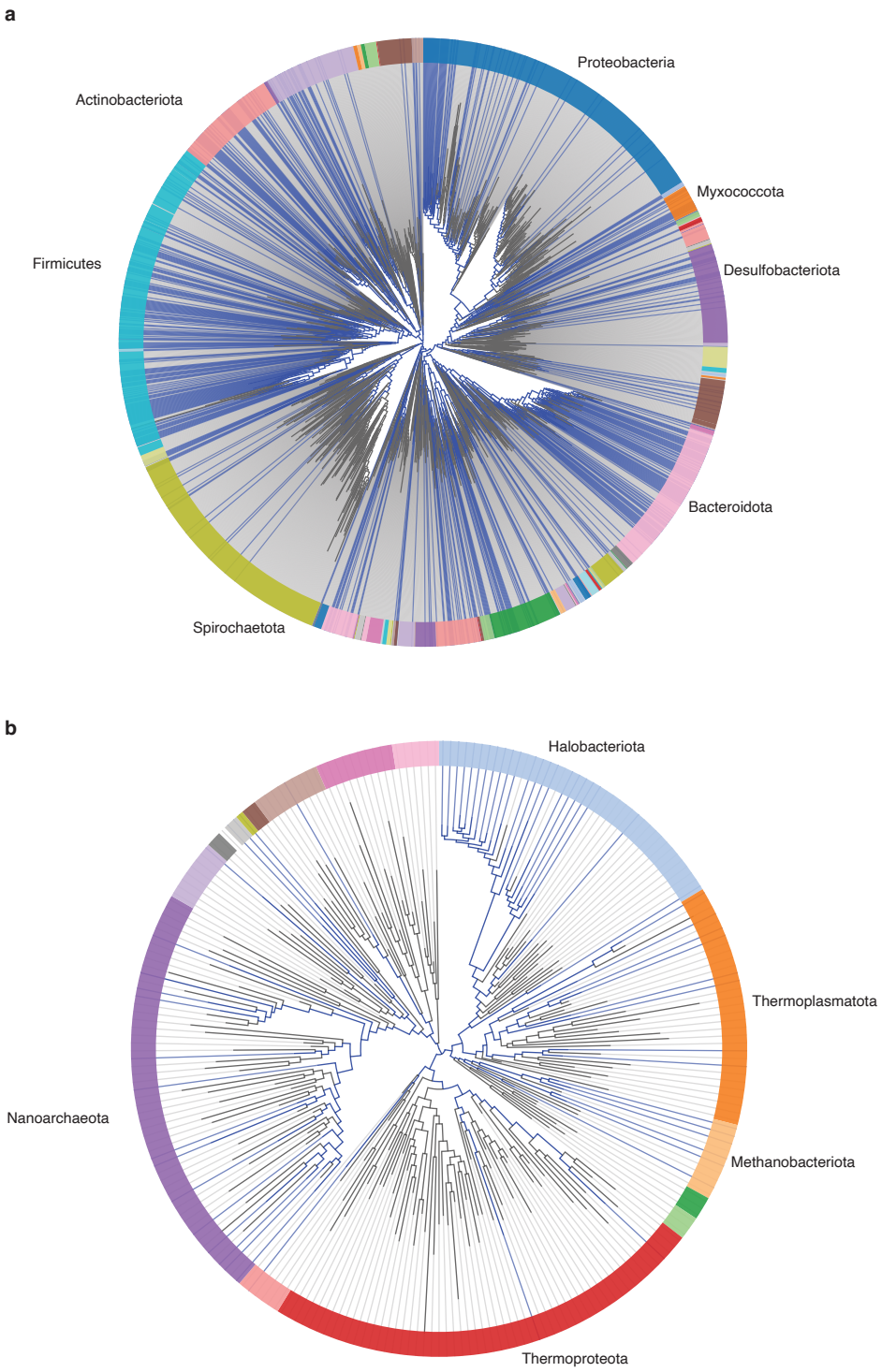


Supplemental Figure 1. Uncropped gel source data for Extended data 2c. **(a)** Uncropped western blot of the his tagged yngC protein with cropped region indicated. **(b)** Spliced image of visible and chemiluminescent channels to highlight molecular weight. **(c)** Uncropped western blot of the sigA protein with cropped region indicated. **(d)** Spliced image of visible and chemiluminescent channels to highlight molecular weight.



Supplemental Figure 2. Multiple sequence alignments of DedA (UptA) and PopT homologs that provide MX2401 resistance. **(a)** Multiple sequence alignment of DedA (UptA) homologs that provide MX2401 resistance. Two highly conserved arginines are boxed in black and two membrane re-entrant helices that are commonly found in membrane embedded transporters13 are boxed in red and blue. **(b)** Multiple sequence alignment of DUF368 (PopT) homologs that provide MX2401 resistance. Homologous re-entrant helices that are commonly found in membrane embedded transporters13 are boxed in red and blue.



Supplemental Figure 3. DUF368 is broadly conserved in bacteria and archaea. Dendrograms highlighting the distribution of DUF368 domains in a broad range of bacteria **(a)** and archaeal **(b)** species. All members of the pfm04018 (DUF368) were mapped onto representative phylogenetic trees.