



Figures and figure supplements

A cytoplasmic peptidoglycan amidase homologue controls mycobacterial cell wall synthesis

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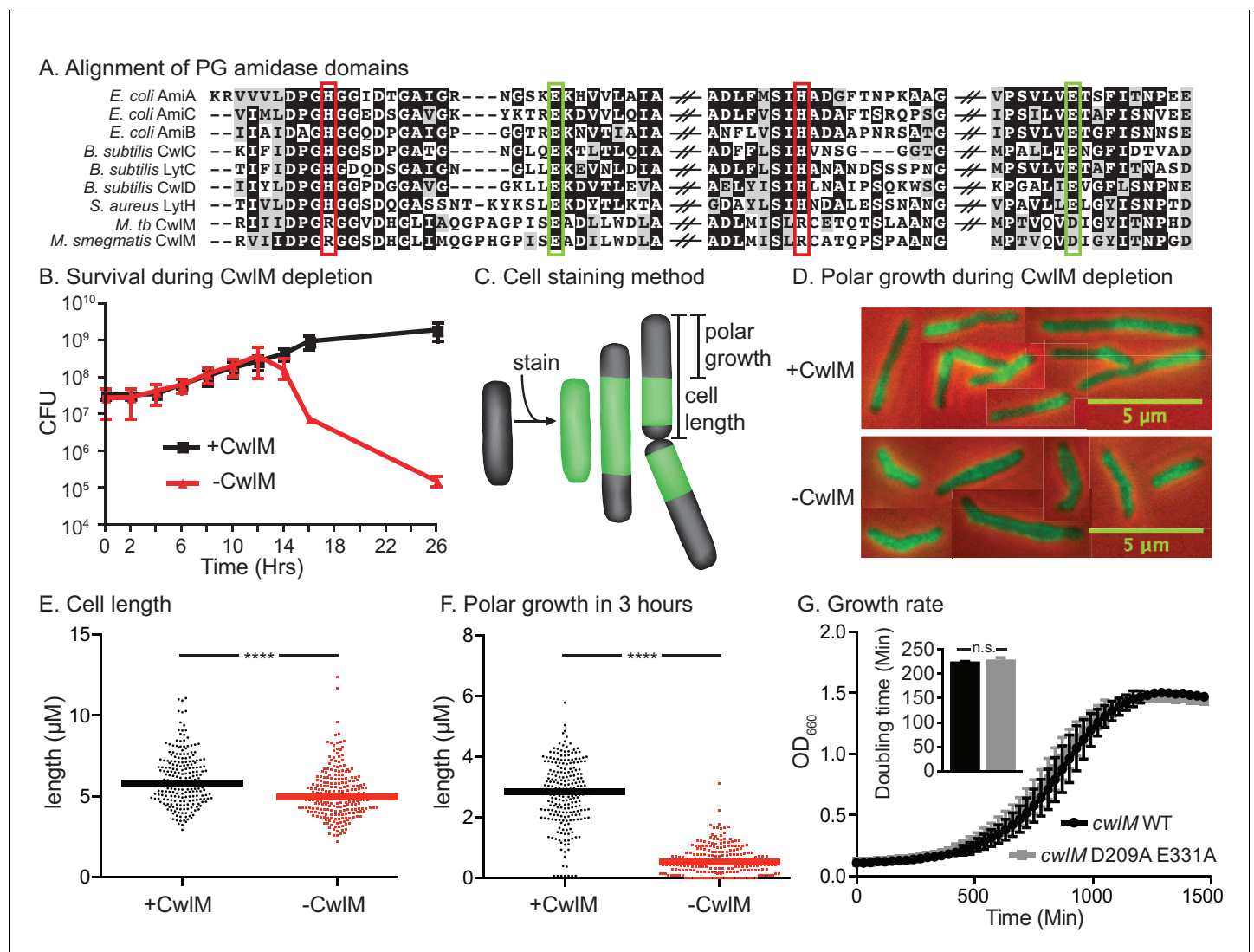


Figure 1. CwlM's essential function promotes polar growth, but not through enzymatic activity. (A) Alignment of the active-site proximal regions of the enzymatic domain of PG amidases. The Zn^{2+} -coordinating residues are boxed: the conserved aspartate/ glutamates in green, and the degenerate histidine/ arginines in red. (B) Colony forming units (CFU) of the Ptet::cwIM (CB82) strain grown with (+CwlM) or without (–CwlM) Atc inducer from Time 0. Data from all experiments throughout is from three biological replicates and error bars are the standard deviation, unless otherwise noted. (C) Cartoon of the amine reactive dye (ARD) staining method showing polar growth after staining, with a diagram of how cell length and polar growth were measured for Figures 1E and 2E. (D) Overlaid micrographs of the Ptet::cwIM strain grown with (+CwlM) or without (–CwlM) Atc for 9 hr, stained with ARD, grown 3 hr and imaged with a 488/530 filter (green) and in phase (red and black). Representative cells from several images used in E and F were pasted together, the scale is conserved between images. (E) The length of ~230 cells from each condition in (D). Two biological replicates were used for all microscopy experiments, unless noted. (F) The length of the longer unstained pole of the cells in (E), line indicates the median in (E) and (F). **** p value < 0.0001. (G) Growth curves showing the OD₆₆₀ of WT (L5::cwIM, CB236) and amidase-ablated (L5::cwIM D209A E331A, CB239) strains growing in 7H9, and a bar graph showing the calculated doubling times of these strains (inset). n.s. – not significant. The p value was 0.51 by the student's t-test
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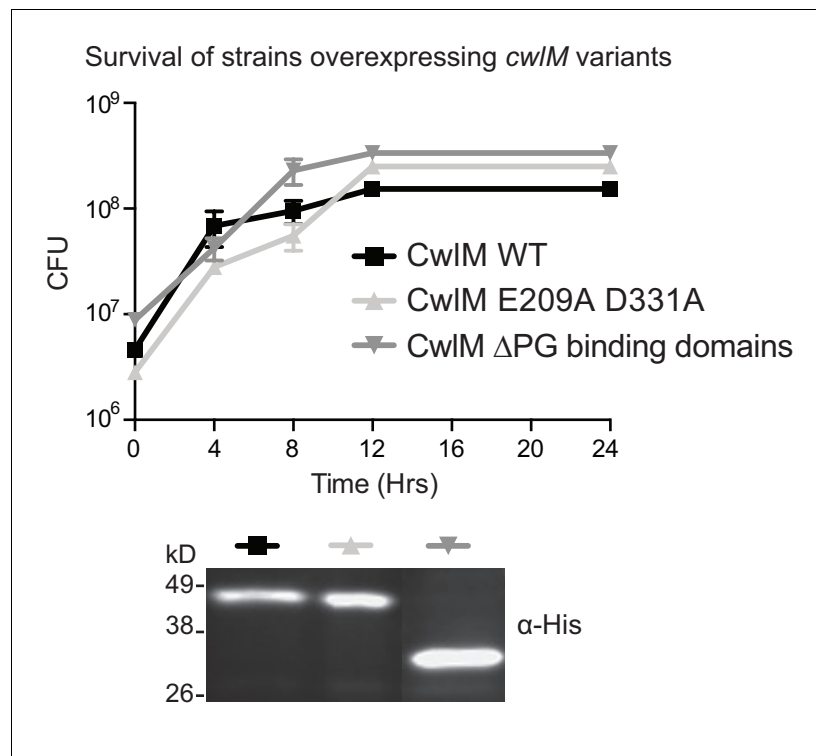


Figure 1—figure supplement 1. Overexpression of CwIM. Colony forming units of *Msmeg* strains overexpressing wild-type (CB193), the amidase ablated allele of *cwIM* (CB194), and the amidase domain alone (CwIM Δ PG binding domains, CB202), and an α -His western blot showing that these proteins are expressed. Proteins were expressed from Atc-inducible promoters on episomal vectors.

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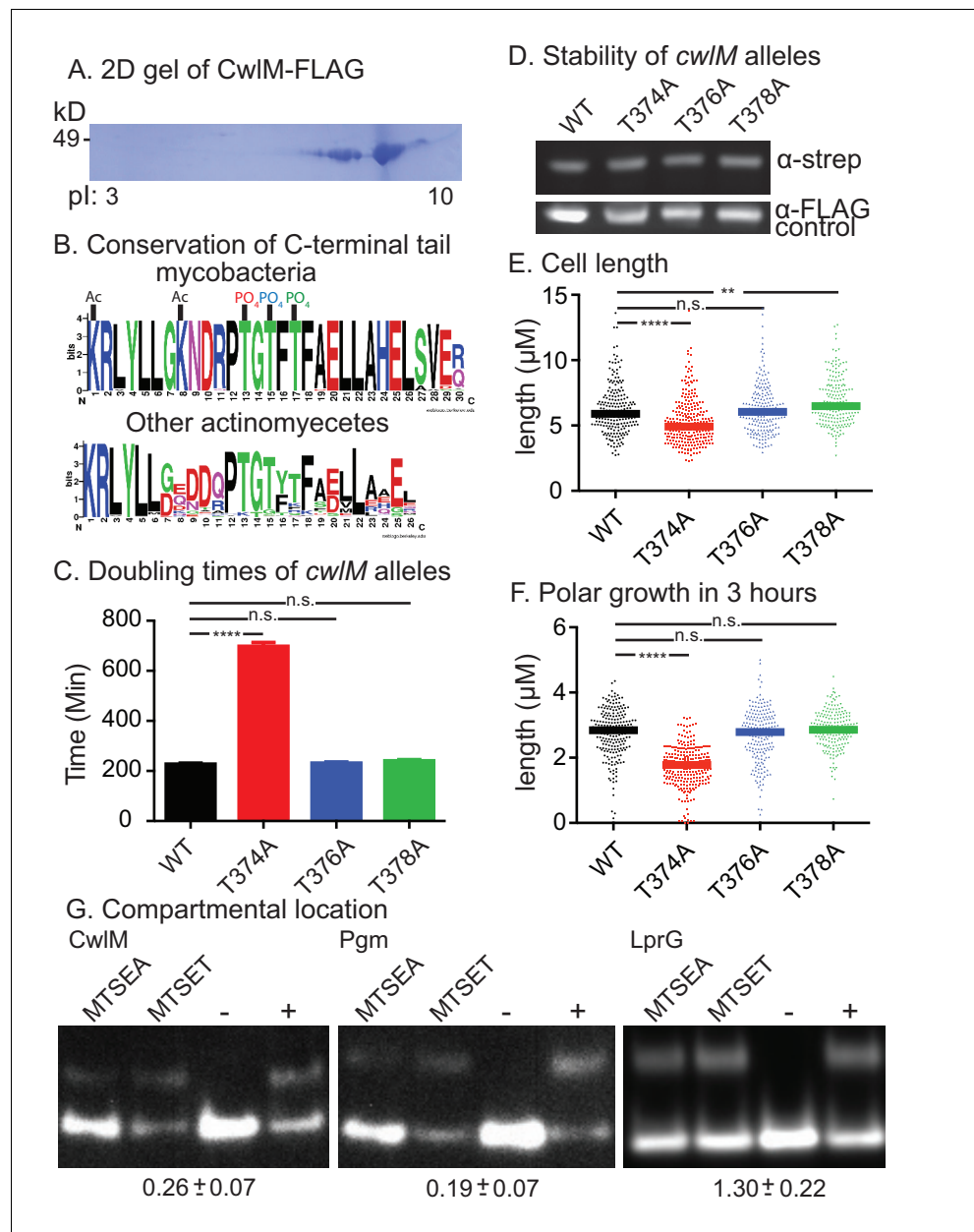


Figure 2. CwlM is phosphorylated and cytoplasmic. (A) 2-D gel of CwlM-FLAG from *Msmeg* (CB100) with the isoelectric point (pI) indicated on the bottom. (B) Weblogo diagrams of the C-terminus of CwlM homologues. Acetylation (Ac) and phosphorylation (PO₄) sites are indicated. The colors of the three phosphates match the colors of the corresponding phospho-ablative strains in C, E and F. (C) Doubling times of L5::cwlM-FLAG WT (CB300) and phospho-ablative mutants (T374A = CB300; T376A = CB345; T378A = CB348). All multiple comparisons throughout were performed by One-way ANOVA with a Dunnett multiplicity correction. The adjusted p values for each mutant compared to WT are: T374A = < 0.0001; T376A = 0.88; T378A = 0.15. (D) α-strep western blot of L5::cwlM-strep WT (CB663) and phospho-ablative mutants (CB666, 669, 672). A background band on the same blot probed with α-FLAG is used as a control. Westerns of the L5::cwlM-FLAG strains could not be used because of high background. (E) Cell length of 200–300 cells from L5::cwlM-FLAG WT and phospho-ablative mutants. The adjusted p values for each mutant compared to WT are: T374A = < 0.0001; T376A = 0.99; T378A = 0.01. (F) Length of the longer unstained pole of cells in (E). Cells were stained with ARD and grown for three hours before imaging. The adjusted p values for each mutant compared to WT are: T374A = < 0.0001; T376A = 0.59; T378A = 0.36. (G) Substituted cysteine accessibility. α-strep western of cells with strep-tagged Pgm (CB886, MSMEG_4579, cytoplasmic control), LprG (CB706, Rv1411, periplasmic control) and CwlM1cys (CB457) that were cysteine-blocked with MTSEA (membrane permeable) or MTSET (membrane impermeable) then alkylated at

Figure 2 continued on next page

Figure 2 continued

unblocked cysteines. Control samples: (–) = not alkylated or blocked, (+) = alkylated but not blocked. % alkylation (%alk.) = high band/ total for each sample. The periplasmic localization score = (%alk._{MTSEA}/ % alk._{MTSET}) and is indicated below the gel for each protein ± the 95% confidence interval from two biological replicates. Scores < 0.5 are cytoplasmic, near 1 are periplasmic.

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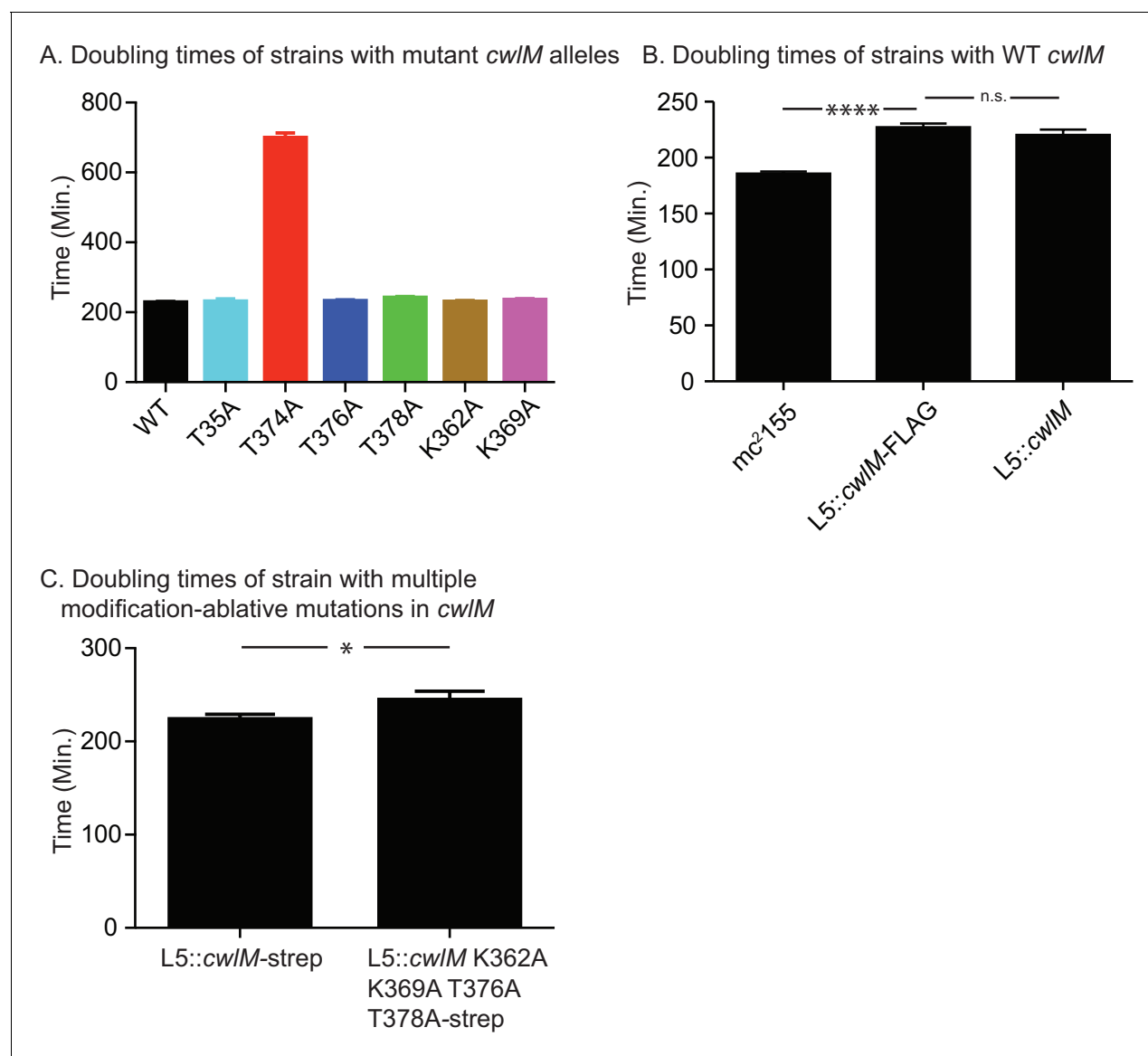


Figure 2—figure supplement 1. Growth rates of strains with different *cwIM* alleles. (A) Doubling times of $\Delta cwIM$ *Msmeg* strains with different *cwIM*-FLAG alleles at the L5 phage integrase site. WT = CB300; T35A = CB265; T374A = CB319; T376A = CB345; T378A = CB348; K362A = CB351; K369A = CB354. p values for each mutant against the WT were calculated with the students t test: all p values were insignificant except T374A > 0.0001. (B) Doubling times of wild-type *M. smegmatis* mc²155 (CB1), mc²155 $\Delta cwIM$ L5::cwIM-FLAG (CB300) and mc²155 $\Delta cwIM$ L5::cwIM (CB236). p values: CB1 vs. CB300 > 0.0001; CB300 vs. CB236 = 0.8. (C) Doubling times of mc²155 $\Delta cwIM$ L5::cwIM-strep (CB663) and mc²155 $\Delta cwIM$ L5::cwIM K362A K369A T376A T378A-strep (CB675), p value = 0.033.

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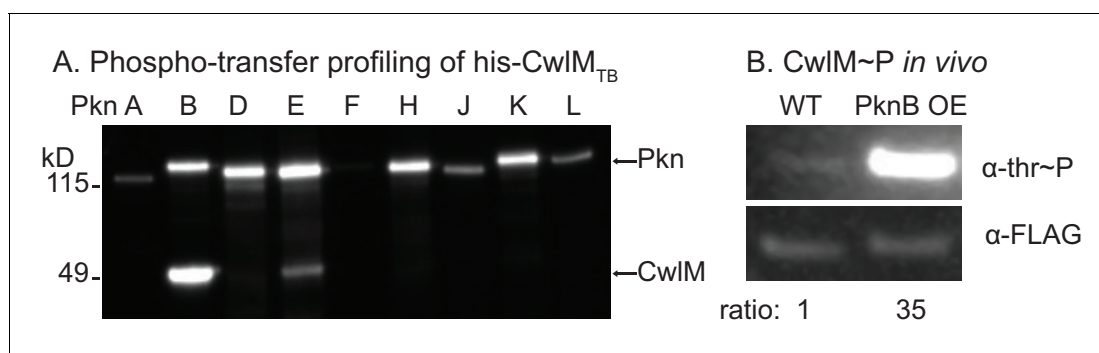


Figure 3. CwIM is phosphorylated by PknB. (A) α -thr~p western blots of *in vitro* kinase reactions with the kinase domains of 9 STPKs from *Mtb* fused to MBP and his-CwIM from *Mtb*. Reactions were stopped at 2 min. Longer time points are in **Figure 3-figure supplement 1B**. (B) α -thr~p and α -FLAG western blot of lysates from a strain expressing CwIM-FLAG in a PknB_{TB} overexpression (CB418) background. PknB_{TB} was uninduced or induced for 30 min with 100 ng/ml Atc. Ratio = signal of (α -thr~p / α -FLAG)_{PknB OE} / (α -thr~p / α -FLAG)_{uninduced}. Experiments in (A) and (B) were both performed twice, representative images are shown.

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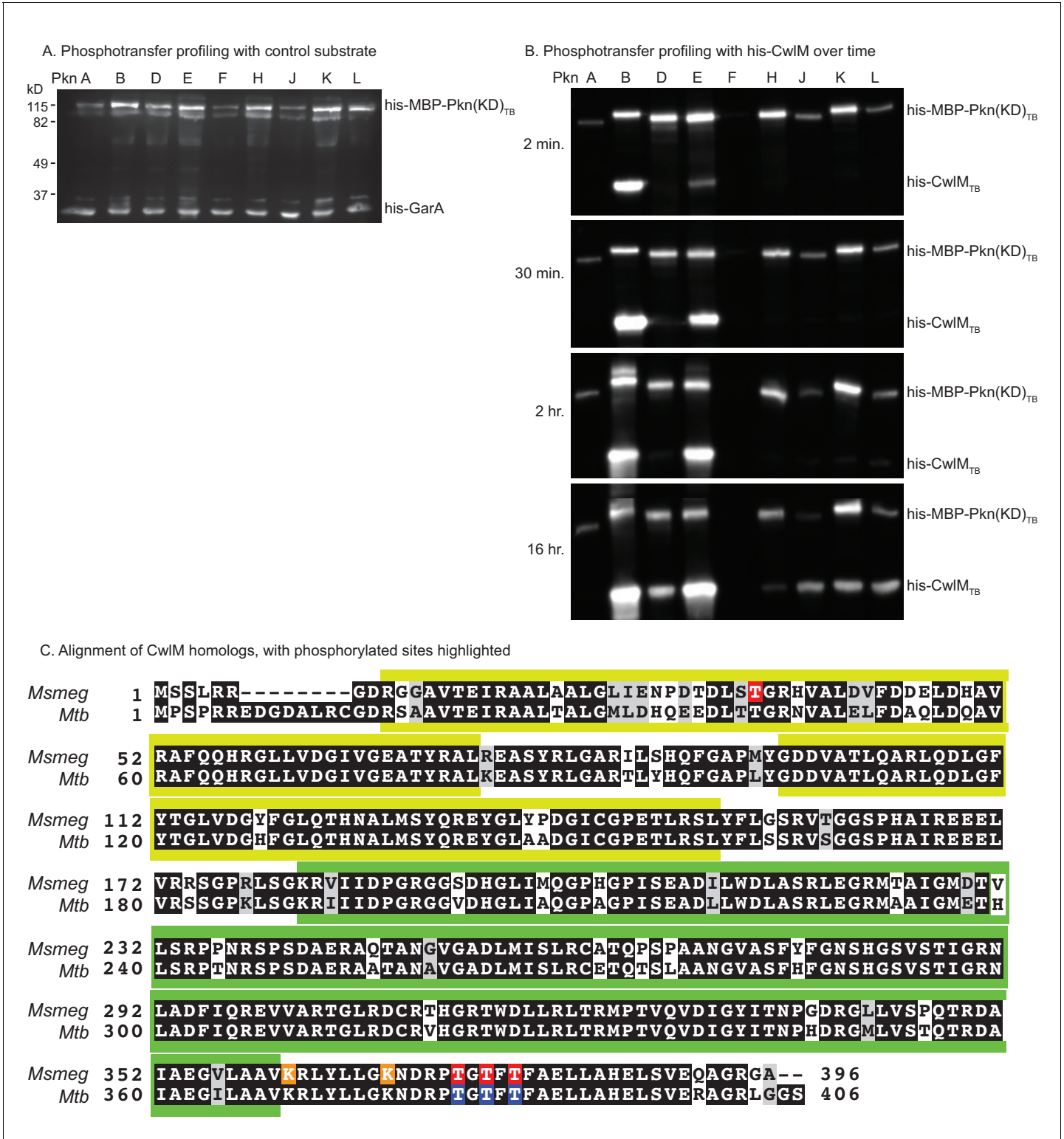


Figure 3—figure supplement 1. Phosphorylation of CwlM. (A) α -phospho-threonine western blot of kinase reactions of the kinase domains (KD) of STPKs from *Mtb* fused to his-MBP incubated overnight at room temperature with his-GarA_{TB}, a non-specific substrate. (B) α -phospho-threonine western blot of kinase reactions of his-CwlM_{TB} incubated with the kinase domains of STPKs from *Mtb* fused to his-MBP. (C) Alignment of CwlM from *Mtb* and *Msmeg*. Yellow boxes indicate the position of the PG binding 1 domains, green boxes indicate the position of the PG amidase domain. Residues highlighted in red were found to be phosphorylated in CwlM-FLAG pulled down from *Msmeg* cell lysates. Residues in orange were acetylated in these

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same lysates. Residues highlighted in blue were found to be phosphorylated on his-CwlM_{TB} expressed and purified from *E. coli* and incubated with his-MBP-PknB(KD)_{TB}. All the modifications were identified by mass spectrometry.

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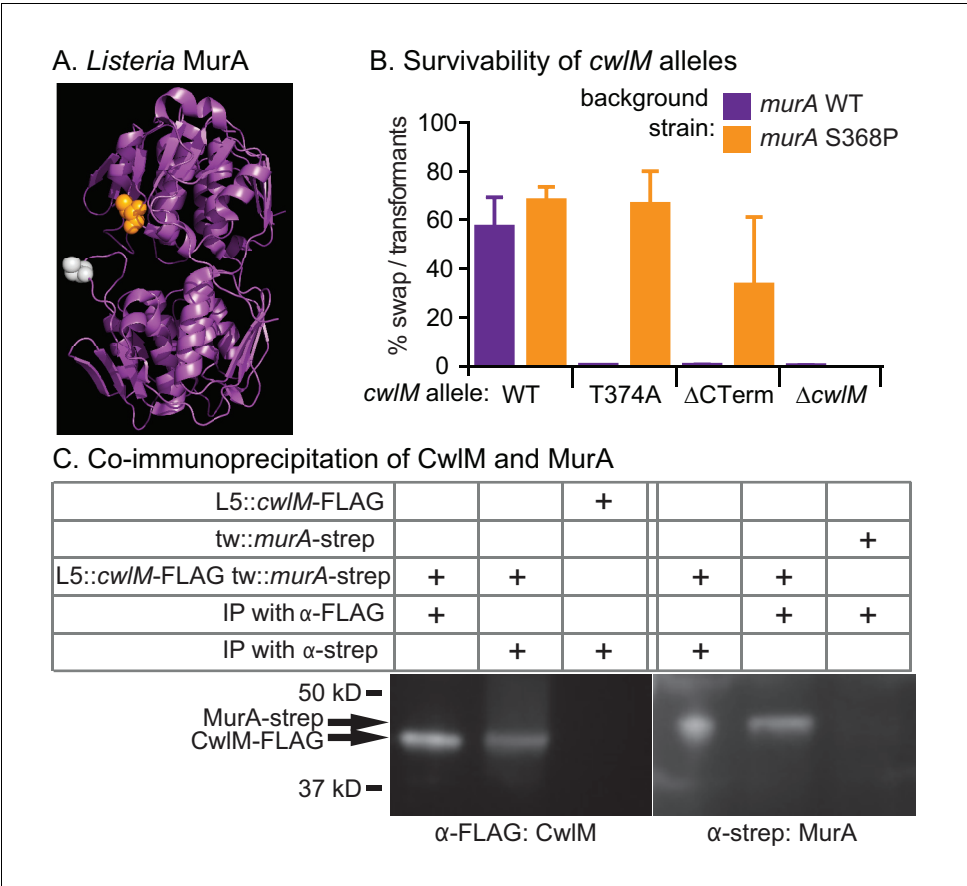


Figure 4. CwlM binds to and regulates MurA. (A) Crystal structure of MurA from *L. monocytogenes* (Halavaty et al., 2011). Orange: alanine that corresponds to S368 in *Msmeg* and *Mtb* MurA; gray: catalytic cysteine (aspartate in *Msmeg* and *Mtb*). (B) Fitness of *cwlM* alleles in *murA* WT (CB737) and S368P (CB762) backgrounds, assessed by the percentage of colonies in which the WT *cwlM* allele was replaced by the indicated allele (WT = pCB277; T374A = pCB255; ΔCT = pCB557; Δ*cwlM* = pCB558). Four biological replicates of each transformation were performed, and 96–198 colonies were counted for each replicate. The Sidak correction for multiple comparisons was used to calculate p values using one-way ANOVA. The adjusted p values among the *murA* WT strains were calculated compared to *cwlM* WT: T374A = < 0.0001; ΔCT = < 0.0001; Δ*cwlM* = < 0.0001. The adjusted p values among the *murA* S368P strains were calculated compared to *cwlM* WT: T374A = 0.99; ΔCT = 0.006; Δ*cwlM* = < 0.0001. (C) α-FLAG and α-strep western blots of immunoprecipitates from the indicated strain (top three rows, from top: CB300, CB737, CB779) with the indicated antibody-conjugated beads (bottom two rows). The pulldown was performed twice, a representative image is shown.
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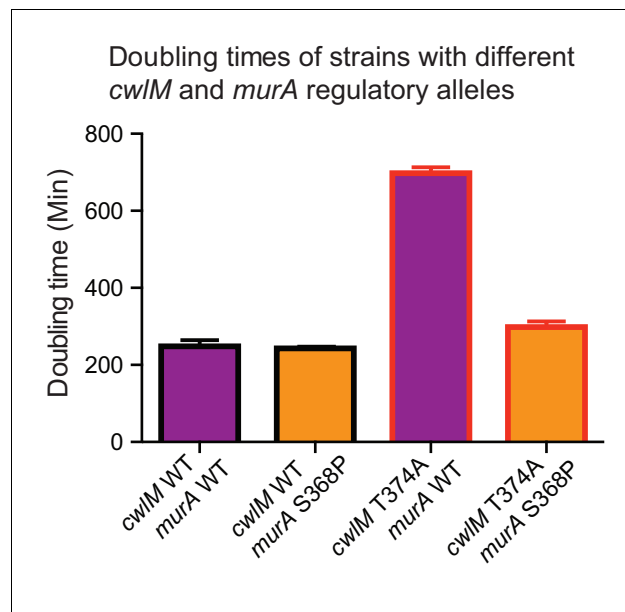


Figure 4—figure supplement 1. Growth rates of strains with different *cwIM* and *murA* regulatory alleles. Doubling times of strains. *cwIM* WT *murA* WT = CB779; *cwIM* WT *murA* S368P = CB782; *cwIM* T374A *murA* WT = CB319; *cwIM* T374A *murA* S368P = CB785. In the experiment described in **Figure 4**, we were not able to attain any *cwIM* T374A *murA* WT transformants, so the strain with this genotype is not isogenic with the other strains presented here. We were only able to attain colonies with the *cwIM* T374A *murA* WT genotype when the L5 allele swap was conducted in such a way that the correct swap was genetically forced. CB319 and the other strains presented in **Figure 2** were made by using a parental strain (CB233) in which the only copy of *cwIM* was under the control of an Atc-inducible promoter on an L5 vector that contained the *tetR* gene: this strain is Atc-dependent, because TetR will repress transcription of *cwIM* unless Atc is added. The alternate alleles of *cwIM* were under the control of Atc-inducible promoter on L5-integrating vectors that do not contain the *tetR* gene; they were transformed into CB233 and plated on plates that do not contain Atc. If the original L5 vector remains at the L5 site in addition to the new vector with the alternate allele, the TetR expressed from the original vector will repress transcription of both alleles of *cwIM* and cause cell death: thus, the loss of the original vector is forced, and we were able to recover colonies even with very slow growth rates. In the transformations described in **Figure 4**, resulting in strains CB779, 782 and 785, there was no *tetR* on either of the L5 vectors; thus, the swap was not forced, and at least half of the colonies from any transformation had both the original L5 vector with WT *cwIM* and the newly-transformed vector.

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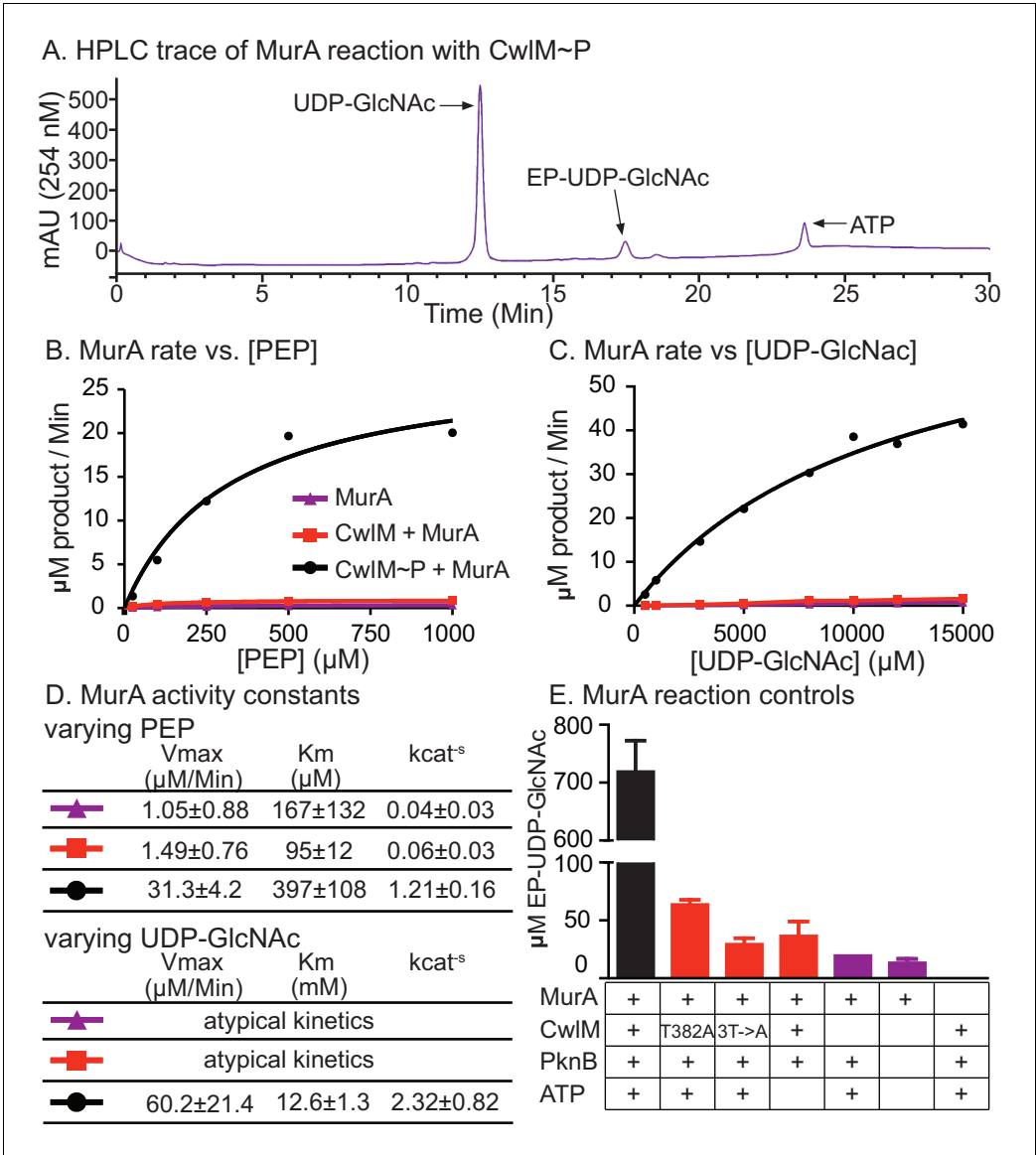


Figure 5. CwlM~P activates MurA *in vitro*. (A) An HPLC trace from the MurA_{TB} kinetic assays, with substrate UDP-GlcNAc and product EP-UDP-GlcNAc indicated. Assays were done on his-MurA_{TB} alone, his-MurA_{TB} with equimolar his-CwlM_{TB}~P in an active kinase reaction (+ATP) with his-MBP-PknB_{TB}(KD) (a his-Maltose binding protein fusion to the kinase domain (KD) of PknB from *Mtb*), or his-MurA_{TB} with his-CwlM_{TB} in an inactive kinase reaction (-ATP) with his-MBP-PknB_{TB}(KD). (B) Rate of MurA activity vs. the concentration of phosphoenol pyruvate (PEP). Lines are the fit to the Michaelis Menten model. (C) Rate of MurA activity vs. the concentration of UDP-GlcNAc for each MurA reaction condition. MurA and MurA+CwlM did not follow Michaelis Menten kinetics, (lines only connect the dots), for MurA+CwlM~P the line is the Michaelis Menten fit. The assays shown in (B) and (C) were done twice with two separate preps of his-MurA_{TB} and his-CwlM_{TB}. Data from one replicate is shown here, full data in **Figure 5 - figure supplement 1A–D**. (D) Michaelis Menten kinetic constants from experiments in (B) and (C). Constants are the average ± the 95% confidence interval from the two replicates. (E) Amount of EP-UDP-GlcNAc produced after 30 min at 37°C in reactions with 2 mM PEP and 15 mM UDP-GlcNAc and his-MurA_{TB} at 20 μg/ml; his-CwlM_{TB} at 20.2 μg/ml; his-MBP-PknB_{TB}(KD) at 2 μg/ml and ATP at 1 mM where indicated. his-CwlM_{TB} was incubated with his-MBP-PknB_{TB} and ATP for 1 hr at RT before initiating the MurA reaction. T382A indicates that the singly-phosphoablative mutant protein his-CwlM_{TB} T382A was used. 3T->A indicates that the triply-phosphoablative mutant protein his-CwlM_{TB} T382A T384A T386A was used. Experiment was performed 2 times. DOI: 10.7554/eLife.14590.011

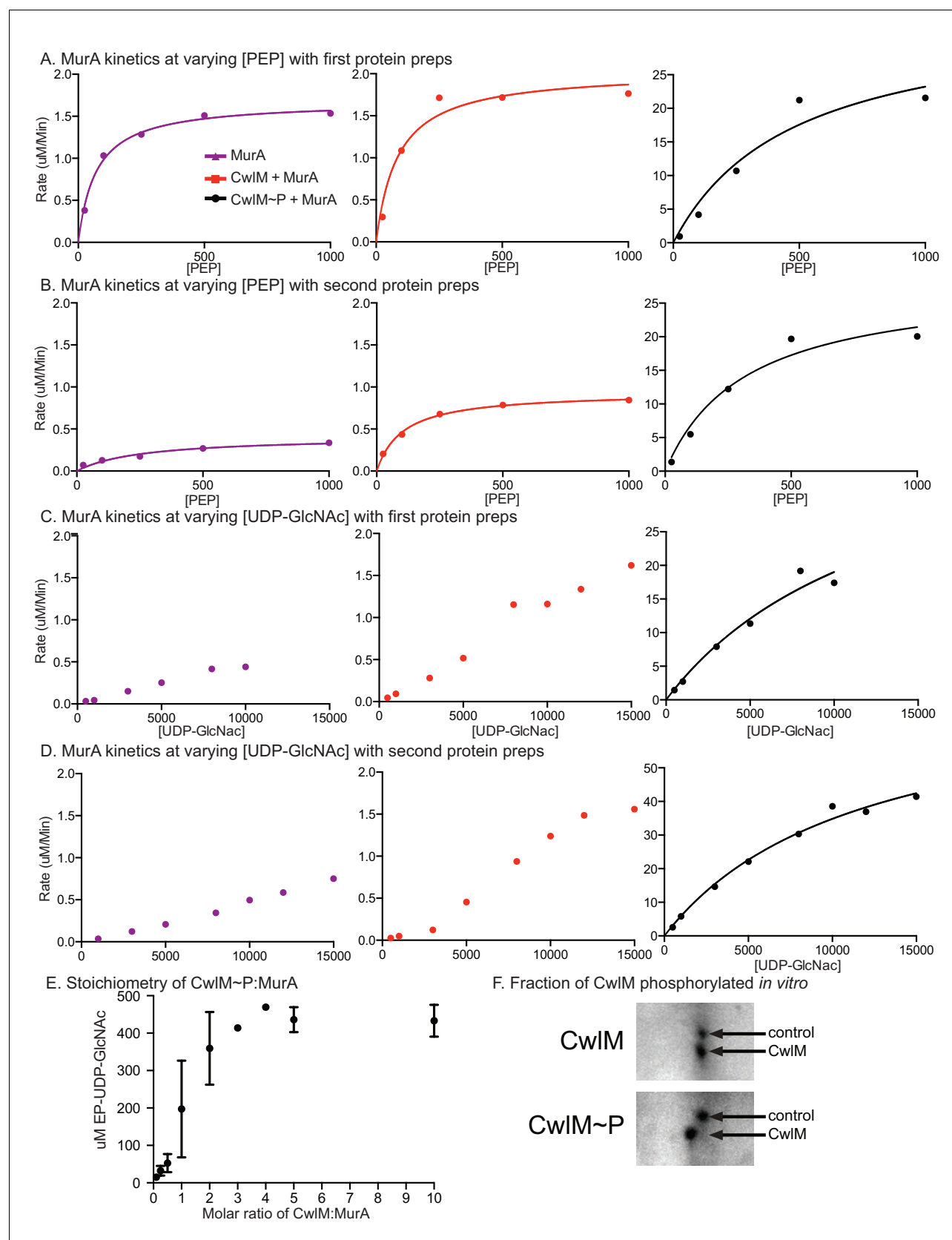


Figure 5—figure supplement 1. MurA enzyme kinetics. Rates of MurA activity vs. [PEP] concentration with (A) the first preparation of his-MurA_{TB} and his-CwlM_{TB}. (B) the second prep. Rates of MurA activity vs. [UDP-GlcNAc] concentration with (C) the first preparation of his-MurA_{TB} and his-CwlM_{TB} and Figure 5—figure supplement 1 continued on next page

Figure 5—figure supplement 1 continued

(D) the second prep. Data in ABC and D were fit to the Michaelis Menten formula where possible, the lines indicate this fit. In C and D, the data for MurA alone and MurA +CwIM could not be fit to the Michaelis Menten formula. These reactions apparently operate under atypical kinetic parameters. (E) Concentration of EP-UDP-GlcNAc formed in a 30 min incubation at 37°C of 2 mM PEP, 15 mM UDP-GlcNAc, 20 µg/ml his-MurA_{TB} and his-CwIM_{TB} ~ P in an active kinase reaction at a range of CwIM:MurA molar ratios. (F) Two-dimensional gel of purified His-CwIM_{TB} after 1 hr incubation with his-MBP-PknB_{TB} in the absence (top) or presence (bottom) of ATP. The shift of the entire CwIM spot to a higher pI indicates that ~ 100% of the protein is phosphorylated.

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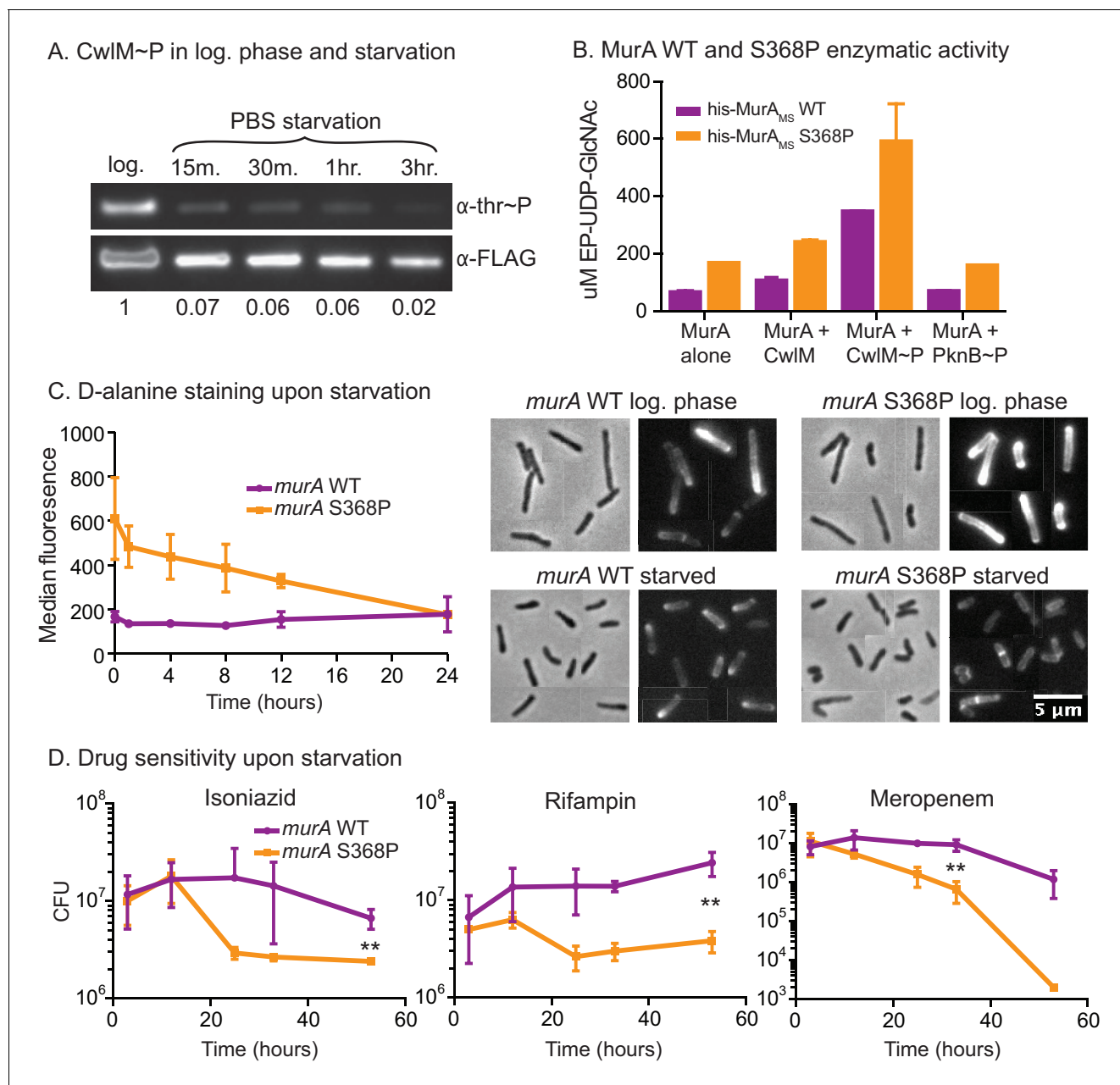


Figure 6. MurA is downregulated in starvation, contributing to drug tolerance. (A) α -thr~P western blot showing the level of CwIM phosphorylation in the *cwIM::FLAG* strain (CB100) in log. phase and upon starvation in PBS. Ratio = signal of (α -thr~p / α -FLAG)_{starvation} / (α -thr~p / α -FLAG)_{log phase} (B) Amount of EP-UDP-GlcNAc produced after 30 min in a reaction with 20 μ g/ml of his-MurA_{MS}WT or his-MurA_{MS}S368P, 2 mM PEP, 15 mM UDP-GlcNAc and either no or equimolar his-CwIM_{TB} or his-CwIM_{TB}~P. (C) Fluorescent intensity of WT (CB779) and *murA* S368P (CB782) cells pulse stained with TADA in log. phase HdB culture ($t = 0$) and at time points after initiation of starvation in PBS+tween 80, as measured by flow cytometry. The median fluorescence for 10K+ cells is shown. On the right are representative images of cells from the $t = 0$ (log. phase) and $t = 24$ (starved) time points. Phase and red fluorescent images of each are shown. The images were taken with identical exposure settings and the brightness and contrast was adjusted to identical levels. Pictures of several cells from images processed identically were pasted together. The scale bar applies to all the images. (D) Colony forming units per ml of strains with the *murA* WT (CB779) and S368P (CB782) alleles after transfer to PBS+tween80 starvation media and treatment with 100 μ g/ml isoniazid, 20 μ g/ml meropenem or 50 μ g/ml rifampin. The p value for the indicated time point in each experiment was calculated by the student's t test. Isoniazid = 0.0087; meropenem = 0.0082; rifampin = 0.0067.

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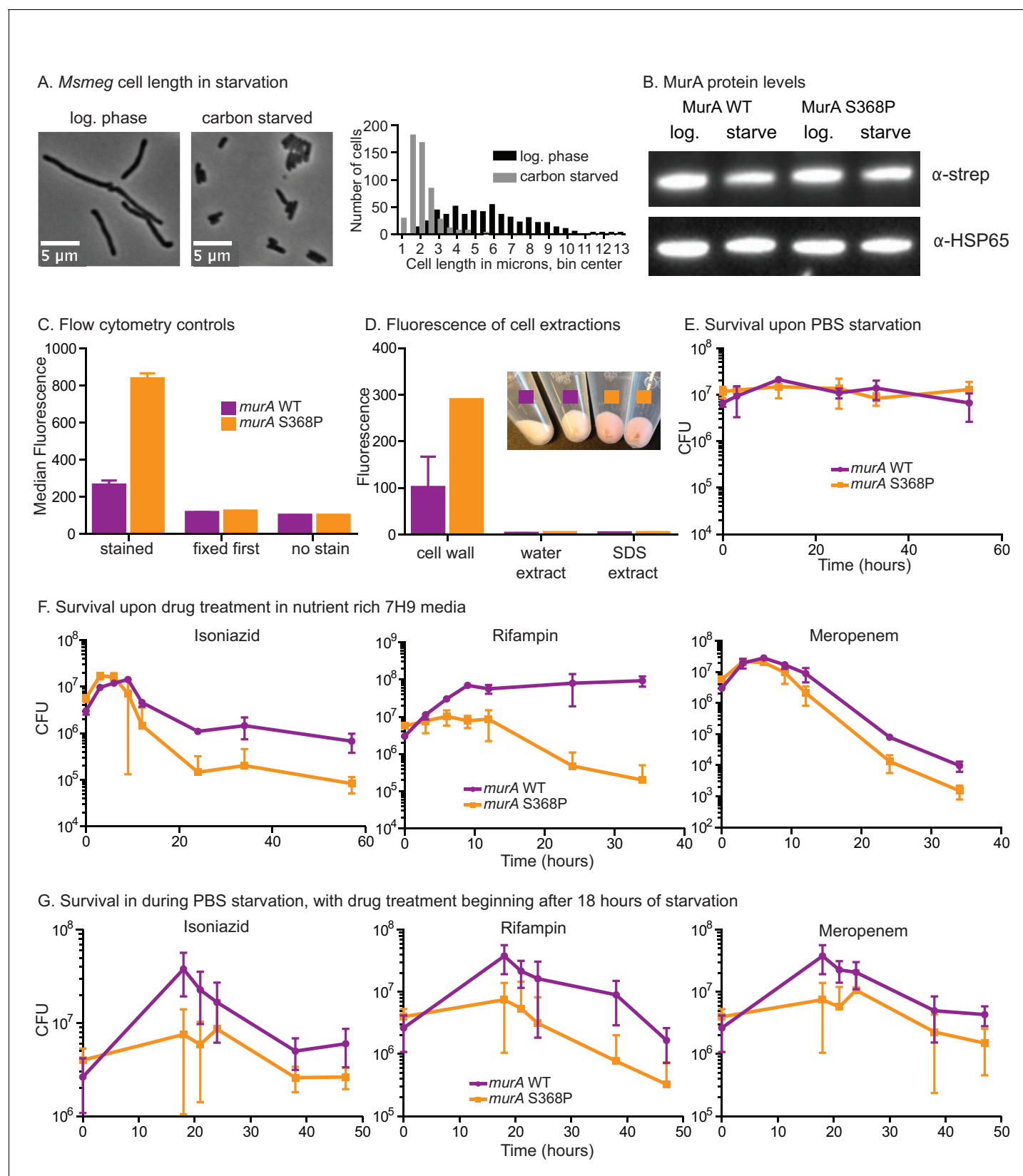


Figure 6—figure supplement 1. Phenotypes of *murA* S368P. (A) Phase micrographs and quantification of *Msmeg* mc²155 (CB1) cell lengths in log. phase 7H9 and after 5 hr in HdB-C carbon starvation media. 500 cells from single cultures were measured for each condition. $p < 0.0001$ by the student's *t*-test. Figure 6—figure supplement 1 continued on next page

Figure 6—figure supplement 1 continued

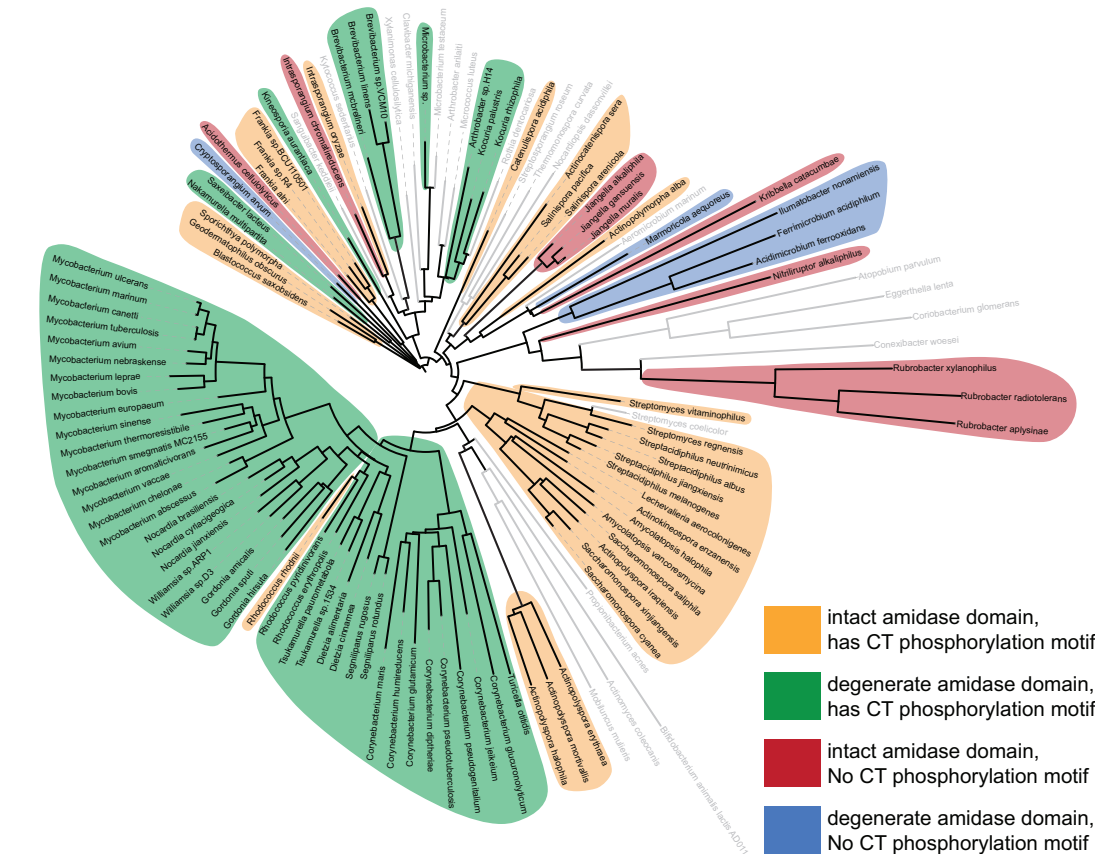
t test. (B). α -strep and α -Hsp65 western blots of A_{280} -normalized lysates from *murA* WT (CB779) and S368P (CB782) cultures starved for four hours in Hdb-C. The *murA* allele in both these strains has a C-terminal strep tag. (C) Controls for flow cytometry. Cells were grown in HdB as in the $t = 0$ time point of **Figure 6C**, and either stained and then fixed as in **Figure 6C**, or fixed first and then stained, or fixed but not stained, and then subjected to flow cytometry. The median fluorescence for 10K+ cells is shown. (D) Fluorescence intensity of TADA stained *murA* WT and *murA* S368P cell fractions. Cells were extracted first with water and then SDS. The remaining cell wall pellets were photographed (inset), and all fractions measured by fluorimetry. Two replicates of each culture were used. (E) Colony forming units (CFU) of *murA* WT and S368P strains after being grown to log phase in 7H9, then pelleted and resuspended in PBS+tween80. (F) CFU of *murA* WT and S368P strains grown to log phase in 7H9 then treated with either 50 μ g/ml isoniazid, 20 μ g/ml rifampin or 5 μ g/ml meropenem. (G) Colony forming units of *murA* WT and S368P strains after being grown to log phase in 7H9, then pelleted and resuspended in in PBS+tween80. At the 18 hr time point either 100 μ g/ml isoniazid, 40 μ g/ml rifampin or 20 μ g/ml of meropenem was added. Error bars represent the standard deviation.

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A. CwIM domain structure



B. Phylogeny of *cwIM* in actinobacteria



C. Molecular model of PknB-CwIM-MurA regulation

Nutrient rich conditions:

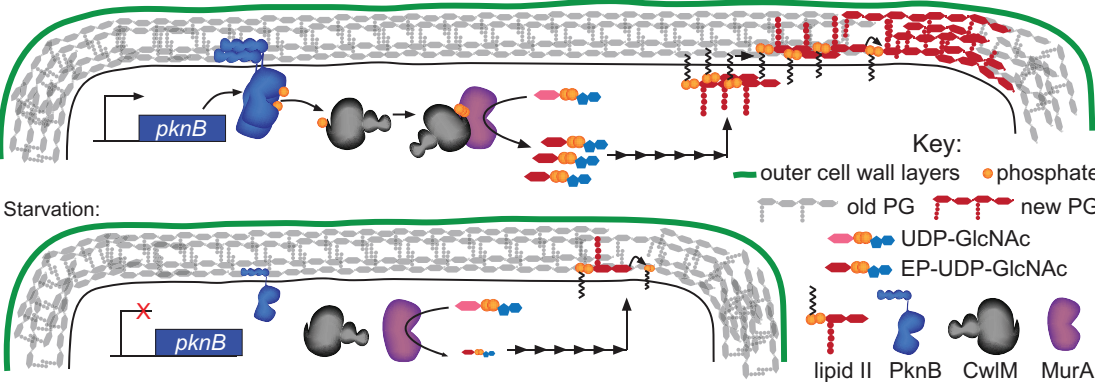


Figure 7. Phylogenetics and model for the PknB-CwIM-MurA regulatory pathway. (A) Domain structure of CwIM from mycobacteria. (B) Phylogenetic analysis of *cwIM* in the actinobacteria. Tree is based on a 16S rRNA alignment for each species. Species indicated in gray do not contain *cwIM* Figure 7 continued on next page

Figure 7 continued

homologues, and are representatives of larger clades. All homologues lack a predicted secretion signal. The conservation of an intact or degenerate amidase active site and the presence of a PTG or TGT phosphorylation motif are indicated by color. (C) Model for the regulation of MurA. In nutrient rich conditions PknB phosphorylates CwlM, which can then promote the activity of MurA, resulting in increased flux through the PG biosynthetic pathway (indicated by the series of arrows) and promoting cell growth. In starvation, PknB is poorly expressed, CwlM is not phosphorylated, and MurA is therefore not activated, resulting in reduced production of PG precursors, which helps to halt cell growth.

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