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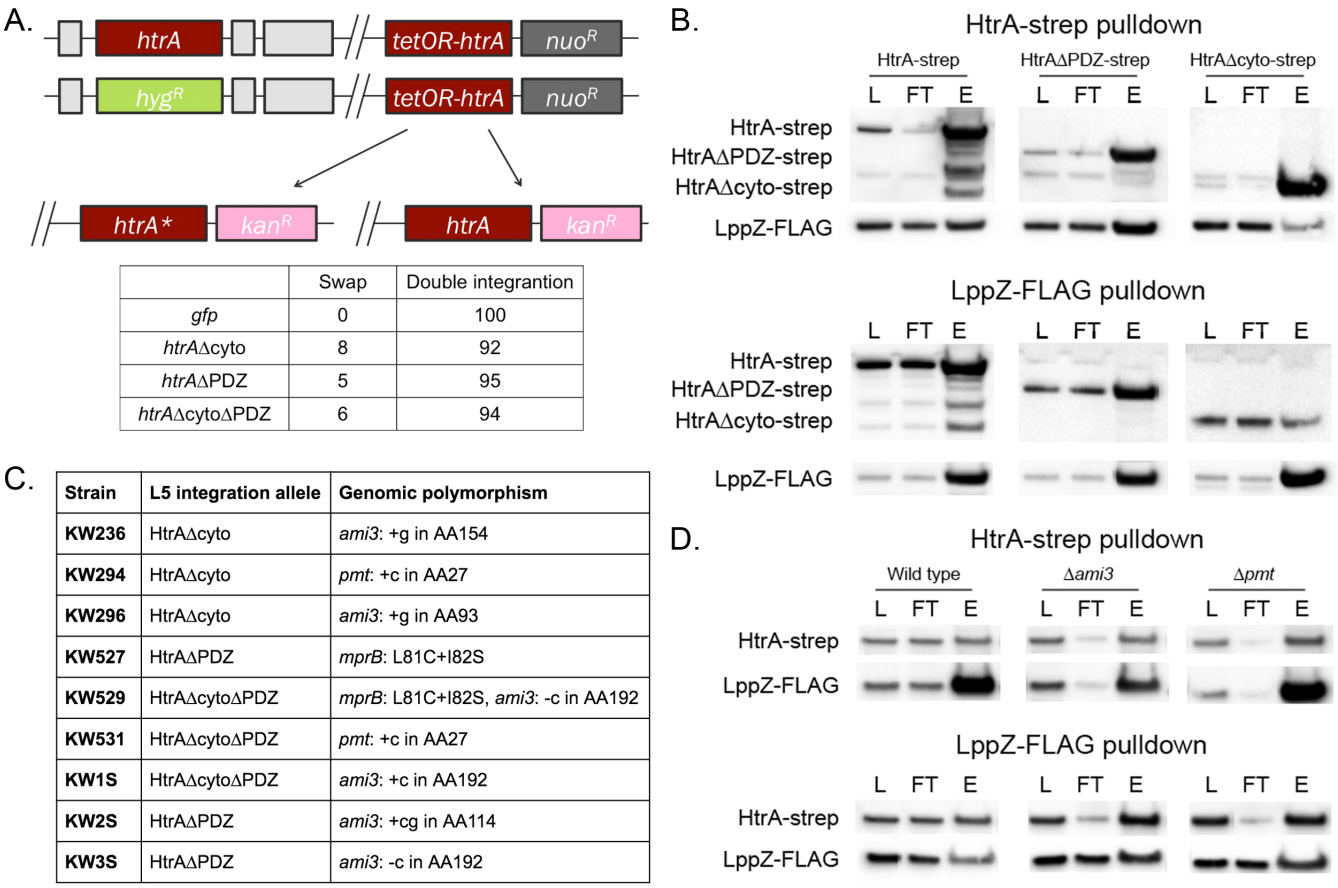
Supplemental Information

Mycobacterium smegmatis HtrA Blocks the Toxic Activity of a Putative Cell Wall Amidase

Katherine J. Wu, Cara C. Boutte, Thomas R. Ioerger, and Eric J. Rubin

SUPPLEMENTAL FIGURES

Figure S1



	Swap	Double integrantion
gfp	0	100
*htrA*Δcyto	8	92
*htrA*ΔPDZ	5	95
*htrA*ΔcytoΔPDZ	6	94

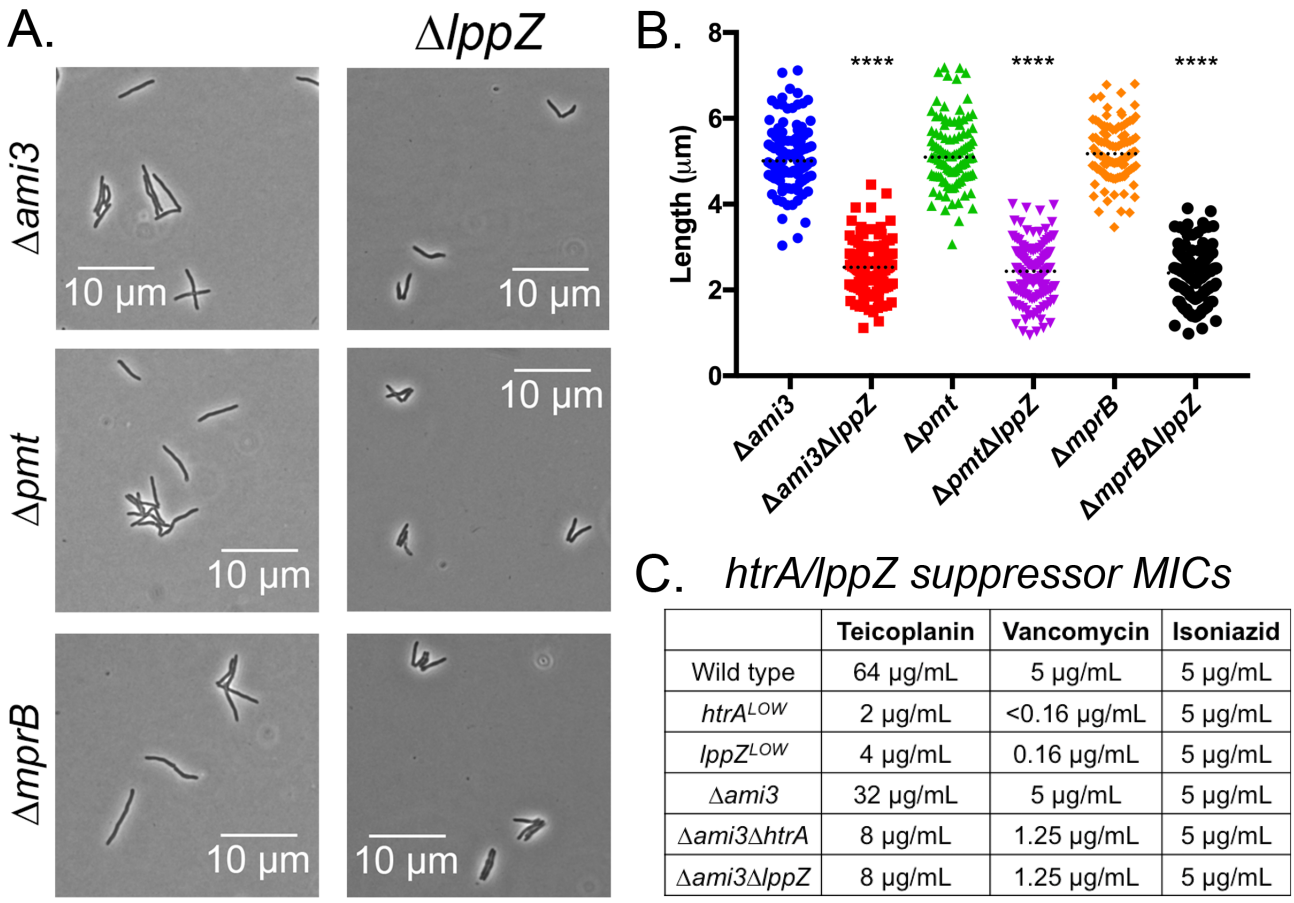
C.

Strain	L5 integration allele	Genomic polymorphism
KW236	HtrAΔcyto	*ami3*: +g in AA154
KW294	HtrAΔcyto	*pmt*: +c in AA27
KW296	HtrAΔcyto	*ami3*: +g in AA93
KW527	HtrAΔPDZ	*mprB*: L81C+I82S
KW529	HtrAΔcytoΔPDZ	*mprB*: L81C+I82S, *ami3*: -c in AA192
KW531	HtrAΔcytoΔPDZ	*pmt*: +c in AA27
KW1S	HtrAΔcytoΔPDZ	*ami3*: +c in AA192
KW2S	HtrAΔPDZ	*ami3*: +cg in AA114
KW3S	HtrAΔPDZ	*ami3*: -c in AA192

Figure S1, related to Figure 2: HtrA suppressor screen and HtrA-LppZ interactions. A. The cytoplasmic and PDZ domains of HtrA are essential for viability. Top: a schematic of the L5 essentiality swap. Placing a second copy of *htrA,* along with a nourseothricin resistance cassette, at the L5 phage integration site allows replacement of endogenous *htrA* with a hygromycin resistance cassette. The L5-integrated copy of *htrA* can be swapped for another copy of *htrA* attached to another antibiotic resistance marker, but not for truncations of *htrA* missing the cytoplasmic and/or PDZ domains (*htrA**). Bottom: quantification of *htrA* swaps. A total of 100 transformants were tested for antibiotic resistance. **B. HtrA and LppZ still interact even when the PDZ or cytoplasmic domains of HtrA are removed.** Different alleles of HtrA-Strep and LppZ-FLAG were individually immunoprecipitated using anti-Strep and anti-FLAG magnetic beads, respectively, and the following fractions were analyzed by Western blot: L = lysate, FT = flow through, E = elution. **C. Successful HtrA truncation swaps were whole genome sequenced for extragenic suppressors.** All strains sequenced carried mutations in *ami3, pmt*, and/or *mprB.* **D. HtrA and LppZ interact even in the absence of Ami3 or Pmt.** In the indicated genetic backgrounds, HtrA-Strep and LppZ-FLAG were individually immunoprecipitated using anti-Strep and anti-FLAG magnetic beads, respectively, and the

following fractions were analyzed by Western blot: L = lysate, FT = flow through, E = elution. Western blot images were cropped, but display all relevant lanes and reactive bands.

Figure S2



C. *htrA/lppZ suppressor MICs*

	Teicoplanin	Vancomycin	Isoniazid
Wild type	64 μg/mL	5 μg/mL	5 μg/mL
htrA^{LOW}	2 μg/mL	<0.16 μg/mL	5 μg/mL
lppZ^{LOW}	4 μg/mL	0.16 μg/mL	5 μg/mL
Δ*ami3*	32 μg/mL	5 μg/mL	5 μg/mL
Δ*ami3*Δ*htrA*	8 μg/mL	1.25 μg/mL	5 μg/mL
Δ*ami3*Δ*lppZ*	8 μg/mL	1.25 μg/mL	5 μg/mL

Figure S2, related to Figure 2: Suppressors of *htrA* essentiality also suppress *lppZ* essentiality and produce morphologically similar cells. A and B. Morphology of *lppZ* suppressor strains. Single suppressor knockouts and *lppZ* double knockouts were grown to log phase and analyzed for total cell length. At least 100 cells were quantified in each condition. Dotted black lines indicate median values. **** = p-value <0.0001. **C. Loss of *htrA* or *lppZ* in a suppressor background partially rescues antibiotic susceptibility.** The indicated strains were grown in teicoplanin, vancomycin, and isoniazid.

Figure S3

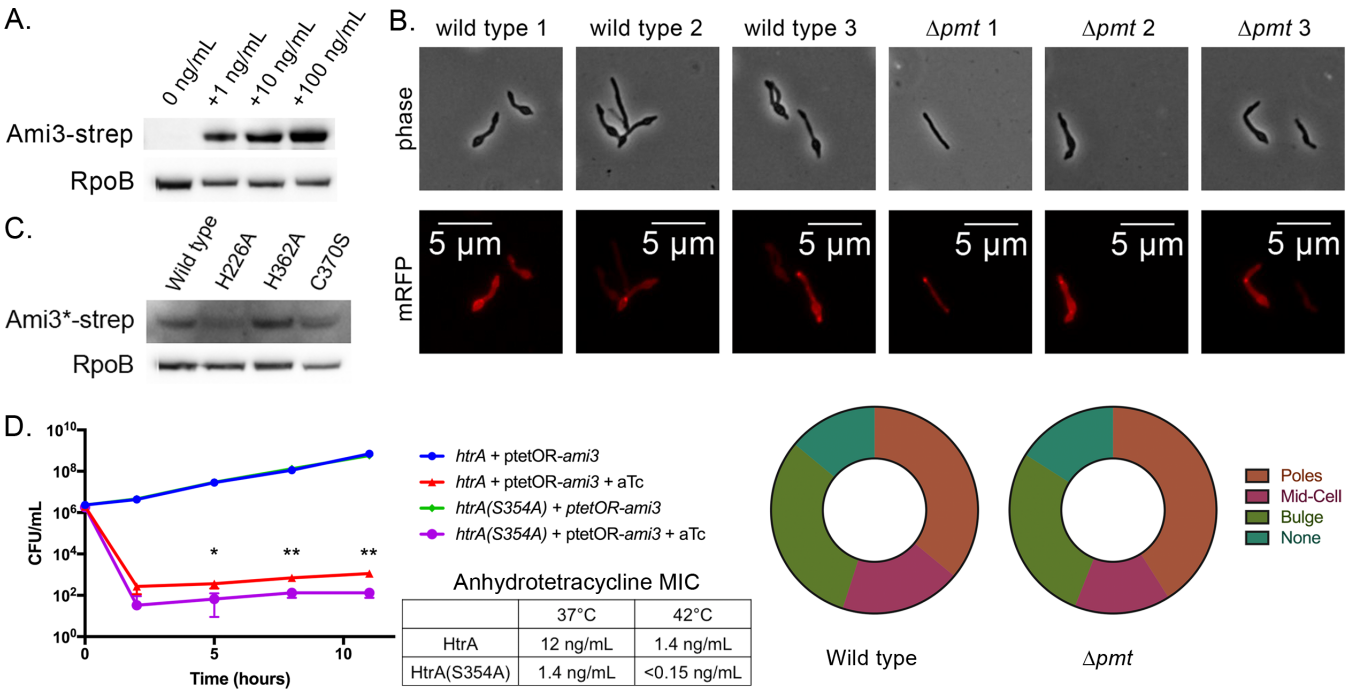


Figure S3, related to Figure 3 and Figure 4: Variable toxicity of Ami3. A. Different amounts of aTc induce different amounts of Ami3. A strain carrying an aTc-inducible copy of *ami3* was grown in the indicated concentrations of aTc for two hours. Cell lysate was analyzed by Western blotting using anti-Strep and anti-RpoB as a loading control. **B. Ami3-mRFP localization.** Ami3-mRFP was expressed under an aTc-inducible promoter on an episomal vector in either wild-type or Δ*pmt* cells. Strains were analyzed by microscopy after 2 hours of induction with 100 ng/mL aTc. The localization of Ami3-mRFP to the poles, mid-cell, or bulges was quantified for both strains. In some cases, cells did not exhibit clear Ami3-mRFP localization; these were marked as "None." 100 cells were counted in each strain. **C. Catalytic mutants of Ami3 accumulate to varying degrees.** Whole cell lysate of the indicated strains was analyzed by Western blotting using anti-Strep and anti-RpoB as a loading control. Ami3* indicates the respective Ami3 allele. **D. Killing dynamics of Ami3 overexpression in different HtrA genetic backgrounds.** Left: Strains expressing either wild-type *htrA* or *htrA(S354A)* and *ami3* under an aTc-inducible episomal construct were grown in the presence or absence of 100 ng/mL aTc. Aliquots were taken at the indicated time points for CFU analysis. *p<0.05, **p<0.01. Error bars represent standard deviation of the mean. Right: the aTc MIC of strains expressing either wild-type *htrA* or *htrA(S354A)* and *ami3* under an aTc-inducible episomal construct was measured at two different temperatures. Western blot images were cropped, but display all relevant lanes and reactive bands.

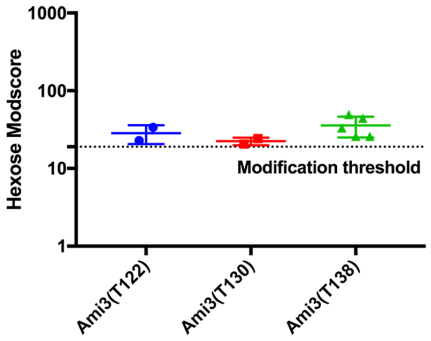
Figure S4

A.

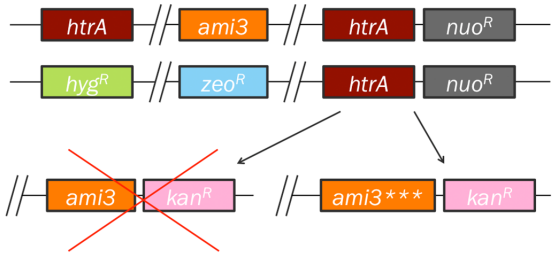
Background	Total Peptides	Unique Peptides
Wild type	1400	62
Δ*pmt*	1524	60

Residue	XCorr	ΔCorr	# Ions	Peptide	Max	PPM
T122	1.284	0.314	15/84	R.T#NTVQIAITRPENAAPTAPAPK.N	2.01E+05	0.44
T130	2.324	0.018	25/84	R.TNTVQIAIT#RPENAAPTAPAPK.N	1.73E+07	-0.4
T138	2.139	0.265	23/84	R.TNTVQIAITRPENAAPT#APAPK.N	1.73E+07	-0.57
T138	3.164	0.406	27/84	R.TNTVQIAITRPENAAPT#APAPK.N	1.73E+07	-0.41

B.



C.



	Swap	Double integration
Wild type	0	60
T122A	0	60
T130A	0	60
T138A	0	60
T122A/T130A	33	27
T122A/T138A	0	60
T130A/T138A	0	60
T122A/T130A/T138A	26	34

D.

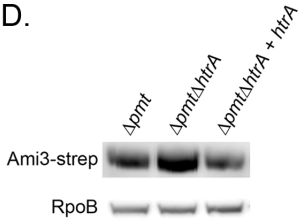


Figure S4, related to Figure 5: Ami3 is mannosylated. A. Hexose modified peptides of Ami3. Ami3-Strep was immunoprecipitated from wild-type and Δ*pmt* backgrounds and analyzed by mass spectrometry for hexose modifications. Total peptides and unique peptides from each strain's samples are listed. Only the wild-type sample yielded modifications, which are scored below. **B. Hexose modification scores of Ami3.** Scores over 19 signify a confident assignment of modification. **C. Toxicity of Ami3 mannosylation mutants.** Top: the endogenous copies of *ami3* and *htrA* were replaced with zeocin and hygromycin resistance cassettes, respectively, and a copy of *htrA* was integrated at the L5 site. *ami3* or mannosylation mutant alleles of *ami3* (*ami3****) were transformed into this background. Full swaps that acquire kanamycin resistance at the expense of noursethricin resistance render strains devoid of *htrA* and must thus carry a suppressor mutation. Bottom: quantification of *ami3* and *ami3**** swaps. A total of 60 transformants were tested for antibiotic resistance. **C. Ami3 stability is still dependent on HtrA in the absence of Pmt.** Whole cell lysate of the indicated strains was analyzed by Western blotting using anti-Strep and anti-RpoB as a loading control. Western blot images were cropped, but display all relevant lanes and reactive bands.

Figure S5

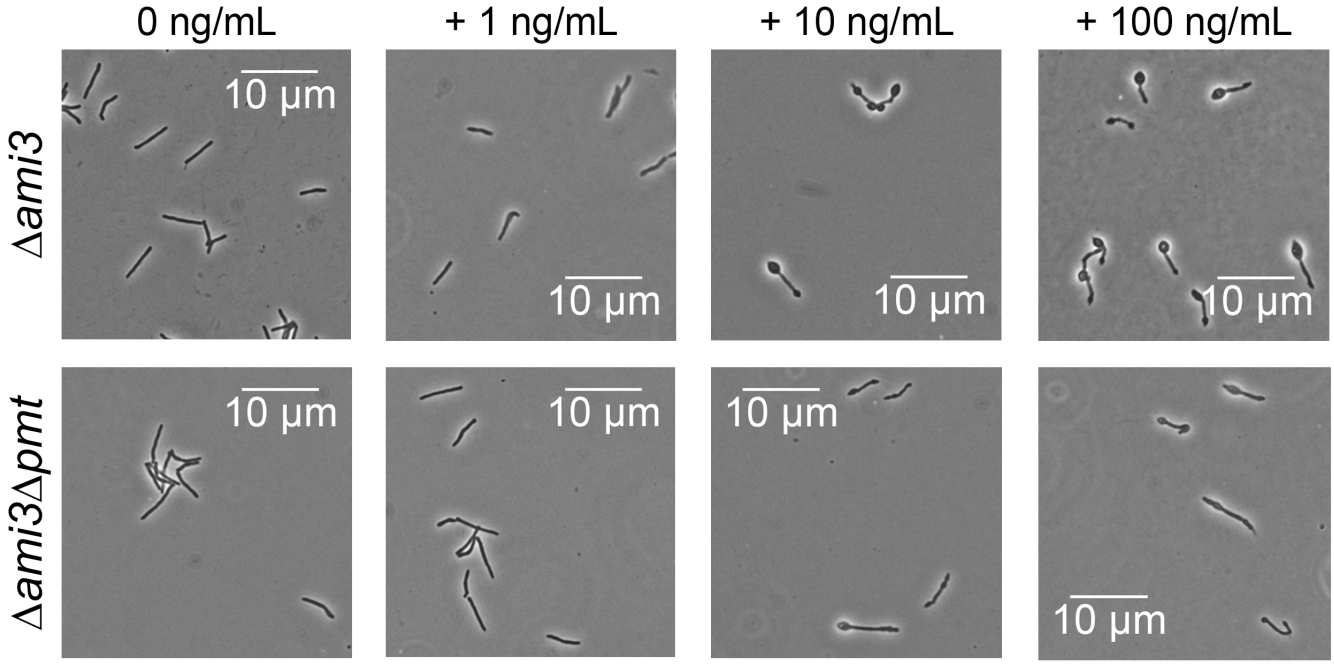
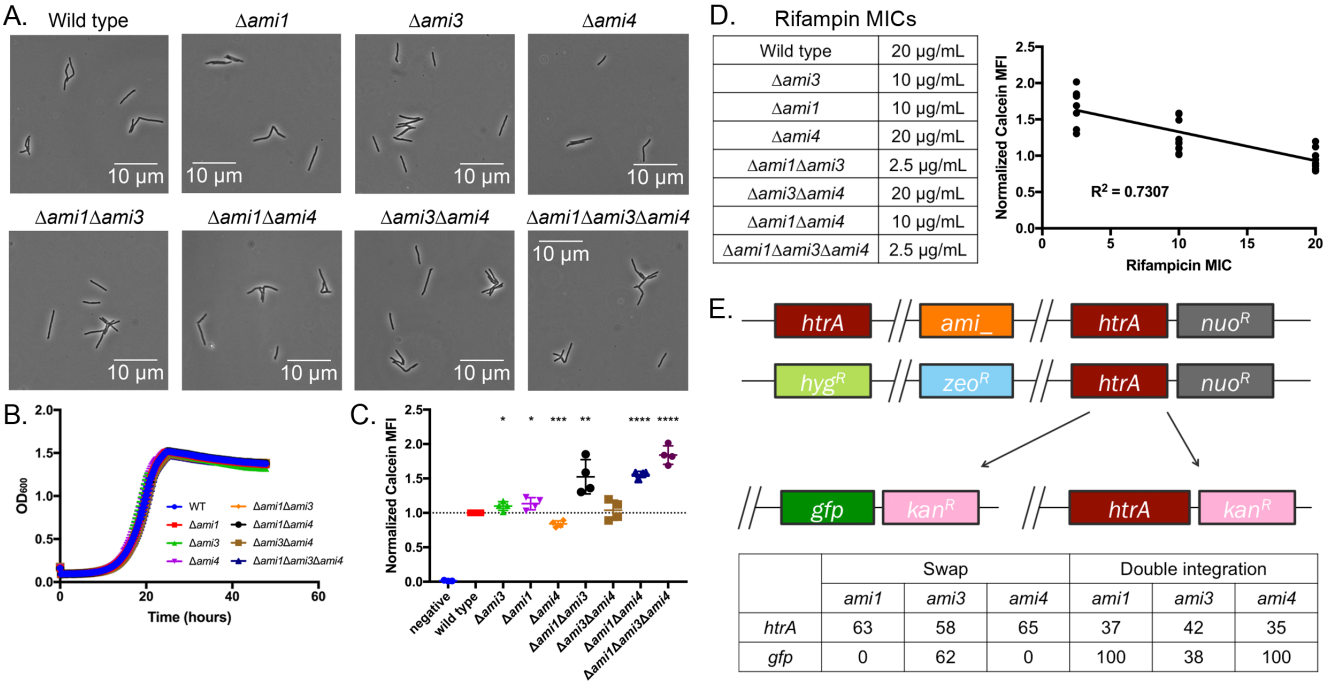


Figure S5, related to Figure 6: Loss of *pmt* relieves morphological defects in Ami3 overexpressions in a dose-dependent manner. Strains expressing *ami3* in a wild-type or Δ*pmt* background under an aTc-inducible promoter were grown in the indicated concentrations of aTc.

Figure S6



Wild type	20 μg/mL
Δ*ami3*	10 μg/mL
Δ*ami1*	10 μg/mL
Δ*ami4*	20 μg/mL
Δ*ami1*Δ*ami3*	2.5 μg/mL
Δ*ami3*Δ*ami4*	20 μg/mL
Δ*ami1*Δ*ami4*	10 μg/mL
Δ*ami1*Δ*ami3*Δ*ami4*	2.5 μg/mL

	Swap			Double integration		
	ami1	*ami3*	*ami4*	*ami1*	*ami3*	*ami4*
htrA	63	58	65	37	42	35
gfp	0	62	0	100	38	100

Figure S6, related to Figure 2: Ami1, Ami3, and Ami4 contribute to cell wall impermeability. A and B. Single and combinatorial knockouts of *ami1, ami3,* and *ami4* grow normally. The indicated strains were grown to log phase and observed by microscopy, or grown at 37°C. Error bars represent standard deviation of the mean. **C and D. Combinatorial amidase knockouts exhibit increased permeability to calcein and rifampin.** The indicated strains were grown in the presence of calcein or rifampin; calcein permeability and rifampin MIC are negatively correlated. Calcein mean fluorescence intensity (MFI) was measured by flow cytometry and normalized to wild type. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars represent standard deviation of the mean. **E. Knocking out *ami3,* but not *ami1* or *ami4*, suppresses *htrA* essentiality.** Top: the endogenous copies of the indicated amidase allele and *htrA* were replaced with zeocin and hygromycin resistance cassettes, respectively, and a copy of *htrA* was integrated at the L5 site. *htrA* or *gfp* were transformed into this background. Full swaps that acquire kanamycin resistance at the expense of noursethricin resistance render strains devoid of *htrA* and must thus carry a suppressor mutation. Bottom: quantification of amidase suppressor swaps. A total of 100 transformants were tested for antibiotic resistance.