Supporting Information

Structure-guided microbial targeting of antistaphylococcal prodrugs

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WhatsGNU analysis. The *S. aureus* database was used to produce WhatsGNU proteomic reports for all the strains using WhatsGNU_main.py script in the ortholog mode. Eighteen *S. aureus* (9 atopic dermatitis (AD) and 9 soft and skin tissue infection (SSTI)) isolates from an ongoing project representing different clonal complexes (CC1/5/8/22/30) were used for the comparison. The CC details for the 18 isolates are provided in Supplementary Table 10. The reports were then used to produce a heat map of the GNU scores of GloB and FrmB using the heat map function in the WhatsGNU_plotter.py script. The heatmap was annotated with the ortholog variant rarity index where 'r' represents a rare GNU score (in the context of other alleles in the same protein ortholog group).

Phylogenetic tree construction. Sequences of GloB, FrmB, and RpoB orthologs were retrieved from NCBI using the BlastP function with each organism on the tree as an individual search set. Of the returned sequences, the first complete sequence with the lowest E-value was selected for further analysis. Organisms were selected to include a wide variety of pathogenic and commensal microbes[1]. In one instance, several of the top *E. coli* sequences were found to be highly similar to *S. aureus*, and on further analysis we discovered that the original sequencing samples had high levels of *S. aureus* reads. These contaminated sequences were disregarded in our analysis. Sequence alignment was performed using MUSCLE using the default parameters, and the unrooted phylogenetic trees were visualized using iTOL[2,3].

Glyoxalase II activity assay. Glyoxalase II activity was assessed as previously with minor changes[4,5]. Briefly, reactions were mixed to form a final concentration of 25 mM Tris pH 7.5, 250 mM NaCl, 1 mM $MnCl_2$, 10% glycerol, 200 µM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma D8130), 1 mM D-lactoylglutathione (Sigma L7140) and 0.15-0.63 µg protein (130-550 nM GloB, 100-430 nM FrmB). Protein concentrations were varied to ensure the reaction was linear across

protein concentrations. Reactions without D-lactoylglutathione were pre-incubated at 37ºC for 10 min prior to assay initiation with the addition of substrate. Release of glutathione from D-lactoylglutathione was quantified spectrophotometrically at 37ºC and 412 nm through the conversion of DTNB to TNB. Experiments were performed in triplicate with technical duplicates.

4-nitrophenyl ester substrate activity assays. 4-Nitrophenyl substrate specific activity was determined in 50 µL reactions containing 25 mM Tris pH 7.5, 250 mM NaCl, 1 mM $MnCl_2$, 10% glycerol, 1 µM protein, and 1 mM 4-nitrophenyl substrate. The tested substrates, 4-nitrophenyl acetate (Sigma, N8130), 4-nitrophenyl butyrate (Sigma N9876), and 4-nitrophenyl trimethylacetate (Sigma 135046) were resuspended in acetonitrile at 100 mM. Reactions without 4-nitrophenyl substrate were preincubated at 37ºC for 10 min prior to assay initiation via substrate addition. Conversion of 4-nitrophenyl substrates to 4-nitrophenol was tracked photometrically at 37ºC and A_{405nm}. Experiments were performed in triplicate with technical duplicates.

NMR characterization of GloB and FrmB activation products. 200 or 400 µM POM-HEX was incubated with 4 nmol GloB, FrmB, or 4 nmol each GloB and FrmB in 500 µL reactions. Reactions were buffered to a final concentration of 50 mM Tris pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$. Reactions were allowed to proceed for 1 h at 37˚C prior to analysis. Samples were prepared for NMR studies by resuspending them in water and 10% (50 µL) D_2O (deuterium oxide 99.9% D, contains 0.75 wt% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt, Sigma–Aldrich). NMR spectra are acquired on a Bruker Avance III HD 500 MHz spectrometer equipped with a cryoprobe. Two-dimensional (2D) ^{1}H-^{31}P heteronuclear single quantum correlation (HSQC) measurements were obtained using hsqcetgp pulse program (with duration

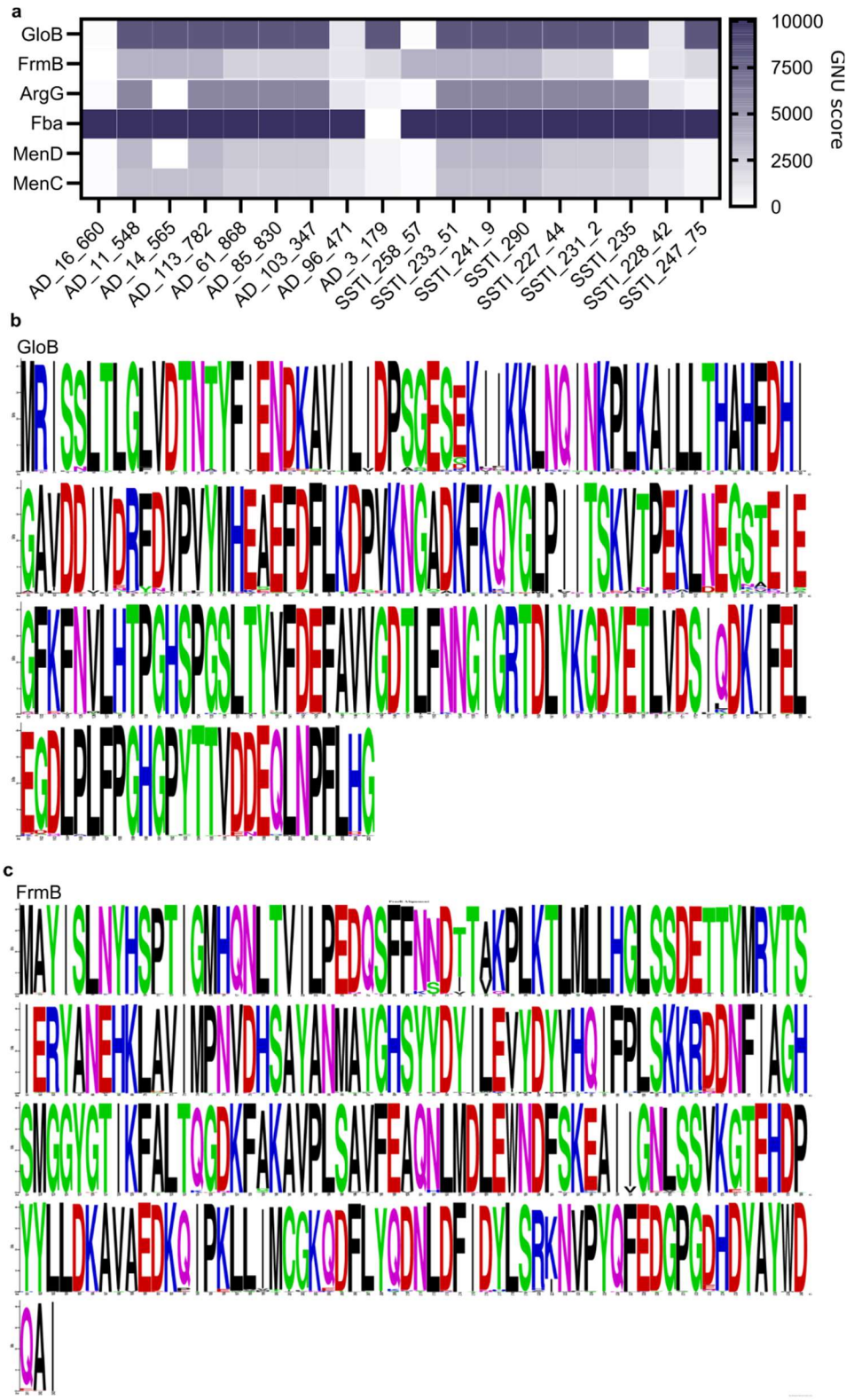
of 15 min and scan parameters of 2 scans, td=1024 and 256, gpz2 %=32.40, ^{31}P SW= 40 ppm, O2p=20 ppm, cnst2=22.95) and analyzed using 3.1 TopSpin. The 1D projection of columns excluding the water signal was obtained from the 2D ^{1}H-^{31}P HSQC spectrum by obtaining spectra of positive projection of columns 1 to 600 and 650 to 1024 and adding them.

Esterase substrate specificity determination using fluorogenic SAR library. Kinetic measurements were performed according to White *et al.* with minor variation[6]. Lyophilized human and mouse sera were resuspended according to manufacturer instructions in highly pure, filtered water at protein concentrations of 85 mg/mL and 70 mg/mL, respectively. 1 mL of resuspended serum was added to a 24 mL mastermix for a final concentration of 31.25 mM Tris pH 7.5, 312.5 mM NaCl, 1.25 mM $MgCl_2$, 12.5% glycerol, and 3.4 mg/mL or 2.8 mg/mL protein for human and mouse serum, respectively. For purified proteins, 5 mL of a 75 µg/mL stock was added to yield a 20 mL mastermix containing 31.25 mM Tris pH 7.5, 312.5 mM NaCl, 1.25 mM $MgCl_2$, 12.5% glycerol, and 18.75 µg/mL protein. Mastermix was stored on ice when not in use. 20 µL of mastermix was transferred to a black, 96-well half area microplate (Corning, CLS3993) and prewarmed at 37°C. Fluorogenic substrates were prepared as 10 mM stock solutions in 100% DMSO and were diluted in water to a starting concentration of 500 µM. Enzyme catalyzed substrate hydrolysis was initiated by addition of 5 µL substrate dilution in technical duplicate to the prewarmed serum or protein solution. Final assay concentrations were: 25 mM Tris pH 7.5, 250 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, and protein at a concentration of 2.72 mg/mL (human serum), 2.24 mg/mL (mouse serum), or 15 µg/mL (FrmB, GloB). The resulting change in fluorescence (λ_{ex} = 485 nm, λ_{em} = 520 nm) was followed for 15 min at 37°C, collecting data every 30 sec on a FLUOstar Omega microplate reader (BMG Labtech). Fluorescence measurements were converted to molar concentrations using a fluorescein standard curve (2.5 nmol-0.6 pmol). The initial rates of reaction were measured three independent times with two

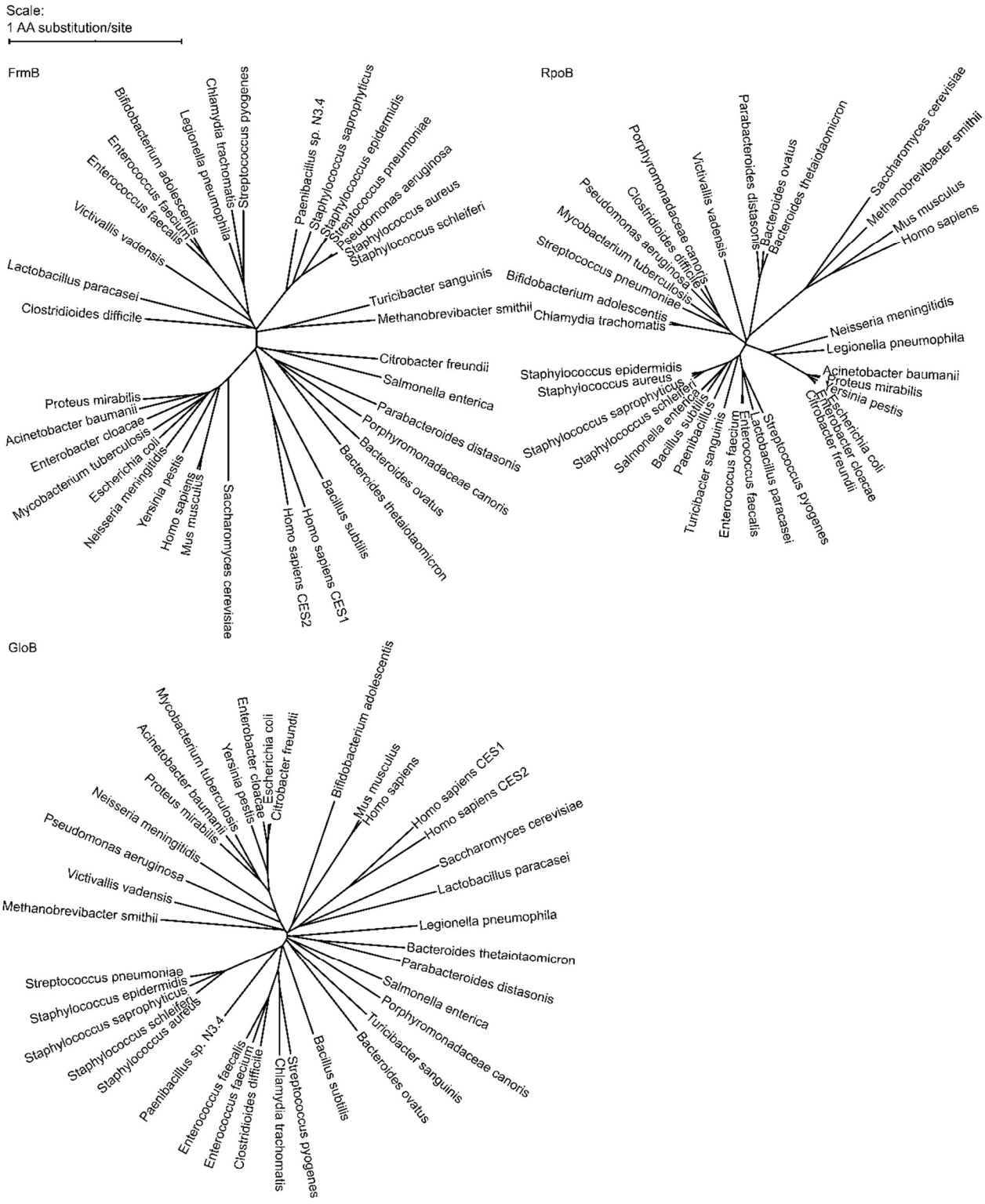
technical replicates per measurement and fit to a line using GraphPad Prism (GraphPad Software, La Jolla, CA). Initial rates of reaction were plotted versus the concentration of substrate and fit to a standard Michaelis-Menten equation ($v = V_{max}[S]/(K_m + [S])$, yielding estimates of V_{max} and K_m. Values for k_{cat} and k_{cat}/K_m were calculated based on amount of enzyme added when purified enzymes were used. For substrates that did not display saturation in the velocity versus substrate experiment (i.e., $K_m >>> [S]$), V_{max}/K_m was estimated based on v/[S].

Serum half-life determination. Lyophilized human sera was obtained from Rockland Inc. and resuspended in pure water. 20 µL lyophilized sera or fresh sera was prewarmed at 37ºC in a 96-well half-area microplate (Corning, CLS3993). Following plate warming, 5 µL of the fluorogenic substrates were added to the plate for a final concentration of 25 µM. Substrate hydrolysis was tracked over a period of three h at 37ºC, with fluorescence measurements (λ_{ex} = 485 nm, λ_{em} = 520 nm) being taken every two min on a FLUOstar Omega microplate reader (BMG Labtech). The resulting fluorescence values were converted to % substrate hydrolyzed using a fluorescein standard curve and fit to a one-phase decay model using GraphPad Prism. Experiments were performed in technical and biological duplicate.

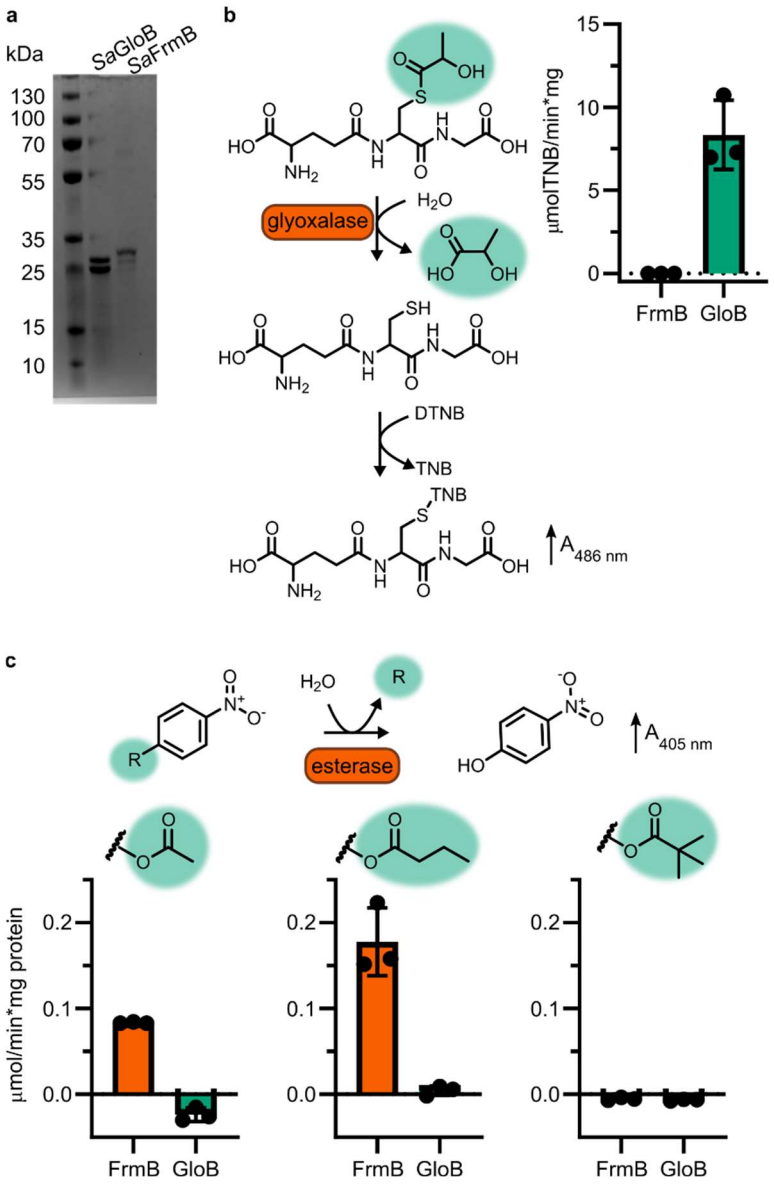
Supplementary Figures



Supplementary Figure 1 Conservation of FrmB and GloB within *S. aureus*. (a) WhatsGNU analysis of GloB and FrmB. Control proteins: ArgG, argininosuccinate synthase;, Fba, fructose-bisphosphate aldolase; MenD, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1carboxylate synthase; and MenC, o-succinylbenzoate synthase. GNU stands for gene novelty unit and is a count of how many protein sequences in the database have an exact match to the queried sequence, with higher counts indicating sequence conservation. Strains across the x-axis are representative strains from the 18 *S. aureus* colony complexes which were used to query the *S. aureus* database. (b, c) MAFFT alignment of GloB (b) and FrmB (c) protein sequences across the *S. aureus* sequence database.

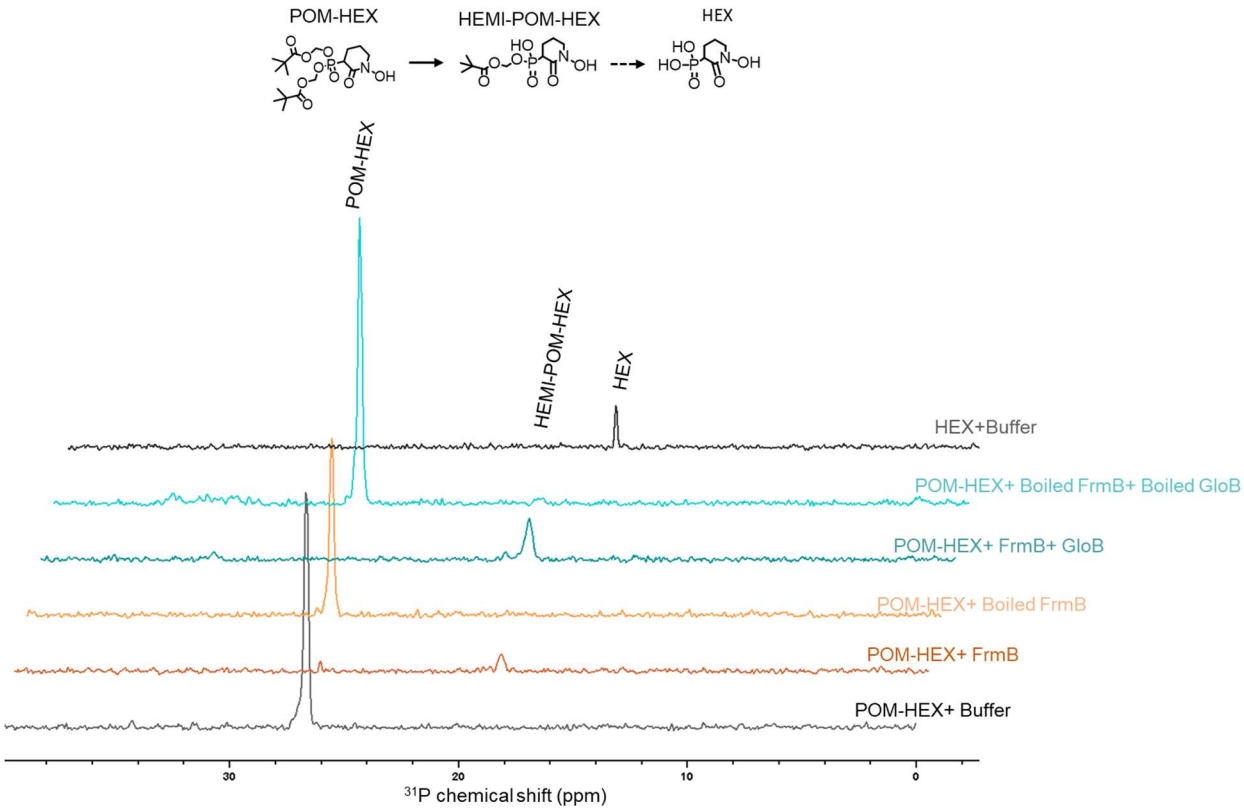


Supplementary Figure 2 Phylogenetic tree of FrmB and GloB. Sequences of GloB, FrmB, and RpoB were retrieved from NCBI using BlastP against each organism. Sequence alignment performed using MUSCLE and alignment visualized using the interactive Tree of Life (iTOL).

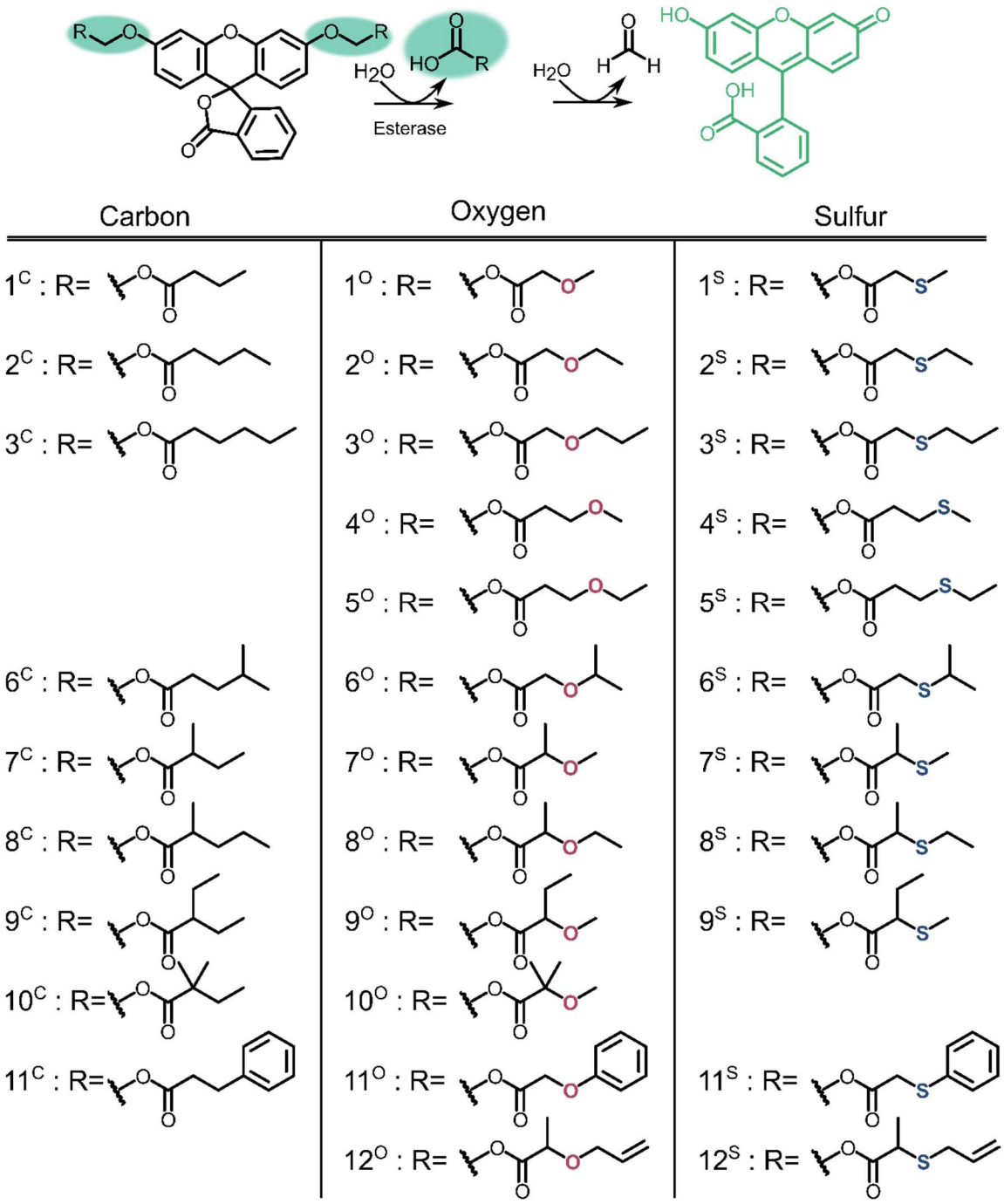


Supplementary Figure 3 Enzymatic characterization of GloB and FrmB. (a) SDS-PAGE gel of GloB and FrmB protein preparations. Expected molecular weights are 23.3 kDa and 29.5 kDa, respectively. (b) Glyoxalase II activity assay, enzymatic conversion of S-lactoylglutathione releases free glutathione and reacts with DTNB resulting in increased absorbance at 412 nm. (c) 4-Nitrophenyl activation results in increased absorbance at 405 nm. Left to right, activity when supplied 4-nitrophenyl acetate, 4-nitrophenyl butyrate, and 4-nitrophenyl trimethyl acetate. Displayed in points is the mean of two technical replicates for individual experiments, bars

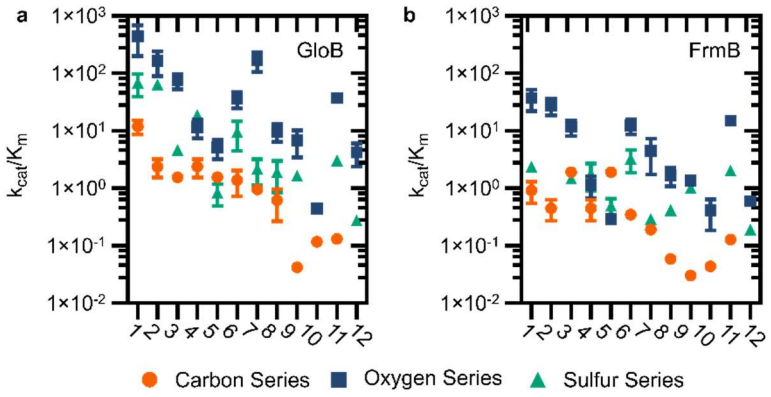
indicate mean ± SD of three independent biological experiments performed in technical duplicate.



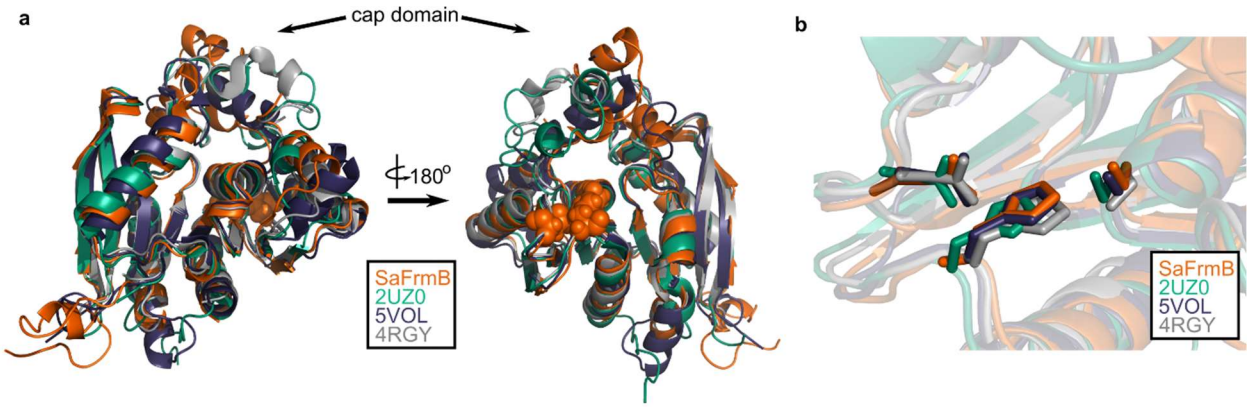
Supplementary Figure 4 NMR characterization of POM-HEX activation by GloB and FrmB. Two-dimensional (2D) ^{1}H-^{31}P HSQC NMR spectra of products following incubation of FrmB, GloB, catalytically inactive (boiled) GloB and FrmB, or buffer alone. Also included are the ^{1}H-^{31}P HSQC NMR spectra of POM-HEX and HEX. Displayed are representative traces of three independent experiments. HEMI-POM HEX peak inferred by predicted shift.



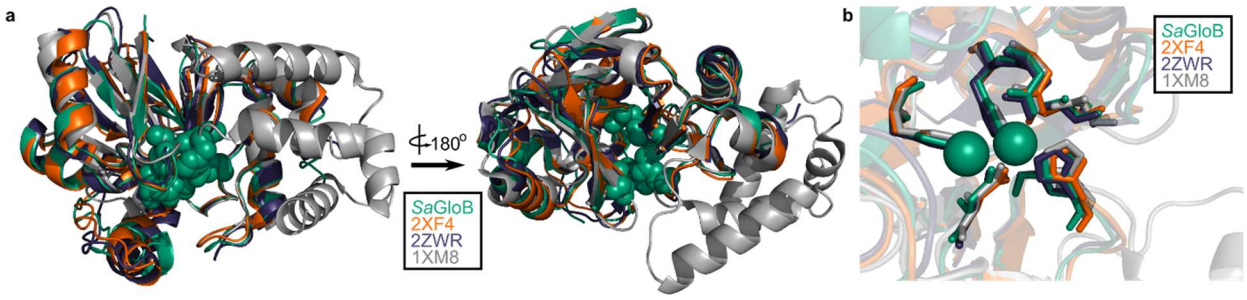
Supplementary Figure 5 Profluorescent substrate library. Activation of substrates via esterase action results in fluorescence.



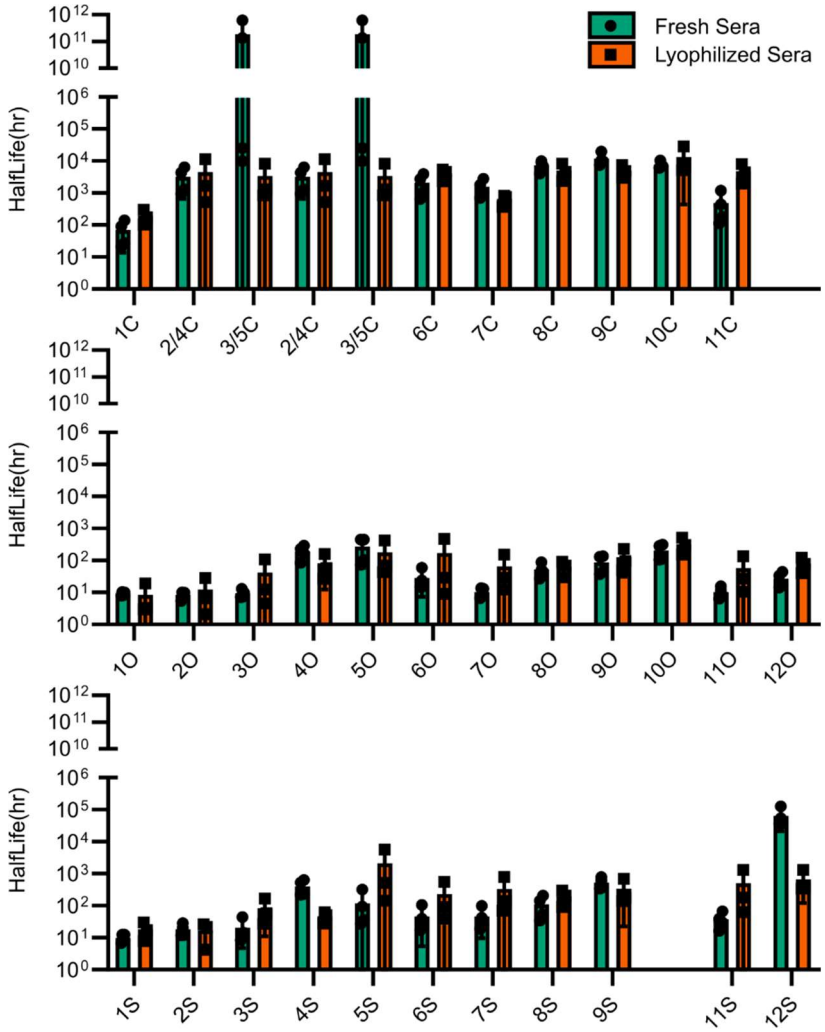
Supplementary Figure 6 Catalytic efficiency of GloB and FrmB. Numbers correspond to the structures displayed in Figure S5, compounds in the carbon series denoted in orange, oxygen series in blue, and sulfur series in green.



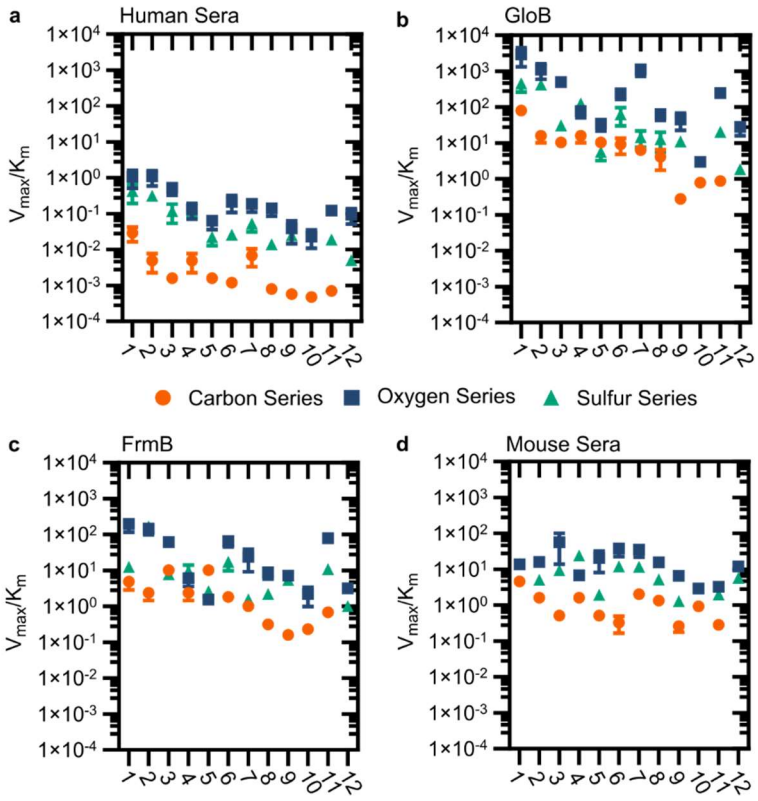
Supplementary Figure 7 Structural conservation of FrmB. (a) Overall structural alignment of FrmB (orange) with *S. pneumonia* EstA (PDB:2UZ0), *B. intestinalis* ferulic acid esterase (PDB:5VOL), and deep sea bacteria Est12 (PDB4RGY). (b) Conservation of the serine hydrolase catalytic triad in FrmB and related proteins.



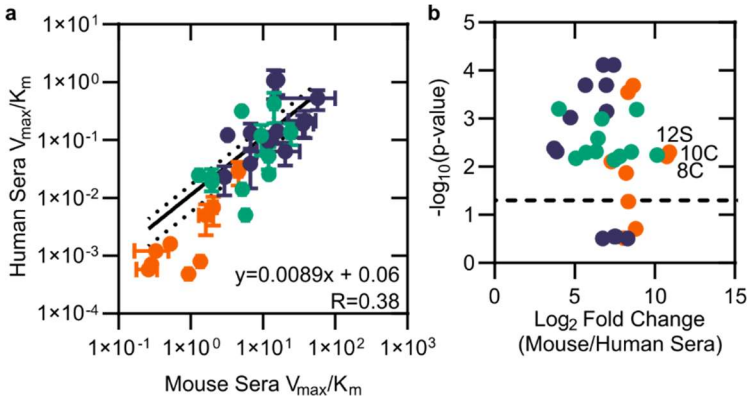
Supplementary Figure 8 Structural conservation of GloB. (a) Overall structural alignment of GloB (green) with *S. enterica* YcbI (PDB:2XF4), *T. thermophilus* TTHA1623 (PDB:2ZWR), and *A. thaliana* glyoxalase II (PDB:1XM8). Zinc coordinating residues are colored in green spheres. (b) Positioning of the zinc coordinating residues (green spheres).



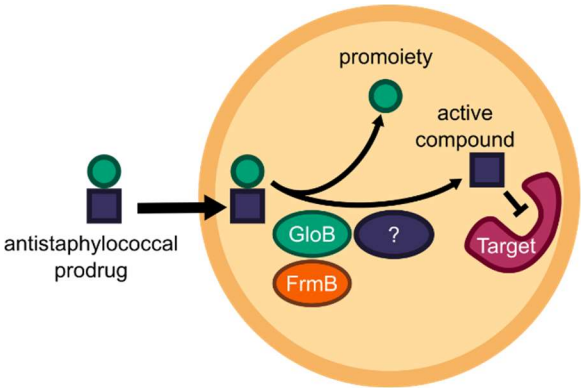
Supplementary Figure 9 Comparison of esterase activity between fresh and lyophilized human sera. Points represent individual experiments; bars represent the mean ± SD of the four replicates.



Supplementary Figure 10 Modified vatalytic efficiency (pmol fluorescein produced * min^{-1}*μg^{-1} protein) of human sera, GloB, FrmB, and mouse sera. X-axis corresponds to compound identities in Figure S5. Carbon containing compounds indicated in orange, oxygen in blue, and sulfur in green. Displayed are the means ± SD of three independent biological experiments.



Supplementary Figure 11 Comparison of mouse and human sera. (a) Modified catalytic efficiency (pmol fluorescein produced * min^{-1}*μg^{-1} protein) of human and mouse sera. Displayed is a linear regression of the fit between mouse and human sera. (b) Volcano plot of catalytic efficiency. Displayed are the means of three independent experiments. P-values calculated as pairwise t-tests with Holm-Sidak correction for multiple comparisons. Dashed line indicates a p-value of 0.05.



Supplementary Figure 12 Model of antistaphylocccal prodrug activation. Lipophilic carboxy ester prodrugs transit the cell membrane, are first activated by either GloB or FrmB and at least one additional enzyme, before inhibiting the cellular target.

Supplementary Table 1 Half-maximal inhibitory concentration (IC_{50}) values for POM-HEX against predicted prodrug activating esterases. IC_{50} values are the result of three independent biological experiments with technical duplicates. P-values calculated as a one-way ANOVA with Dunnett's correction for multiple comparisons.

SAUSA300 gene	Newman gene	StrID (NARSA)	Pan gene symbol	POM-HEX IC_{50} (µM)	POM-HEX IC_{50} SD (µM)	Significantly different from WT (JE2)?	Adjusted P-value	Predicted function	GO term
Parental Strain		JE2		1.54	0.40			N/A	Parental Strain
SAUSA300_0742	NWMN_0727	NE145	*uvrA*	1.46	0.17	ns	0.9999	Exonuclease ABC, A subunit	carboxylic ester hydrolase activity, carboxylesterase activity
SAUSA300_1902	NWMN_1859	NE202		0.700	0.066	ns	0.9101	Hypothetical protein	phosphoric diester hydrolase activity
SAUSA300_2173	NWMN_2121	NE223	*truA*	1.39	0.29	ns	0.9997	tRNA pseudouridine synthase A	phosphatase
SAUSA300_1285	NWMN_1303	NE293		1.41	0.64	ns	0.9997	ABC transporter, ATP-binding protein	phosphatase
SAUSA300_2515	NWMN_2477	NE355	*gbaA*	1.73	0.48	ns	0.9997	Transcriptional regulator, TetR family	carboxylic ester hydrolase activity
SAUSA300_1505	NWMN_1449	NE377	*gloB*	10.6	3.2	****	<0.0001	Hydroxyacylglutathione hydrolase	hydroxyacylglutathione hydrolase
SAUSA300_0142	NWMN_0084	NE478	*phnE*	1.61	0.81	ns	0.9999	Phosphonate ABC transporter, permease protein	phosphoric diester hydrolase activity
SAUSA300_2564	NWMN_2528	NE503	*estA, frmB*	6.28	2.25	****	<0.0001	Tributyrin esterase	carboxylic ester hydrolase activity
SAUSA300_2473	NWMN_2434	NE532		1.56	0.51	ns	>0.9999	Hypothetical Alkaline Phosphatase	phosphatase
SAUSA300_0581	NWMN_0561	NE621		1.62	0.55	ns	0.9999	Hypothetical protein	phosphatase activity
SAUSA300_0299		NE812		1.64	0.56	ns	0.9998	Hypothetical protein	phosphatase activity
SAUSA300_1993	NWMN_1947	NE937	*fruC*	1.67	0.52	ns	0.9998	PfkB family kinase	phosphatase
SAUSA300_1752	NWMN_1700	NE949	*hsdM2*	1.73	0.53	ns	0.9996	type I restriction-modification system, M subunit	phosphoric diester hydrolase activity
SAUSA300_0312	NWMN_0254	NE1039	*psuG*	1.73	0.51	ns	0.9997	Hypothetical protein	carboxylic ester hydrolase activity
SAUSA300_0538	NWMN_0515	NE1071	*capD*	1.54	0.41	ns	>0.9999	NAD-dependent epimerase/dehydratase family protein	phosphatase activity
SAUSA300_1792	NWMN_1735	NE1173		1.84	0.40	ns	0.9994	Hypothetical protein	phosphatase activity

SAUSA300 gene	Newman gene	StrID (NARSA)	Pan gene symbol	POM-HEX IC_{50} (μM)	POM-HEX IC_{50} SD (μM)	Significantly different from WT (JE2)?	Adjusted P-value	Predicted function	GO term
SAUSA300_0421	NWMN_0414	NE1225		2.15	0.48	ns	0.9938	Hypothetical protein	carboxylic ester hydrolase activity, carboxylesterase
SAUSA300_0214	NWMN_0156	NE1238		2.08	0.54	ns	0.9986	Hypothetical protein	phosphoric diester hydrolase activity
SAUSA300_0690	NWMN_0674	NE1296	*saeS*	2.07	0.53	ns	0.9986	Sensor histidine kinase SaeS	phosphatase
SAUSA300_1639	NWMN_1586	NE1486	*phoP*	2.18	0.41	ns	0.9932	Alkaline phosphatase synthesis transcriptional regulatory protein	phosphoric diester hydrolase activity
SAUSA300_0840	NWMN_0808	NE1505		2.20	0.54	ns	0.9927	Hypothetical protein	phosphoric diester hydrolase activity
SAUSA300_1563	NWMN_1507	NE1519	*accC*	1.75	0.70	ns	0.9996	Acetyl-CoA carboxylase, biotin carboxylase	phosphoric diester hydrolase activity
SAUSA300_2508		NE1547		1.48	0.23	ns	>0.9999	Hypothetical protein	phosphatase activity
SAUSA300_0996	NWMN_962	NE1610	*pdhD*	1.23	0.81	ns	0.9993	Dihydrolipoamide dehydrogenase	phosphoric diester hydrolase activity
SAUSA300_2367	NWMN_2320	NE1682	*hlgB*	1.37	0.19	ns	0.9997	Gamma-hemolysin component B	phosphatase

Supplementary Table 2 Genotype and phenotype of POM-HEX resistant *S. aureus*. Displayed are the whole genome sequencing mutations that have been verified. Called mutations that were not observed via confirmatory Sanger sequencing are excluded. IC_{50} values are the result of three independent biologic replicates with technical duplicates.

Gene (Newman)	**WT_SAUR**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**
NWMN_1449 (*gloB*)							c.376G>T p.Val126Leu		c.430T>A p.Phe144Ile	c.433G>A p.Ala145Thr
NWMN_2528 (*estA*, *frmB*)				c.40G>C p.Gly14Arg						
NWMN_0144					c.1472G>T p.Arg491Met					
NWMN_0240										
NWMN_0309				c.23A>C p.Asn8Thr		c.23A>C p.Asn8Thr				c.23A>C p.Asn8Thr
NWMN_0471 (*tilS*)										
NWMN_0523 (*sdrC*)										
NWMN_0564										
NWMN_0954										
NWMN_1152										
NWMN_1311 (*lysA*)										
NWMN_1425 (*recN*)										
NWMN_1655			c.17G>A p.Arg6His	c.17G>A p.Arg6His						
NWMN_1735										
NWMN_1754										
NWMN_2040 (*pdp*)										
NWMN_2253		c.1412C>T p.Ala471Val	c.1412C>T p.Ala471Val			c.1412C>T p.Ala471Val				
NWMN_2388										
POM-HEX IC_{50} (μM)	1.921	6.337	5.932	9.561	10.54	3.752	11.09	10.71	16.96	17.71
Std. Deviation	1.111	0.8778	0.7325	0.4846	6.261	2.594	4.974	6.602	7.264	10.65

Gene (Newman)	R10	R11	R12	R13	R14	R15	R16	R17	R18
NWMN_1449 (*gloB*)	c.70G>T p.Val24Phe	c.166C>T p.His56Tyr	c.326C>A p.Pro109His	c.70G>T p.Val24Phe	c.70G>T p.Val24Phe	c.289C>T p.Gln97*			
NWMN_2528 (*estA*, *frmB*)							c.218_219insCATATGCCATGTTAGCA p.Met74fs	c.366G>A p.Met122Ile	
NWMN_0144									
NWMN_0240				c.432G>T p.Met144Ile					
NWMN_0309	c.23A>C p.Asn8Thr				c.23A>C p.Asn8Thr	c.23A>C p.Asn8Thr		c.23A>C p.Asn8Thr	
NWMN_0471 (*tilS*)								c.760_765dupTTTAAT p.Phe254_Asn255dup	
NWMN_0523 (*sdrC*)		c.2299A>G p.Asn767Asp							
NWMN_0564							c.230_231delCT p.Ser77fs		
NWMN_0954			c.359C>A p.Pro120His						
NWMN_1152									
NWMN_1311 (*lysA*)									
NWMN_1425 (*recN*)									
NWMN_1655		c.17G>A p.Arg6His		c.17G>A p.Arg6His	c.17G>A p.Arg6His	c.17G>A p.Arg6His		c.17G>A p.Arg6His	
NWMN_1735				c.2754C>A p.Phe918Leu					
NWMN_1754								c.232T>A p.Leu78Ile	
NWMN_2040 (*pdp*)		c.426G>T p.Leu142Phe							
NWMN_2253									c.1412C>T p.Ala471Val
NWMN_2388				c.195G>T p.Gln65His					
POM-HEX IC_{50} (μM)	8.353	31.66	11.47	6.746	4.033	5.61	5.559	3.882	2.788
Std. Deviation	3.673	32.94	4.009	2.199	1.276	5.394	2.96	1.153	0.538

Gene (Newman)	R19	R20	R21	R22	R23	R24	R25
NWMN_1449 (*gloB*)						c.401C>T p.Pro134Leu	
NWMN_2528 (*estA*, *frmB*)	c.40G>C p.Gly14Arg		c.366G>A p.Met122Ile		c.356G>A p.Gly119Asp		c.40G>C p.Gly14Arg
NWMN_0144							
NWMN_0240							
NWMN_0309	c.23A>C p.Asn8Thr					c.23A>C p.Asn8Thr	c.23A>C p.Asn8Thr
NWMN_0471 (*tilS*)							
NWMN_0523 (*sdrC*)							
NWMN_0564							
NWMN_0954							
NWMN_1152						c.1570G>T p.Asp524Tyr	
NWMN_1311 (*lysA*)						c.1145C>A p.Ser382Tyr	
NWMN_1425 (*recN*)	c.1130T>C p.Leu377Ser						
NWMN_1655	c.17G>A p.Arg6His	c.17G>A p.Arg6His	c.17G>A p.Arg6His	c.17G>A p.Arg6His	c.17G>A p.Arg6His		
NWMN_1735							
NWMN_1754			c.232T>A p.Leu78Ile				
NWMN_2040 (*pdp*)							
NWMN_2253							
NWMN_2388							
POM-HEX IC_{50} (μM)	5.678	3.847	3.923	4.568	3.794	8.396	3.519
Std. Deviation	2.073	1.061	1.087	4.194	0.903	1.049	0.112

Supplementary Table 3 Half-maximal inhibitory concentration (IC_{50}) values for POM-HEX against transposon mutations in genes identified by whole genome sequencing. Assays performed in biological triplicate with technical duplicates. P-value calculated as a one-way ANOVA with Dunnett correction for multiple comparisons.

SAUSA300 gene	Newman gene	StrID (NARSA)	Pan gene symbol	POM-HEX IC_{50} (µM)	POM-HEX IC_{50} SD (µM)	Significantly different from WT (JE2)?	Adjusted P-value	Predicted function
Parental Strain		JE2		1.54	0.40			N/A
SAUSA300_1781	NWMN_1723	NE64	*hemY*	1.43	0.15	ns	0.9998	protoporphyrinogen oxidase
SAUSA300_0671	NWMN_0654	NE364		1.65	0.72	ns	0.9998	ABC transporter, ATP-binding protein, MsbA family
SAUSA300_1505	NWMN_1449	NE377	*gloB*	10.6	3.2	****	<0.0001	hydroxyacylglutathione hydrolase
SAUSA300_1708	NWMN_1655	NE386	*rot*	1.40	0.15	ns	0.9997	staphylococcal accessory regulator Rot
SAUSA300_2564	NWMN_2528	NE503	*estA*, *frmB*	6.28	2.25	****	<0.0001	tributyrin esterase
SAUSA300_1452	NWMN_1410	NE520	*proC*	1.47	0.75	ns	0.9999	pyrroline-5-carboxylate reductase
SAUSA300_0201	NWMN_0144	NE541		1.60	0.52	ns	>0.9999	peptide ABC transporter, permease protein
SAUSA300_1085	NWMN_1101	NE874		1.64	0.57	ns	0.9998	conserved hypothetical protein
SAUSA300_2105	NWMN_2057	NE929	*mtlF*	1.70	0.55	ns	0.9997	PTS system, mannitol specific IIBC component
SAUSA300_0778	NWMN_0762	NE1051		1.46	0.25	ns	0.9999	hypothetical protein
SAUSA300_1290	NWMN_1308	NE1118	*dapD*	1.69	0.59	ns	0.9997	tetrahydrodipicolinate acetyltransferase
SAUSA300_0414	NWMN_0407	NE1127	*lpl4*	1.72	0.62	ns	0.9997	tandem lipoprotein
SAUSA300_0028		NE1283	*tnp*	2.10	0.36	ns	0.9986	putative transposase

Supplementary Table 4 Michaelis-Menten parameters for *Sa*GloB. Displayed are the results of three independent biological replicates in technical duplicate.

	V_{max} (pmol*min^{-1}*µg protein^{-1})		K_m (µM)		V_{max}/K_m (pmol*min^{-1}*mg GloB^{-1}µM^{-1})		k_{cat} (10^{-3} s^{-1})		k_{cat}/K_m (M^{-1} s^{-1})		k_{cat}/k_{uncat} (10^3)		((k_{cat}/K_m)/k_{uncat}) (10^9 M^{-1})		((V_{max}/K_m)/k_{uncat}) (10^{12}pmol*mg GloB^{-1}µM^{-1})	
Substrate	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**
1C	0.497	0.074	6.23	3.39	79.9	22	0.0744	0.0111	11.9	3.28	21.2	16.3	3.41	4.81	380	536
2C	0.292	0.051	18.3	9.0	15.9	5.6	0.0436	0.0076	2.38	0.845	17.2	7.9	0.940	0.881	105	98
3C			>50		10.4	1.0			1.55	0.15			0.256	0.061	28.5	6.8
6C	0.444	0.130	48.0	29.5	9.24	4.40	0.0663	0.0194	1.38	0.658	12.7	33.4	0.265	1.133	29.6	126.3
7C			>50		6.43	0.31			0.961	0.046			0.109	0.041	12.2	4.6
8C	0.351	0.113	84.9	47.9	4.13	2.36	0.0524	0.0169	0.618	0.353	79.1	26.3	0.932	0.549	104	61
9C			>50		0.28	0.01			0.042	0.002			0.0244	0.0020	2.72	0.22
10C			>50		0.79	0.07			0.118	0.011			0.0752	0.0392	8.38	4.37
11C			>50		0.88	0.04			0.132	0.005			0.261	0.006	29.1	0.7
1O	217	41.5	73.5	25.7	2960	1620	32.5	6.2	442	242	38.6	15.3	0.525	0.595	58.6	66.5
2O	46.4	9.95	42.0	19.8	1103	503	6.93	1.49	165	75.3	19.4	11.8	0.462	0.595	51.5	66.4
3O	4.21	0.515	8.41	3.52	500.	146	0.630	0.077	74.8	21.9	4.91	1.41	0.584	0.400	65.0	44.6
4O	1.39	0.15	18.0	5.37	77.2	27.3	0.208	0.022	11.5	4.08	8.42	5.18	0.468	0.964	52.1	107.5
5O	0.731	0.15	21.2	11.8	34.5	12.7	0.109	0.023	5.15	1.91	17.0	24.1	0.803	2.038	89.5	227.2
6O	2.91	0.26	12.0	3.3	242	76.8	0.435	0.038	36.1	11.5	5.76	1.09	0.479	0.325	53.4	36.3
7O	23.1	4.50	20.5	10.9	1130	412	3.45	0.67	168	61.7	23.6	21.2	1.15	1.95	129	217
8O	1.01	0.149	15.5	6.8	64.9	22.0	0.151	0.022	9.71	3.30	6.80	1.80	0.437	0.267	48.7	29.7
9O	2.54	0.83	55.5	36.5	45.7	22.8	0.380	0.125	6.84	3.41	14.6	16.0	0.264	0.439	29.4	48.9
10O			>50		2.96	0.22			0.442	0.033			0.0715	0.0346	7.97	3.85
11O			>50		248	21.9			37.1	3.27			0.0411	0.0369	4.58	4.12
12O	1.04	0.190	36.9	15.4	28.2	12.3	0.156	0.028	4.21	1.84	1.28	0.67	0.0348	0.0438	3.87	4.88
1S	14.9	3.92	32.5	20.4	457	192	2.22	0.59	68.4	28.8	11.4	13.6	0.351	0.666	39.2	74.3
2S			>50		422	39.5			63.1	5.91			0.0529	0.0665	5.90	7.41
3S			>50		31.0	2.34			4.63	0.350			0.0259	0.0088	2.89	0.98
4S			>50		126	15.2			18.8	2.27			0.278	0.057	30.9	6.4
5S	0.18	0.049	31.7	21.1	5.60	2.34	0.0266	0.0074	0.840	0.351	4.35	2.45	0.137	0.116	15.3	12.9
6S	4.19	1.17	65.8	34.9	63.7	33.6	0.627	0.176	9.53	5.03	12.9	7.8	0.197	0.224	21.9	25.0
7S	0.722	0.150	49.5	21.4	14.6	7.01	0.108	0.022	2.18	1.05	7.86	3.04	0.159	0.142	17.7	15.9
8S	1.02	0.40	79.8	56.2	12.8	7.19	0.153	0.060	1.92	1.08	9.01	7.64	0.113	0.136	12.6	15.2
9S			>50		11.1	0.86			1.66	0.128			0.0215	0.0613	2.40	6.84
11S			>50		20.5	2.87			3.07	0.429			0.0662	0.0219	7.38	2.44
12S			>50		1.86	0.03			0.278	0.005			0.0310	0.0005	3.46	0.06

Supplementary Table 5 Michaelis-Menten parameters for *Sa*FrmB. Displayed are the results of three independent biological replicates in technical duplicate.

	V_{max} (pmol*min^{-1}*µg protein^{-1})		K_m (µM)		V_{max}/K_m (pmol*min^{-1}*mg FrmB^{-1}µM^{-1})		k_{cat} (10^{-3} s^{-1})		k_{cat}/K_m (M^{-1} s^{-1})		k_{cat}/k_{uncat} (10^3)		((k_{cat}/K_m)/k_{uncat}) (10^9 M^{-1})		((V_{max}/K_m)/k_{uncat}) (10^{12} pmol*mg FrmB^{-1}µM^{-1})	
Substrate	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**
1C	0.149	0.059	30.4	29.5	4.89	2.01	0.0280	0.0112	0.921	0.379	7.99	16.5	0.263	0.557	23.3	49.3
2C	0.0637	0.0274	26.5	28.9	2.40	0.95	0.0120	0.0052	0.452	0.179	4.74	5.38	0.179	0.186	15.8	16.5
3C			>50		10.2	0.64			1.92	0.12			0.316	0.050	28.0	4.4
6C			>50		1.84	0.14			0.347	0.026			0.0666	0.0444	5.90	3.93
7C			>50		1.01	0.08			0.191	0.015			0.0217	0.0133	1.92	1.17
8C			>50		0.314	0.012			0.0591	0.0022			0.0892	0.0034	7.89	0.30
9C			>50		0.160	0.009			0.0301	0.0018			0.0177	0.0019	1.56	0.17
10C			>50		0.233	0.029			0.0438	0.0055			0.0279	0.0194	2.47	1.72
11C			>50		0.682	0.062			0.128	0.012			0.254	0.014	22.5	1.2
1O	5.98	0.677	30.3	8.33	197	81.3	1.13	0.127	37.2	15.3	1.34	0.31	0.0442	0.0377	3.91	3.34
2O	2.04	0.328	13.9	6.76	147	48.6	0.385	0.062	27.7	9.14	1.08	0.49	0.0777	0.0723	6.88	6.40
3O	0.621	0.191	9.95	10.06	62.4	18.9	0.117	0.036	11.7	3.57	0.911	0.656	0.0916	0.0653	8.11	5.78
4O	0.183	0.038	30.0	15.2	6.12	2.51	0.0345	0.0072	1.15	0.47	1.40	1.70	0.0467	0.1118	4.14	9.89
5O			>50		1.54	0.10			0.290	0.020			0.0452	0.0210	4.00	1.86
6O	0.597	0.091	9.17	4.66	65.2	19.4	0.112	0.017	12.3	3.66	1.49	0.48	0.163	0.104	14.4	9.2
7O	2.57	0.96	107	64.8	24.0	14.8	0.485	0.180	4.52	2.78	3.31	5.69	0.0309	0.0877	2.73	7.77
8O	0.163	0.031	18.3	9.66	8.92	3.17	0.0307	0.0058	1.68	0.60	1.38	0.47	0.0757	0.0482	6.70	4.27
9O			>50		7.24	0.31			1.36	0.06			0.0525	0.0076	4.65	0.67
10O	0.163	0.053	74.2	43.6	2.20	1.21	0.0307	0.0099	0.414	0.227	4.97	10.26	0.0671	0.2351	5.94	20.81
11O			>50		79.1	2.7			14.9	0.51			0.0165	0.0058	1.46	0.51
12O			>50		3.18	0.26			0.598	0.048			0.00493	0.00115	0.44	0.10
1S			>50		12.5	0.6			2.36	0.11			0.0121	0.0026	1.07	0.23
2S			>50		169	14			31.8	2.58			0.0266	0.0290	2.36	2.57
3S			>50		7.79	0.19			1.47	0.04			0.0082	0.0009	0.728	0.081
4S	0.505	0.147	52.7	31.3	9.58	4.70	0.0950	0.0277	1.80	0.89	1.40	0.70	0.0266	0.0223	2.36	1.97
5S	0.0295	0.0030	11.1	3.61	2.66	0.83	0.00556	0.00056	0.500	0.156	0.910	0.187	0.0818	0.0517	7.24	4.58
6S	0.629	0.169	36.3	22.3	17.3	7.5	0.118	0.032	3.27	1.42	2.44	1.42	0.0674	0.0633	5.97	5.61
7S			>50		1.55	0.12			0.292	0.023			0.0213	0.0032	1.88	0.28
8S			>50		2.20	0.24			0.415	0.046			0.0244	0.0058	2.16	0.51
9S			>50		5.38	0.48			1.01	0.09			0.0131	0.0435	1.16	3.85
11S			>50		10.8	1.1			2.04	0.21			0.0440	0.0105	3.89	0.93
12S			>50		1.02	0.07			0.191	0.013			0.0213	0.0013	1.89	0.12

Supplementary Table 6 Summary of crystallographic data collection and refinement statistics.

Data Collection	*Sa*FrmB	*Sa*GloB (SeMet)
Space Group	C2	P 1 21 1
Cell dimensions	a= 128.3Å, b= 80.5Å c=66.7Å, β=113.8°	a= 93.7Å, b= 44.8Å c=105.0Å, β=96.7°
Wavelength (Å)	1.000	1.000
Resolution (Å) (highest shell)	36.8-1.60 (1.63-1.60)	48 - 1.71 (1.74 - 1.71)
Reflections (total/unique)	145,907 / 78193	536,305 / 97,666
Completeness (highest shell)	96.4% (99.1%)	96.1 % (88.8 %)
<I/σ> (highest shell)	27.1 (4.1)	21.3 (1.4)
R_{sym} (highest shell)	6.6% (57.3%)	10.4% (54.8%)
Refinement		
R_{cryst} / R_{free}	0.156 / 0.179	0.225 / 0.251
No. of protein atoms	4164	6323
No. of waters	496	431
No. of ligand atoms	3	28
R.m.s.d., bond lengths (Å)	0.009	0.007
R.m.s.d., bond angles (°)	1.34	1.21
Avg. B-factor (Å^2): protein, water, ligand	25.8, 37.6, 15.9	39.6, 44.9, 47.5
Stereochemistry: most favored, allowed, disallowed	98.4, 1.6, 0 %	96.2, 3.8, 0 %

Supplementary Table 7 Michaelis-Menten parameters for human sera. Displayed are the results of three independent biological replicates in technical duplicate.

	V_{max} (pmol*min^{-1}*mg sera^{-1})		K_m (µM)		V_{max}/K_m (pmol*min^{-1}*mg sera^{-1}µM^{-1})		$((V_{max}/K_m)/k_{uncat})$ (10^{12} pmol*mg sera^{-1}µM^{-1})	
Substrate	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**
1C	1.06	0.15	36.2	11.5	0.0293	0.0128	0.139	0.315
2C	0.356	0.191	71.5	70.6	0.00498	0.00271	0.0328	0.0471
3C			>50		0.00161	0.00022	0.00443	0.00151
6C			>50		0.00121	0.00013	0.00389	0.00381
7C	0.441	0.029	64.18	8.03	0.00688	0.00355	0.0130	0.0531
8C			>50		0.000791	0.000062	0.0199	0.0016
9C			>50		0.000581	0.000026	0.00568	0.00045
10C			>50		0.000483	0.000042	0.00513	0.00251
11C			>50		0.000711	0.000027	0.0234	0.0005
1O	68.6	25.6	64.1	45.8	1.07	0.560	0.0212	0.0230
2O	43.8	10.53	40.4	21.5	1.09	0.489	0.0507	0.0645
3O	11.7	2.81	22.2	14.2	0.529	0.198	0.0687	0.0604
4O	5.88	1.90	44.5	30.9	0.132	0.061	0.0894	0.2420
5O	2.25	0.48	35.4	17.4	0.0635	0.0274	0.165	0.489
6O	11.7	3.82	54.6	35.9	0.215	0.107	0.0475	0.0503
7O	5.73	1.16	30.3	14.9	0.189	0.078	0.0216	0.0411
8O	3.93	0.71	27.7	12.4	0.142	0.057	0.107	0.077
9O	4.35	1.18	111.7	48.9	0.0389	0.0242	0.0250	0.0519
10O	1.46	0.38	63.3	31.8	0.0230	0.0121	0.0621	0.2080
11O			>50		0.122	0.008	0.00225	0.00158
12O	4.00	0.68	42.2	15.7	0.0948	0.0433	0.0130	0.0171
1S	30.5	9.90	71.8	43.0	0.425	0.230	0.0364	0.0890
2S			>50		0.315	0.025	0.00440	0.00472
3S	8.80	2.92	74.3	45.0	0.118	0.065	0.0111	0.0273
4S	3.81	0.39	27.9	7.08	0.137	0.055	0.0336	0.0230
5S	0.690	0.246	31.0	26.7	0.0222	0.0092	0.0606	0.0510
6S			>50		0.0257	0.0025	0.00886	0.00182
7S	1.60	0.43	30.2	19.7	0.0528	0.0216	0.0641	0.0490
8S			>50		0.0140	0.0007	0.0138	0.0015
9S			>50		0.0247	0.0021	0.00532	0.01651
11S			>50		0.0190	0.0017	0.00682	0.00149
12S			>50		0.00504	0.00036	0.00937	0.00062

Supplementary Table 8 Michaelis-Menten parameters for mouse sera. Displayed are the results of three independent biological replicates in technical duplicate.

	V_{max} (pmol*min^{-1}*mg sera^{-1})		K_m (µM)		V_{max}/K_m (pmol*min^{-1}*mg sera^{-1}µM^{-1})		$((V_{max}/K_m)/k_{uncat})$ (10^{12} pmol*mg sera^{-1}µM^{-1})	
Substrate	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**	**Value**	**SEM**
1C			>50		4.56	0.51	21.7	12.4
2C			>50		1.62	0.33	10.7	5.7
3C			>50		0.52	0.02	1.43	0.13
6C	17.6	9.4	53.5	58.1	0.33	0.16	1.05	4.65
7C			>50		2.04	0.27	3.86	4.04
8C			>50		1.35	0.13	34.0	3.3
9C	3.18	0.66	12.3	8.0	0.26	0.08	2.53	1.46
10C			>50		0.93	0.08	9.87	4.74
11C			>50		0.28	0.01	9.34	0.18
1O			>50		14.01	0.83	0.28	0.03
2O			>50		15.90	1.12	0.74	0.15
3O			>50		57.0	43.2	7.41	13.17
4O			>50		6.76	0.21	4.57	0.82
5O			>50		20.3	12.0	52.6	214.7
6O	437	135	11.5	8.65	38.1	15.6	8.42	7.39
7O	929	103	25.9	7.30	35.8	14.1	4.08	7.40
8O			>50		15.7	0.4	11.8	0.5
9O			>50		6.69	0.17	4.30	0.36
10O			>50		2.93	0.14	7.91	2.36
11O			>50		3.24	0.16	0.06	0.03
12O			>50		11.9	0.4	1.64	0.15
1S			>50		14.2	1.4	1.22	0.54
2S			>50		5.07	0.21	0.07	0.04
3S			>50		9.47	0.76	0.88	0.32
4S			>50		24.2	2.6	5.96	1.08
5S			>50		1.93	0.13	5.27	0.71
6S			>50		12.1	0.6	4.16	0.41
7S			>50		11.9	1.1	14.4	2.6
8S			>50		5.17	0.43	5.07	0.91
9S			>50		1.29	0.11	0.28	0.89
11S			>50		1.97	0.10	0.71	0.09
12S			>50		5.74	0.53	10.7	0.9

Supplementary Table 9 Primers used during this study.

No	Name	Sequence	Use
1	NWMN_0144_F	TTTTCCTGATCCTGATTCAC	Sanger Sequencing
2	NWMN_0144_R	ATGATGCTTCCATGTTTGTT	Sanger Sequencing
3	NWMN_0306_F	AATACACCGGGTAACACAAC	Sanger Sequencing
4	NWMN_0306_R	CGTTTTGTTGAGCTAATTCC	Sanger Sequencing
5	NWMN_0309_F	ACCATGCTTAAAGGGATTTT	Sanger Sequencing
6	NWMN_0309_R	TGTCACCTAAGTCAACACCA	Sanger Sequencing
7	NWMN_0407 (*lpl4nm*) _F	CCGTTGGAGATAGGAAGTTA	Sanger Sequencing
8	NWMN_0407 (*lpl4nm*) _R	TTTGTGCTTCTTTTGAACCT	Sanger Sequencing
9	NWMN_0654_F	GAAAATGGAAGACTGATTGC	Sanger Sequencing
10	NWMN_0654_R	TAATGCATCTGACAAAGTCG	Sanger Sequencing
11	NWMN_0762_F	GGTGAAGTTTTGGACGATAA	Sanger Sequencing
12	NWMN_0762_R	TTTTCATCTGTCCGACTTTT	Sanger Sequencing
13	NWMN_1101_F	TCCACCTATTGGAATTATCG	Sanger Sequencing
14	NWMN_1101_R	AGACGTTCAATTTCAGTGCT	Sanger Sequencing
15	NWMN_1192 (*pgsA*) _F	TGGGACGAAGTAATTACAGT T	Sanger Sequencing
16	NWMN_1192 (*pgsA*) _R	ATATCCCCCTTGTATCGTTT	Sanger Sequencing
17	NWMN_1308 (*dapD*) _F	TCTATTCGTGGAGGTACGAT	Sanger Sequencing
18	NWMN_1308 (*dapD*) _R	ATCGTATGTGAGCCATTACC	Sanger Sequencing
19	NWMN_1410_F	CGATAAACCTAAACCACTCG	Sanger Sequencing
20	NWMN_1410_R	ATAAACAATGCTTGCCAAAT	Sanger Sequencing
21	NWMN_1505_F	TGAAGGTGAATTAAGCGATG	Sanger Sequencing
22	NWMN_1505_R	TGCTATTCCCAATTTGTTCA	Sanger Sequencing
23	NWMN_1655_F	GAATTGTTGCAATTTAATGGT	Sanger Sequencing
24	NWMN_1655_R	AACGTAATCATGCTCCATTC	Sanger Sequencing
25	NWMN_1679_F	CCATGGGAAAAATTAGACAA	Sanger Sequencing
26	NWMN_1679_R	AAATATCGCCTCACCTTTTT	Sanger Sequencing
27	NWMN_1723 (*hemY*) _F	GCCGAATACACATCCATTAT	Sanger Sequencing
28	NWMN_1723 (*hemY*) _R	AACCTTTGTCTCTGCTTCAA	Sanger Sequencing
29	NWMN_1851 (*nadC*) _F	AGCCATTTTAGCACCATAAA	Sanger Sequencing
30	NWMN_1851 (*nadC*)_R	TAGAATCCTGTCCTCCTGAA	Sanger Sequencing
31	NWMN_2057 (*mtlF*)_F	TGTACAACGGTGTTGTTTTG	Sanger Sequencing
32	NWMN_2057 (*mtlF*)_R	CGGTGAATAGTACGAGAGGA	Sanger Sequencing
33	NWMN_2528_F	ACTGATGCTTTACCAGAAAC	Sanger Sequencing
34	NWMN_2528_R	TCAGCGGTAGTAATAAAGGT	Sanger Sequencing

Supplementary Table 10 Accession numbers for the isolates used in WhatsGNU analysis.

Isolate	Bioproject	Biosample	WGS	SRA
AD_3_179	PRJNA512846	SAMN10689346	VYMI00000000	SRR8389007
AD_11_548	PRJNA512846	SAMN10689354	VYMN00000000	SRR8389002
AD_14_565	PRJNA512846	SAMN10689355	VYMO00000000	SRR8389003
AD_16_660	PRJNA512846	SAMN10689358	SJAX00000000	SRR8389044
AD_61_868	PRJNA512846	SAMN10689401	VYNS00000000	SRR11016776
AD_85_830	PRJNA512846	SAMN10689428	VYOK00000000	SRR8389056
AD_96_471	PRJNA512846	SAMN10689447	VYOW00000000	SRR8389016
AD_103_347	PRJNA512846	SAMN10689453	VYPC00000000	SRR8389035
AD_113_782	PRJNA512846	SAMN10689463	VYPL00000000	SRR8389099
SSTI_227_44	PRJNA563582	SAMN12642230	VUGB00000000	SRR11016228
SSTI_228_42	PRJNA563582	SAMN12642226	VUGF00000000	SRR11016232
SSTI_231_2	PRJNA563582	SAMN12642218	VUGN00000000	SRR11016241
SSTI_233_51	PRJNA563582	SAMN12642215	VUGQ00000000	SRR11016244
SSTI_235	PRJNA563582	SAMN12642210	VUGV00000000	SRR11016250
SSTI_241_9	PRJNA563582	SAMN12642203	VUHC00000000	SRR11016198
SSTI_247_75	PRJNA563582	SAMN12642193	VUHM00000000	SRR11016209
SSTI_258_57	PRJNA563582	SAMN12642177	VUIB00000000	SRR11016226
SSTI_290	PRJNA563582	SAMN12642174	VUIE00000000	SRR11016283

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