

Supplementary Figure Legends

Movie S1. Representative timelapse of wt *V. cholerae* sphere recovery. Cells were exposed to PenG in M9 MM + glucose, followed by washing and transfer to an agarose pad containing M9 MM + glucose. Frames are 5 min apart.

Table S1 Oligonucleotides used in this study

Table S2 Strains and plasmids

Figure S1. Fluorescent protein fusion to PBP1a is functional. Overnight cultures of the indicated strains were plated on LB agar containing $100 \mu\text{g ml}^{-1}$ cefsulodin, an inhibitor of PBP1B (1).

Figure S2. HADA staining in recovering spheres treated with inhibitors of aPBPs or the Rod system. Frames are taken from the experiment as shown in **Figure 4**. The pixel intensity range was set to 100 – 2000 in all images to illustrate the above background fluorescence in moenomycin-treated cells. Note that this results in overexposure of the untreated sample image.

Figure S3. A) TnSeq hits below the cutoff (≥ 10 -fold fitness defect, $p\text{-val} < 0.01$). **B)** representative Artemis plots of the genes encoding Heptosyltransferase I (*vc0225*) and

the Sigma E operon. POST results from analyses Post-antibiotic; OG results from analyses after post-antibiotic outgrowth.

Figure S4. Silver stain of *V. cholerae* isolated outer membranes, comparing wild type and the *vc0225* mutant. The red arrow points to high molecular weight structures, likely core + O-antigen, M, marker.

Figure S5. Polymyxin sensitivity of *alm* and *eptA* mutants. Overnight cultures of the indicated strains were plated on LB agar containing Polymyxin B at 10 $\mu\text{g ml}^{-1}$ and IPTG (100 μM) where applicable.

Supplementary References

1. Dorr T, Moll A, Chao MC, Cava F, Lam H, Davis BM, Waldor MK. 2014. Differential requirement for PBP1a and PBP1b in in vivo and in vitro fitness of *Vibrio cholerae*. *Infect Immun* 82:2115-24.
2. Chao MC, Zhu S, Kimura S, Davis BM, Schadt EE, Fang G, Waldor MK. 2015. A Cytosine Methyltransferase Modulates the Cell Envelope Stress Response in the Cholera Pathogen [corrected]. *PLoS Genet* 11:e1005666.

Primer #	Primer name	sequence (5'-3')
TDP1434	mrcArevmCherry	<u>CCTCCTCGCCCTTGCTCAC</u> gagctcgaggatg tcGAACAGCTCTTCACCAGAGCTGG
TDP1435	mrcAfwCherry	<u>GGCATGGACGAGCTGTACAAG</u> gacatcctcgagctc TAGTAACTCTGGTACAGAAAACCGAAT
TDP1436	mCherryfw	gacatcctcgagctc GTGAGCAAGGGCGAGGAGG
TDP1437	mCherryrev	gagctcgaggatg tcCTTGACAGCTCGTCCATGCC
TDP1362	mrcAfwCVD	<u>aggatatgtgatgggttaaaaaggatcgatcc</u> CTTGACATCCATCAACACTGGTTC
DLP401	vc0225fwGP	<u>gcagatcgcaggtcgacggatccaagctct</u> ccccacactccctctgtatcctg
DLP402	vc0225revpGP	<u>ccgggagagctcgatatcgcatg</u> cggtacctctagaccatctaagacgtgcattttgtcc
DLP397	vc0212fwGP	<u>gcagatcgcaggtcgacggatccaagctct</u> ttggcatcgcttcgcagc
DLP398	vc0212revpGP	<u>ccgggagagctcgatatcgcatg</u> cggtacctctagaccatactgcatacgttggacg
DLP519	almpCVDfw	ATATGTGATGGGTTAAAAAGGATCGATCCTATTGGCTCGGTGGCGACC
DLP520	almrev	TCATCATTATTACTCGAGTGGCGCCGCATTA CAACTAGTTGTGCGAGCAACCGATT
DLP521	almfw	TAATGCGGCCGCACTCGAGTAATAATGATGA TGAAAGCAATTCGGTGTATCTAAGAAGTTGA
DLP522	almrevpCVD	<u>AGAGCTCGATATCGCATGCGGTACCTCTAGT</u> CCCTTTGGCCGACTCTAAAATC
DLP539	eptAfwCVD	ATATGTGATGGGTTAAAAAGGATCGATCCTGGCATCAGTGGTGATAGCGG
DLP540	eptARev	TCATCATTATTACTCGAGTGGCGCCGCATTA GACCACGCTAAATTCTGTGAACGTT
DLP541	eptAfw	TAATGCGGCCGCACTCGAGTAATAATGATGA TAGCCATACCCAAACAACTTAGCC
DLP542	eptarevpCVD	<u>AGAGCTCGATATCGCATGCGGTACCTCTAG</u> AAGTACACAAACCTTAAGGAAACAGCCG
DLP232	pbp1ArevpCVD	<u>AGAGCTCGATATCGCATGCGGTACCTCTAG</u> TTTACTGCCGAAGATCATTCTACTTTG

underlined = homology overhang for ITA cloning

bold = linker

Strain #	Strain name/genotype	antibiotic resistance	source
WT	WT N16961	smR	
TDW1004	N16961 <i>pbp1a::pbp1amcherry</i>	smR	this study
DL-1	TDW1004 <i>vc0225::pGPkan</i>	smR kanR	this study
DL-2	N16961 Δ <i>alm</i> Δ <i>eptA</i>	smR	this study
DL-3	N16961 <i>vc0225::pGPkan</i>	smR kanR	this study
DL-4	N16961 <i>vc0212::pGPkan</i>	smR kanR	this study
DL-5	N16961 <i>vc0225::pGPkan</i>	smR kanR	this study
DL-6	N16961 <i>vc0212::pGPkan</i>	smR kanR	this study
DL-7	DL-2 <i>vc0225::pGPkan</i>	smR kanR	this study
DL-8	DL-2 <i>vc0212::pGPkan</i>	smR kanR	this study
DL-9	DL-3 <i>lacZ::Ptac:vc0225</i>	smR	this study
DL-10	DL-4 <i>lacZ::Ptac:vc0212</i>	smR	this study
	N16961 Δ <i>ompU</i> Δ <i>rpoE</i>	smR	Chao et al. (2015) Plos Genetics 11 (2)
	N16961 Δ <i>ompU</i>	smR	Chao et al. (2015) Plos Genetics 11 (2)

LB

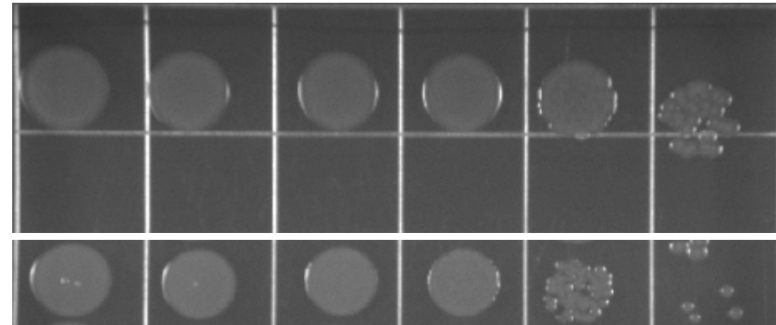
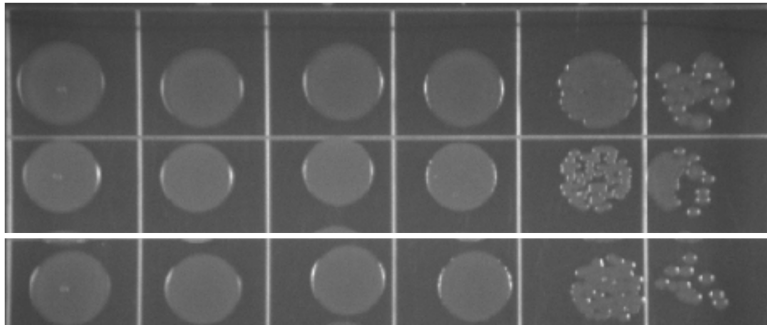
100 $\mu\text{g/ml}$ cefsulodin

WT

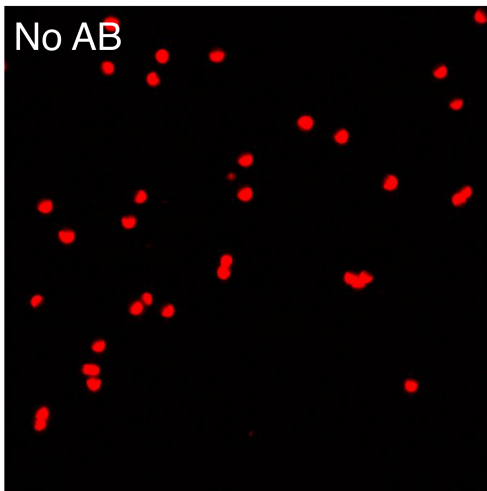
$\Delta pbp1a$

$pbp1a::$

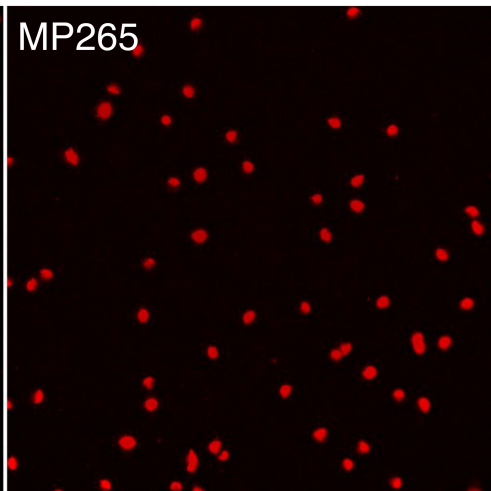
$pbp1amCherry$



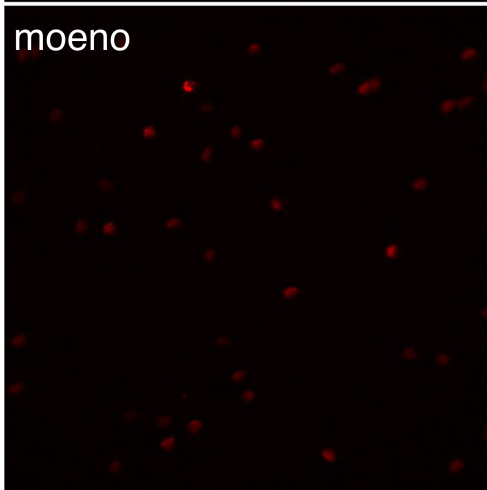
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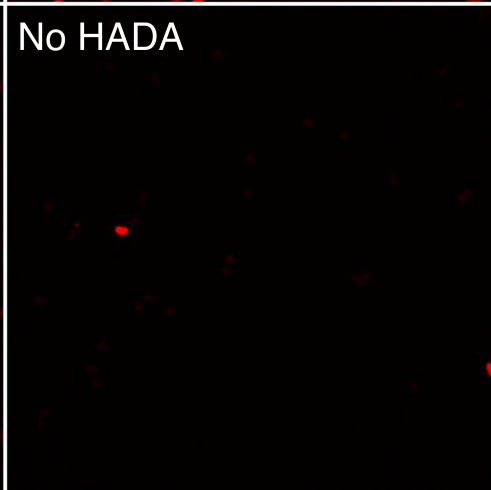
MP265



moeno



No HADA



gene name	annotation	putative role	fold change
VC_2270	riboflavin synthase subunit alpha	Riboflavin biosynthesis	257
VC_2271	riboflavin-specific deaminase	Riboflavin biosynthesis	139
VC_0724	phosphate ABC transporter permease	Phosphate regulation	121
VC_1716	condensin subunit F	Chromosome condensation	109
VC_2153	D,D-carboxypeptidase-like protein	peptidoglycan metabolism	109
VC_1714	cell division protein MukB	Chromosome condensation	90
VC_0212	lipid A biosynthesis (KDO)2-(lauroyl)-lipid IVA acyltransferase	lipopolysaccharide metabolism	89
VC_1263	GTP cyclohydrolase II/ribA	Riboflavin biosynthesis	86
VC_2312	murein transglycosylase A	peptidoglycan metabolism	85
VC_0374	glucose-6-phosphate isomerase	carbon metabolism	81
VC_1894	hypothetical protein VC1894/LpoB	peptidoglycan metabolism	77
VC_1715	condensin subunit E	Chromosome condensation	77
VC_0761	hypothetical protein VC0761/yjgM	envelope stress response	48
VC_0225	lipopolysaccharide biosynthesis protein	lipopolysaccharide metabolism	40
VC_0851	small protein A/BamE	outer membrane metabolism	40
VC_2635	penicillin-binding protein 1A	peptidoglycan metabolism	39
VC_0908	D,D-heptose 1,7-bisphosphate phosphatase	capsule biosynthesis	38
VC_2465	periplasmic negative regulator of sigmaE	envelope stress response	36
VC_0936	polysaccharide export-like protein	capsule biosynthesis	35
VC_0236	ADP-heptose-LPS heptosyltransferase II	lipopolysaccharide metabolism	33
VC_0289	gluconate utilization system gnt-I transcriptional repressor	carbon metabolism	33
VC_A0647	hypothetical protein VCA0647	unknown	29
VC_0034	thiol:disulfide interchange protein	unknown	29
VC_A0424	hypothetical protein VCA0424/DUF3709	unknown	28
VC_2629	shikimate kinase I	amino acid biosynthesis	27
VC_0370	hypothetical protein VC0370/ydIY	salt-induced OM protein	26
VC_1630	ABC transporter ATP-binding protein/LolD	lipoprotein export	23
VC_1021	LuxO repressor protein	quorum sensing	23
VC_0240	ADP-L-glycero-D-manno-heptose-6-epimerase	lipopolysaccharide metabolism	21
VC_0952	hypothetical protein VC0952/rsfS	ribosome downregulation	19
VC_A0804	ATP-dependent RNA helicase DeaD	RNA metabolism	18
VC_0237	O-antigen ligase waaL	lipopolysaccharide metabolism	18
VC_1039	asmA protein	outer membrane metabolism	18
VC_0984	cholera toxin transcriptional activator	pathogenesis	17
VC_0431	arginine repressor ArgR	amino acid biosynthesis	17
VC_2322	exonuclease V subunit gamma-RecC	DNA recombination and repair	16
VC_A0448	hypothetical protein VCA0448	unknown	15
VC_0547	aspartate kinase	amino acid biosynthesis	15
VC_2298	lipoprotein	unknown	15
VC_1697	Short-chain dehydrogenases/reductases	central metabolism	14
VC_0377	hypothetical protein VC0377/CheX	chemotaxis	14
VC_0346	tRNA delta(2)-isopentenylpyrophosphate transferase	tRNA modification	14
VC_A0645	hypothetical protein VCA0645	unknown	14
VC_0378	zinc uptake regulation protein	metal homeostasis	13
VC_0003	tRNA modification GTPase TrmE	tRNA modification	13
VC_A0344	hypothetical protein VCA0344	unknown	13
VC_A0395	hypothetical protein VCA0395	unknown	13
VC_0632	D-alanyl-D-alanine carboxypeptidase/endopeptidase	peptidoglycan metabolism	11
VC_1682	peptide ABC transporter permease	antimicrobial peptide transport	11
VC_0665	Fis family transcriptional regulator	exopolysaccharide synthesis	11
VC_A0573	DamX-like protein	cell division	11
VC_0223	ADP-heptose-LPS heptosyltransferase II	lipopolysaccharide metabolism	11
VC_0727	transcriptional regulator PhoU	Phosphate regulation	10
VC_2320	exodeoxyribonuclease V/RecB	DNA recombination and repair	10
VC_A0405	hypothetical protein VCA0405	unknown	10
VC_1700	intracellular septation protein A	unknown	10

vc0225

OG

POST

vc0225

Sigma E operon

OG

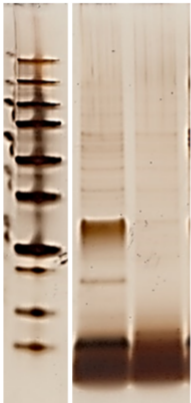
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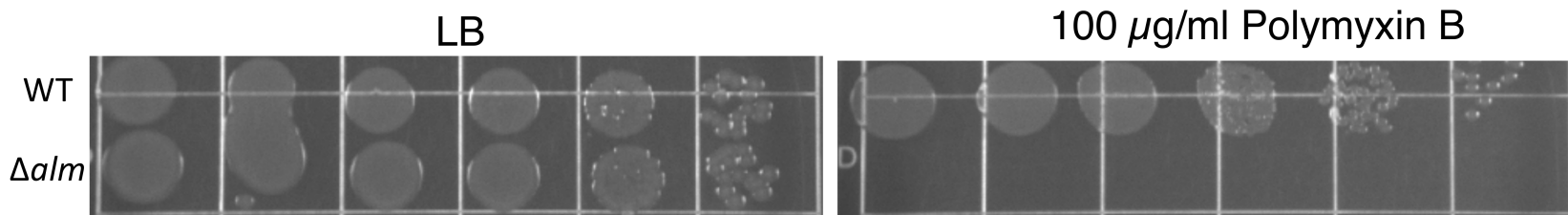


M

WT

vc0225::kan



A**B**