A.Multiple sequence alignment of DivIVA proteins across Actinobacteria and Firmicutes phylum

	Simpletompes, conlicolar/1-32 Simplehoocas, p. v671-199 Statyholocas, p. v671-199 Statyholocas, p. v671-93 Simplehoocas, p. v671-93 Wpcobachirum, markar/1-321 Mpcobachirum, markar/1-320 Mpcobachirum, markar/1-320 Mpcobachirum, markar/1-320 Corpebachirum, markar/1-320 Corpebachirum, markar/1-320 Corpebachirum, markar/1-320 Corpebachirum, markar/1-320 Bealites subBibl'r1-194	1 MPLTPED 1 MALTPDD 1 MPLTPAD 1 MPLTPAD		LLEBELE [LAKLATRAA.ADATRAA.OXAMIKSPE COJOCOCOCOPERGINO COGOCOCOCOPERGINE COLORADA (1) LEBELE [LAKLATRAA.ADATRAA.OXAMIKSPE COJOCOCOCOCOPERGINE COLORADA (1) LEBELE [LAKLATRAA.OXAMIKSPE COJOCOCOCOCOPERGINE COLORADA (1) LEBELE [LAKLATRAA.OXAMIKSPE COJOCOCOCOCOCOCOCOCOCOCOCOCOCOCOCOCOCOC	19 75 59 86 70 82 85 88 88 83 88 83 85 20 59
	Streptomyces coelicolar/1-382 11 Starbytococas ar. J. ASD -199 1 Starbytococas ar. J. ASD -199 1 Starbytococas ar. J. ASD -199 1 Naiamurbis - milgardata -218 1 Micobachima - magnatish -222 1 Micobachima - magnatish -222 1 Micobachima - magnatish -228 1 Micobachima - magnatish -228 1 Micobachima - magnatish -228 1 Micobachima - magnatish -238 1 Corprebachima - Michaneurh -369 1 Corprebachima - Michaneurh -369 1 Corprebachima - Michaneurh -369 1 Corprebachima - Michaneurh -351 1 Corprebachima - Michaneurh -353 11 Corprebachima - Michaneurh -353 11 Corprebachima - Michaneurh -353 11 Corprebachima - Michaneurh -353 11 Bacilhag. subhilin' -154	10 - GGPMGG 76	PPOL PSGAPOL P AGPOGOGOPOGP GPMCDOP GP	DOGPMOCOMOPOGPMOGPMOGPGLPCOGG - POGOS IARVIS LI IXOLTADA I AE IRSEINK I VGE - ARSRAEGLE EDWARKIDALERDIA CE KIR VARIOS LES MA INCOMPAGINATION - CONTRACTOR - CONT	49 48 13 65 00 10 04 06 09 96 15 98 73 13
	Singdomyces coelicolari 342 22 SingdyCoccas ge, ASD1-199 14 SinghyCoccas ge, ASD1-199 14 Natarity Coccas ge, ASD1-199 14 Natarity Coccas ge, ASD1-199 14 Natarity Coccas ge, ASD1-199 14 Mocoachirum, anterpretario 172 21 Mycobachirum, anterpretario 172 21 Mycobachirum, anterpretario 172 21 Mycobachirum, anterpretario 172 21 Carynebachirum, gindaman 734 22 Carynebachirum, gindaman 735 22 Carynebachirum, gindaman 734 22 Carynebachirum, gindaman 735 22 Carynebachirum, gindaman 734 22 Beallau, aubilio'i 194	50 ARATLER 49 ERNTLEA 14 ORSVLES 66 EKVGLEK 01 ORAVLEG 05 ORTVLEG 05 ORTVLEG 10 DKRRLEG 10 DKRRLEG 10 OKRLEA 16 OQNALET 99 QONALET 99 QONALET 14 IAM	KVEDIRGERERVENTENKEN VERSONROLE TOADDOL VERSONROM I FERSTENNI KADUDI KOND VERSONROM I FERSTENNI KADUDI KOND VERSONROM I FERSTENNI KADUDI KOND KUEDIRGERVENTENKEN VERSONROM KADUDO KUEDIRGERVENTENKI VI SOLUCE EN AR KUEDIRGERVENTENKI VI SOLUCE COR KUEDIRGERVENTENKI VI SOLUCE COR KUEDIRGERVENTENKI VI SOLUCE COR VERSONROM KADUDI KUEDIRGERVENTENKI VI SOLUCE COR KUEDIRGERVENTENKI VI SOLUCE COR VEGIARTERERVENTENKI VI SOLUCE COR KUEDIRGERVENTENKI VI SOLUCE COR KUEDIRGERVENTENKI VI SOLUCE COR KUEDIRGERVENTENKI SUBJECCE COR KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTEN KUEDIRGERVENTENKI SUBJECCE COR KUEDIRGERVENTENKI SUBJEC	APPROP AS AAPS LPPSP APSMAP ALL/CLUS YOU CONCOURD/GOP GOPSG PS YOU CONCOURD/GOP SPINOLAPS PANDAP SPINO	32 39 03 81 18 60 72 64 86 75 27 49 65 320 64
	Percentage agreer	nent	Colour		
	> 80%		blue		
	> 60%		lavendar		
	> 40%		light lavendar		
<= 40%			White		

Supplemental Figure 1. Wag31 vs. DivIVA. (A) Multiple sequence alignment of DivIVA proteins across Actinobacteria and Firmicutes phyla using Clustal Omega program (Goujon *et al.*, 2010; Sievers *et al.*, 2011; McWilliam *et al.*, 2013) and visualized with Jalview version 2(Waterhouse *et al.*, 2009; Troshin *et al.*, 2011).

A. Western blot of Wag31 mutants using α -mDHFR and α -Wag31



B. Stability of Wag31-DHFR3 fusions by western blot, quantification



Supplemental Figure 2. Stability of Wag31 mutants. Stability of Wag31 mutant proteins was tested in the context of the Mycobacterial Protein Fragment Complementation Assay (Singh *et al.*, 2006). In this assay, WT *wag31* was fused to the N-terminus (Fragments 1,2) of mDHFR, mutant versions of *wag31* were fused to the C-terminus (Fragment 3) of mDHFR. The interaction of the mDHFR fragments provides trimethoprim resistance, which is an indication of protein-protein interaction. Here we used western blots against Fragment 3 of mDHFR to assess the stability of the Wag31 mutant proteins. (A) Representative western blots of WT and mutants of Wag31-DHFR3 using α -mDHFR antibody (left), Western blots of Wag31 WT and mutant proteins in wag31 allele strains using α -Wag31 antibody (right). RpoB serves as a loading control. (B) Normalized quantification of western blot (as in B) band intensities: ratio of ((Wag31-allele/ matched RpoB)/(Wag31 WT/ matched RpoB))/(DHFR-GCN4/ matched RpoB). Values are the mean of western blots from two independent cultures. Error bars represent SD. The dotted line shows the protein level of the DHFR positive control.



A. Micrographs of HADA stained wag31 allele strains mc²155 ∆wag31 L5:: wag31 WT

Supplemental Figure 3. The formation of rod-shape morphology is affected by NQQR199-**202 residues.** (A) Fluorescence (left) and phase (right) images of *Msmeg wag31* allele strains stained with HADA. The scale bar is 5 microns, and it applies to all images. (B) Western blots of Wag31 NOQR199-202AAA and Wag31 WT proteins in *wag31* allele strains using α -Wag31 antibody. RpoB serves as a loading control. (C) Doubling times of Msmeg cells expressing WT or wag31 NQQR199-202AAAA mutant. The means (on top of bars) are an average of three biological replicates. Error bars represent SD. (D) Cell length of the wag31 NOOR199-202AAAA and wag31 WT strains. Black bars are at the mean. (E) Cell width of the wag31 allele strains. Black bars are at the mean. (F) Relative polar and septal (G) HADA intensity of wag31 allele strains. (H) Length of polar elongation in the wag31 allele strains. Black bar is at the

median. (I) Relative polar intensity of GlfT2-mcherry2B in the Wag31 WT and Wag31 NOOR199-202AAAA mutants at the old pole and the new pole. (J) Relative polar intensity of MurG-Venus in the Wag31 WT and Wag31 NOOR199-202AAAA at the old pole and the new pole. ns, P > 0.05, *, P = < 0.05, ***, P = < 0.005, ****, P = < 0.0001. *P*-values were calculated by the unpaired t-test.



Supplemental Figure 4. Gross cell phenotypes. (A) Doubling time (top), cell length (middle) and cell width (bottom) in *wag31* allele strains. Cells with a mean at least 10% less than the WT

mean, and with a width at least 5% more than the mean of the WT strain, are considered short and wide, respectively. ns, P > 0.05, *, P = < 0.05, **, P = < 0.005, ***, P = < 0.0005, ****, P = < 0.0001. *P*-values were calculated by one-way ANOVA, Dunnett's multiple comparisons test. (B) Mean length of *wag31* allele strains plotted as a function of their mean width. Black line is a linear fit. (C) Residues mutated in the alanine scanning mutagenesis are shown as spheres on the Alphafold2 predicted structure (Jumper *et al.*, 2021) of Wag31. Residues are colored according to phenotype categories in (A). Essential residues (black) are the mutants that were unable to replace the *wag31* WT allele.



Supplemental Figure 5. Polar peptidoglycan metabolism. (A) Mean intensity of HADA signal per cell in *wag31* allele strains. (B), (C) Relative HADA signal at the old and new poles, normalized to the cell mean. Black bars are at the mean. Cells with polar intensity at least 7% less than the mean of the *wag31* WT strain are considered dim at the old pole. Cells with polar intensity at least 5% more than the mean of the *wag31* WT strain are considered bright at the new pole. Cells with polar intensity at least 5% less than the mean of the *wag31* WT strain are considered bright at the new pole. Cells with polar intensity at least 5% less than the mean of the *wag31* WT strain are considered dim at the new pole. (D) Residues mutated in the alanine scanning mutagenesis are shown as spheres on the Alphafold2 predicted structure of Wag31. Residues are colored according to phenotype categories in A, B, C. ns, P >0.05, *, P =< 0.05, **, P=< 0.005, ****, P=<0.0001. *P*-values were calculated by one-way ANOVA, Dunnett's multiple comparisons test.

A. Raw septal signal in *wag31* allele strains





Supplemental Figure 6. Septal peptidoglycan metabolism. (A) Raw and, (B) relative septal HADA intensity of *wag31* allele strains. Black bars are at the mean. (C) Septal location in *wag31* allele strains. (D) Residues mutated in the alanine scanning mutagenesis are shown as spheres on the Alphafold2 predicted structure of Wag31. Residues that have at least 7% less normalized septal intensity than the WT are defined as dim. ns, P >0.05, *, P =< 0.05, **, P=< 0.005, ****, P=< 0.0001. *P*-values were calculated by one-way ANOVA, Dunnett's multiple comparisons test.

A. Micrographs of HADA-stained cells in stationary phase



Supplemental Figure 7. Wag31 phospho-site T73 is not an upstream regulator of peptidoglycan metabolism in stationary phase. (A) Phase (left) and fluorescence (right) micrographs of *Msmeg* cells in stationary phase which are expressing Wag31 WT, Wag31 T73A, and Wag31 T73E. (B) Demographs of HADA intensity (color scale) across the length of the cell (Y axis) of the *wag31* allele strains. The cells were sorted by length, with shortest cells on the left and longest on the right of each demograph. Cells were also pole-sorted according to HADA intensity, such that the brighter pole (presumed to be the old pole) was oriented to the top along the Y axis. At least 100 cells were analyzed from each of three independent biological replicates of each strain. (C) Cell lengths of the *wag31* allele strain. Black bars are at the mean. (D) Cell widths of the *wag31* allele strain. Black bars are at the mean. (E) Percentage of cells in (F) that have septal HADA signal. (F) Relative polar and septal HADA intensity of Wag31 WT, Wag31 T73A, and Wag31 T73E. (G) Septal location in *wag31* allele strains. ns, p >0.05, *, P = < 0.05, ***, P = <0.001. All *P*-values were calculated by one-way ANOVA, Dunnett's multiple comparisons test.

Demographs of HADA intensity of wag31 allele strains



Demographs of HADA intensity of wag31 allele strains



Supplemental Figure 8 and 9. Demographs of HADA intensity (color scale) across the length of the cell (Y axis) of the *wag31* allele strains. The cells were sorted by length, with shortest cells on the left and longest on the right of each demograph. Cells were also pole-sorted according to HADA intensity, such that the brighter pole (presumed to be the old pole) was oriented to the top along the Y axis. At least 100 cells were analyzed from each of three independent biological replicates of each strain.



Supplemental Figure 10. *In vitro* activities of the ACCase complexes. ACCase6 and ACCase5 were reconstituted from their purified component and ACC (A) and PCC (B) activity, respectively, was determined in presence of Wag31. Results are the means of three independent experiments \pm standard deviations (n = 3). ns, P >0.05, *, P =< 0.05, ***, P=< 0.0005. All *P*-values were calculated by the unpaired t-test.