SUPPLEMENTARY INFORMATION

Antibiotic resistance evolved via inactivation of a ribosomal RNA methylating enzyme

Vanja Stojković[1], Lianet Noda-Garcia[2], Dan S. Tawfik[2]*, Danica Galonić Fujimori[1,3]*

SUPPLEMENTARY FIGURES AND TABLES LEGENDS

Supplementary Figure S1. Antibiotics used in this study. Tiamulin, virginiamycin M_1, chloramphenicol and clindamycin are PTC-targeting antibiotics. Streptomycin targets 30S subunit and trimethoprim inhibits dihydrofolate reductase.

Supplementary Figure S2. Tiamulin susceptibility test of Bsub_RlmN evolved variants in *E. coli* BW25113 cells. Survival of the cells in the antibiotic environment is dependent on the expression of Bsub_RlmN variants. Full expression of the enzyme is achieved at concentrations $\geq$10 ng/mL of AHT (1).

Supplementary Figure S3. Tiamulin susceptibility test of *S. aureus* Cfr in *E.coli* BW25113 cells. Each plate contained ampicillin for selection of the plasmid and AHT to induce the expression of the enzymes. Cells were plated at three concentrations, and plates were recorded after 24 hours. Abbreviations: Neg = empty pZA; WT = pZA_WT_Bsub_RlmN; B = pZA_BsubB; Cfr = pZA_Saureus_Cfr.

Supplementary Figure S4. HPLC analysis of the methylation products of the 2447-2625 rRNA fragment by Ecoli_RlmN and Bsub_RlmN. (a) ^{14}C radioactivity chromatogram of digested RNA isolated from the *in vitro* reaction with Bsub_RlmN; (b) ^{14}C radioactivity chromatogram of digested RNA isolated from the *in vitro* reaction with Ecoli_RlmN; (c) UV-Vis chromatogram of synthetic methyladenosine standards at 256 nm. 1. m^2A; 2. m^2m^8A.

Supplementary Figure S5. *In vitro* methylation activity of Bsub_RlmN WT and evolved variants towards potential tRNA substrates. A) WT Bsub_RlmN is an rRNA-specific methylating enzyme and shows no *in vitro* activity towards several tRNA substrates of WT Ecoli_RlmN as well as a non-substrate (tRNA_G (CCC)). Reactions lacking the obligatory reductant, NADPH, serve as negative controls. B) Bsub_RlmN evolved variants do not methylate tRNA_D (GUC) and tRNA_Q2 (UUG), two known substrates of WT Ecoli_RlmN. *Error bars* (n ≥ 2), S.D.

Supplementary Figure S6. Dose-dependent antibiotic susceptibility test towards tiamulin. The plasmids expressing evolved variants were transformed into *E. coli* BW25113/Δ*rlmN* cells, and the experiment was performed on agar plates. Each plate contained kanamycin for selection of the strain, ampicillin for selection of the plasmid and AHT to induce the expression of the enzymes. Cells were plated in three concentrations, and plates were recorded after 24 hours.

Supplementary Figure S7. *In vivo* methylation activity of BsubB towards 23S rRNA. MALDI-TOF mass spectrum of the C2480–C2520 fragment of *E. coli* 23S rRNA isolated from the *E. coli* BW25113/Δ*rlmN* strain carrying an empty plasmid (A), or expressing WT Bsub_RlmN (B), or BsubB variant (C), or *S. aureus* Cfr (D).

Supplementary Figure S8. Expanded phylogenetic tree of RlmN and Cfr sequences from selected Firmicutes species. The numbers correspond to the IMG/JGI database gene identifier. Letters A or B,

added after the scientific name, indicate the existence of paralogues in the specie. Experimentally validated Cfr enzymes were noted by adding term “Cfr” after their scientific names.

Supplementary Figure S9. *In vivo* methylation activity of ClCp towards 23S rRNA. MALDI-TOF mass spectrum of the C2480–C2520 fragment of *E. coli* 23S rRNA isolated from the *E. coli* BW25113/Δ*rlmN* strain expressing ClCp.

Supplementary Table S1. List of all sequenced variants from the Bsub_RlmN library with their mutagenic composition. The most predominant mutations in the library are S168C, G201D and A348T. Another highly representative mutation is C350S, a mutation of the catalytically active residue that usually appeared with S168C, G201D and A348T. In our initial antibiotic susceptibility experiments we tested an additional variant, BsubA, which contained S168C, G201D, A348T and C350S mutations. This variant was more susceptible to tiamulin than the other three variants studied in this paper.

Supplementary Table S2. Percentage of clones containing specific mutations after each round of evolution. The library was void of WT Bsub_RlmN after the first round of evolution.

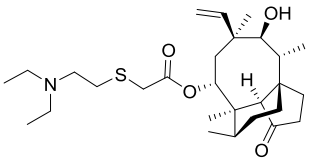
Supplementary Table S3. The MIC values of *E. coli* BW25113 and *E. coli* BW25113/Δ*rlmN* cells harboring the plasmids determined by broth microdilution method. The numbers are an average of at least three independent experiments. Abbreviations: TIA = tiamulin; VIR M_1 = virginiamycin M_1; CLI= clindamycin; CHL = chloramphenicol; STR = streptomycin; TMP = trimethoprim.

Supplementary Table S4. Ribosomal RNA methylating enzymes associated with antibiotic resistance in bacteria. a. Resistance conferred by the presence (+) or absence (-) of methylation; b. $PhLOPS_A$ phenotype includes resistance to phenicols, lincosamides, oxazilidinones, pleuromutlilins, and streptogramin A antibiotics; c. $MILS_B$ phenotype indicates resistance to macrolides, lincosamides, and streptogramin B antibiotics; d. Selected examples of Erm mono- and dimethyltransferases.

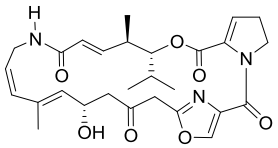
REFERENCES

1. Wellner, A., Raitses Gurevich, M. and Tawfik, D.S. (2013) Mechanism of protein sequence divergence and incompatibility. *PLoS genetics*, **9**, e1003665.

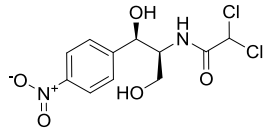
Supplementary Figure S1.



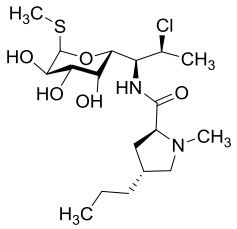
Tiamulin



Virginiamycin M_1



Chloramphenicol



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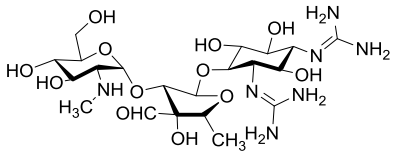
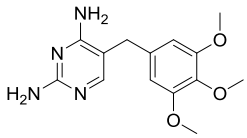
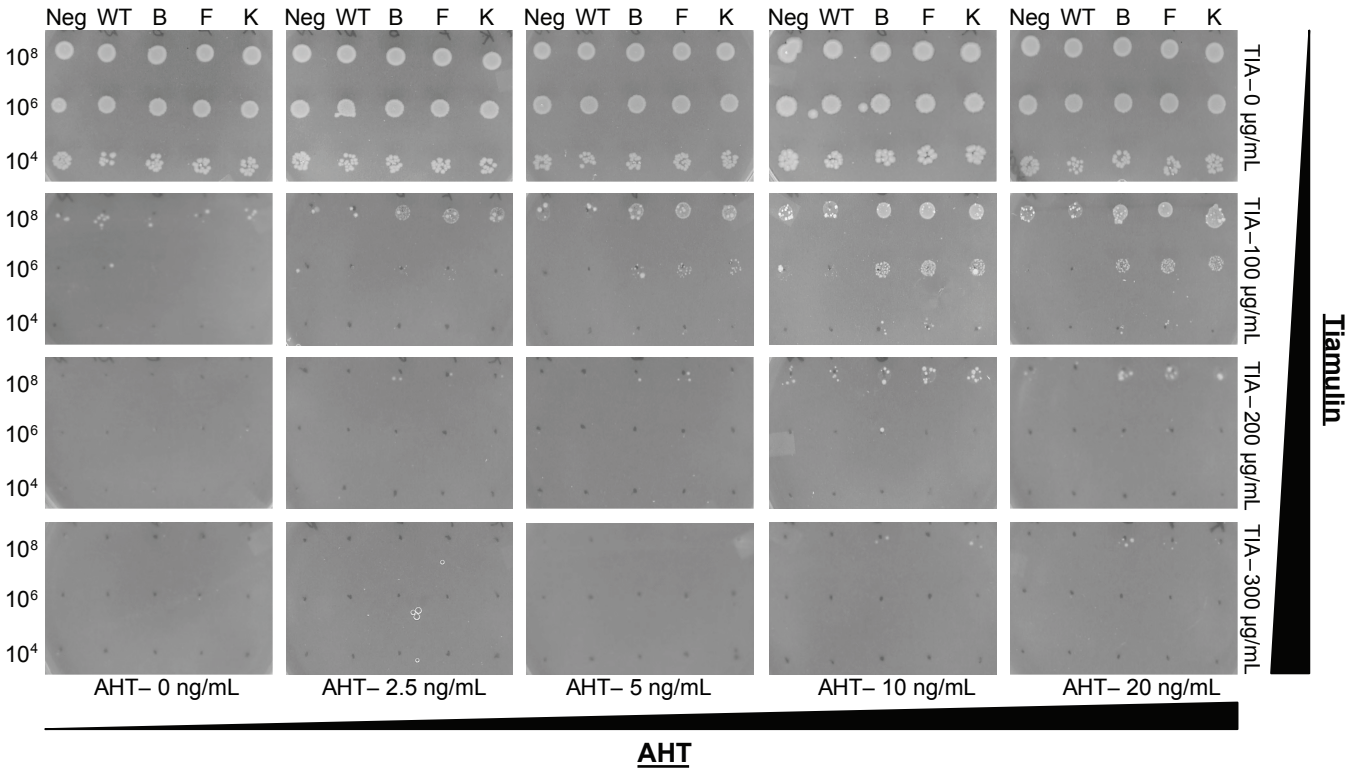


Streptomycin

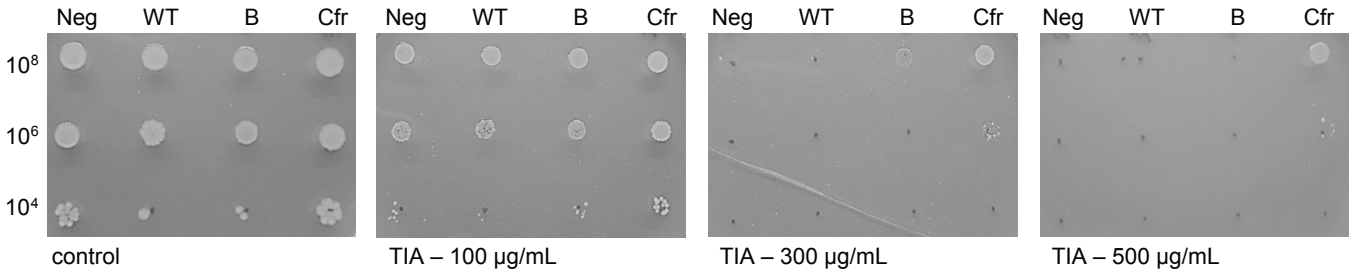


Trimethoprim

Supplementary Figure S2.



Supplementary Figure S3.



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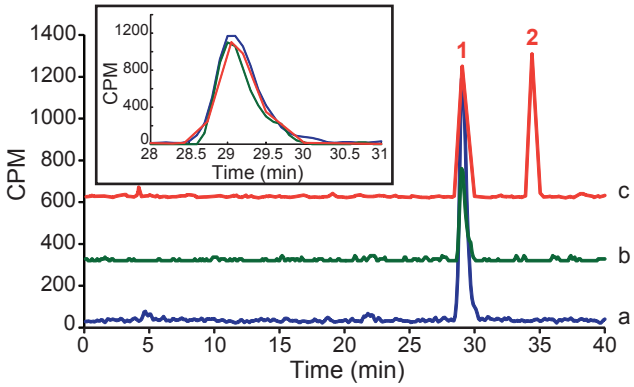

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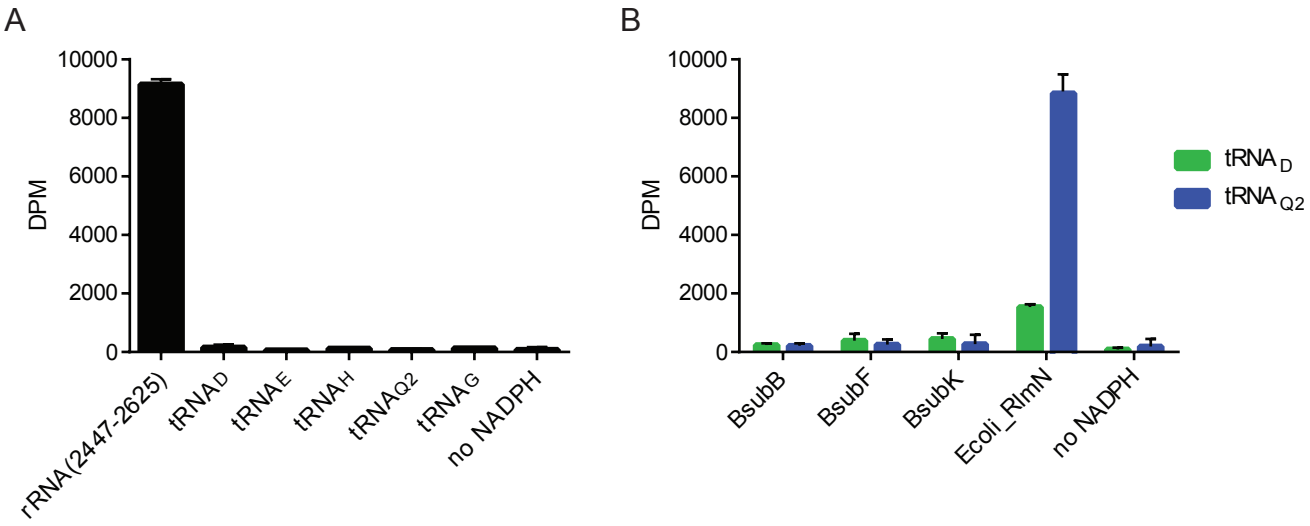

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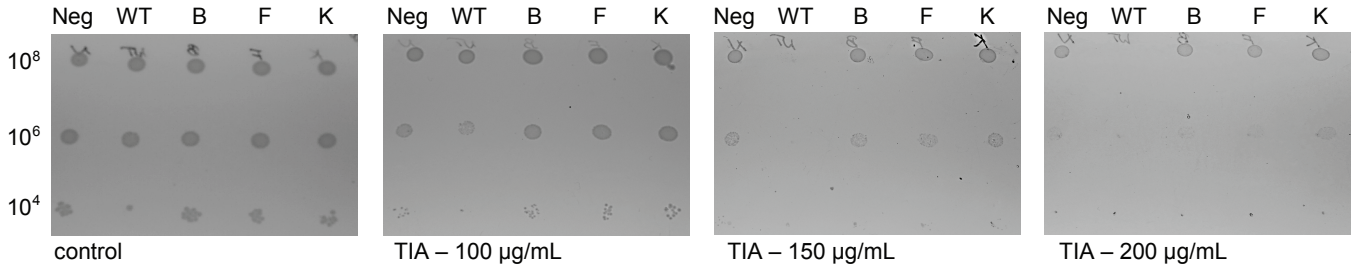
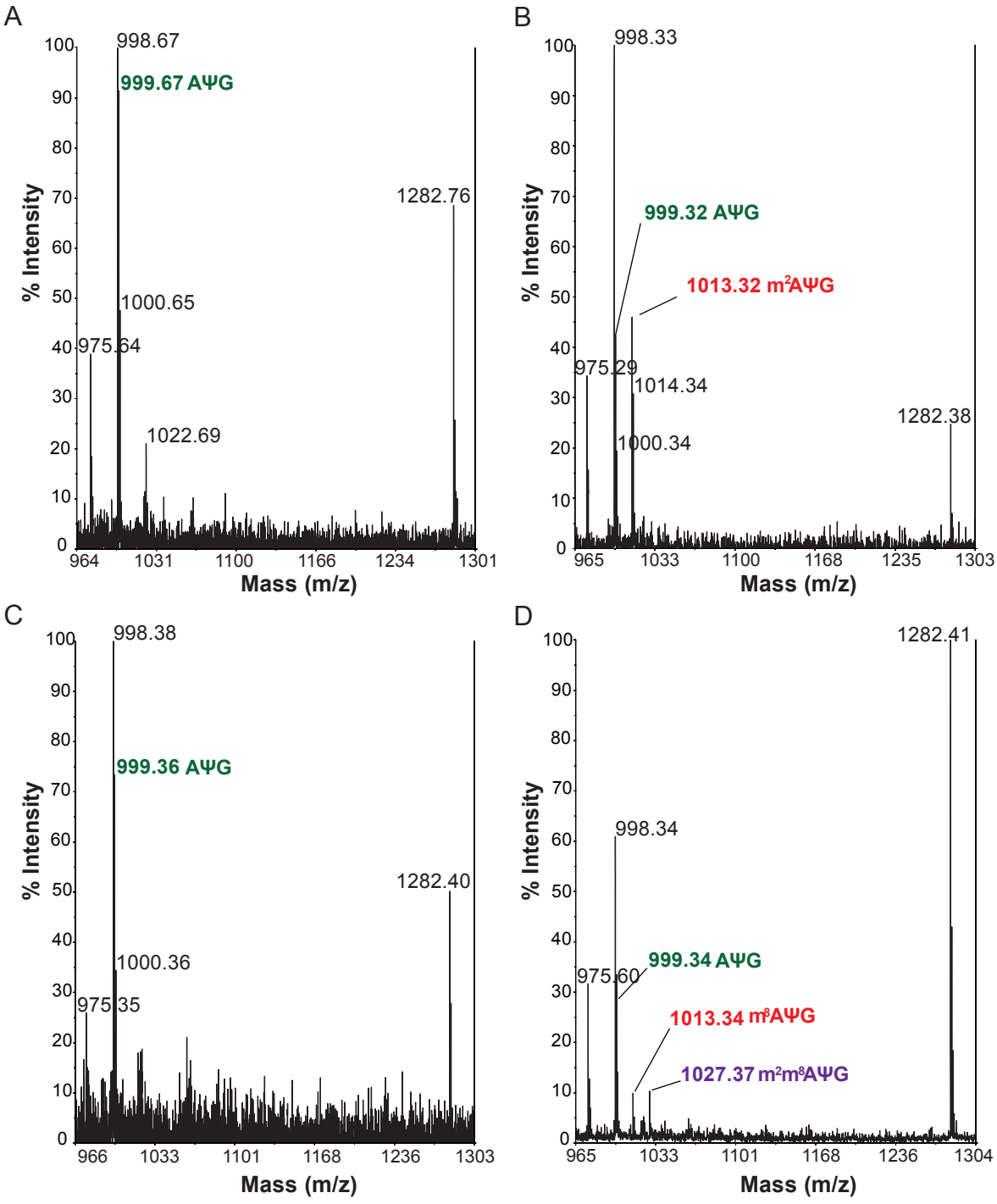
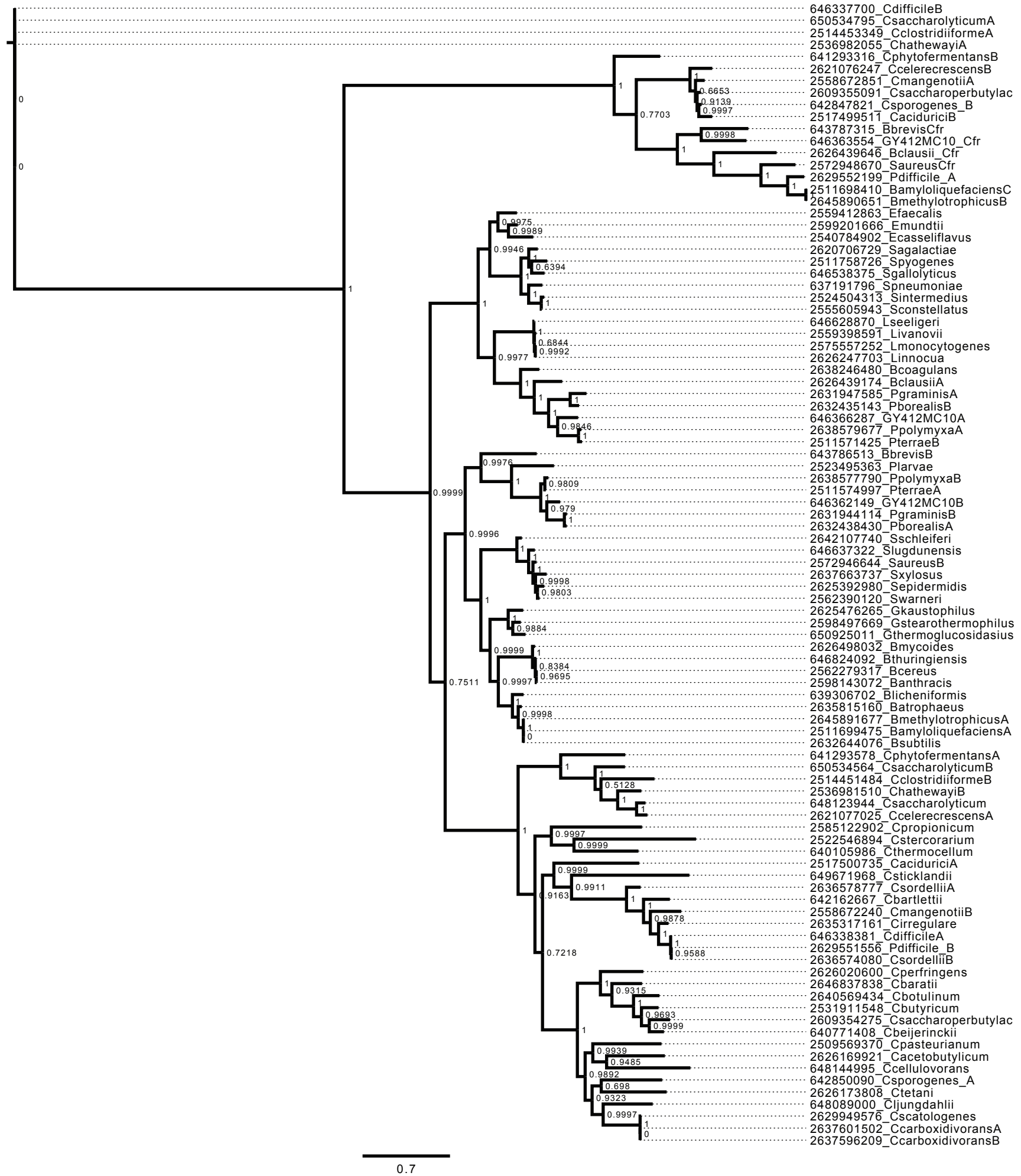


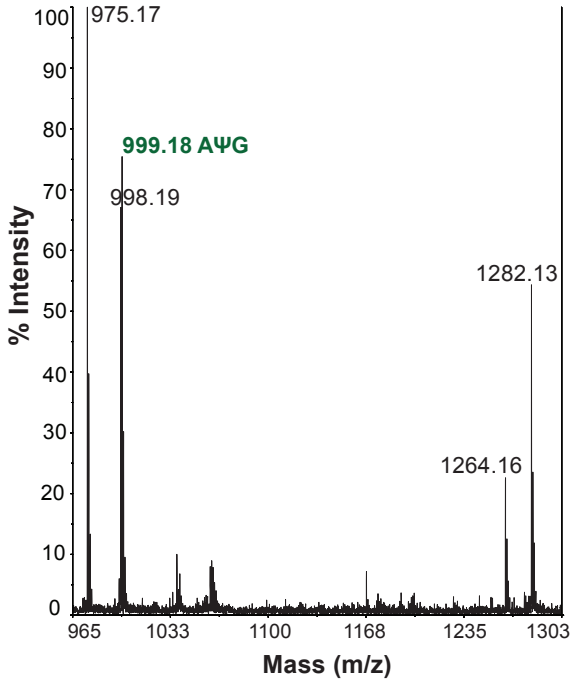
Supplementary Figure S7.



Supplementary Figure S8.



Supplementary Figure S9.



Supplementary Table S1.

Round	Concentration of tiamulin (μg/ml)	Clone number	Mutations
Round #1	100	1	N35S, L67Q, I128N
		2	F188L, F219S, P245T
		3	R242L, A348T, C350S
	125	1	S168C, G201D, A348T, C350S
		2	S168C, G201D, A348T, C350S
		3	S168C, G201D, A348T, C350S
		4 (BsubB)	S168C, G201D
		5	F132L, G139D, F179L, F323I
		6	E16A, K155I, V280I, A348T C350S
		7 (BsubB)	S168C, G201D
Round #1 enrichment	100	1	S231F
		2 (BsubB)	S168C, G201D
		3 (BsubK)	S168C, G201D, A348T
		4	C130F, P245T, Q359K
		5	I128N, S168C, G201D, A348T, C350S
	125	1	G125D, G129D
		2 (BsubB)	S168C, G201D
		3	S168C, G201D, V284M, R316S, Q321L, E341D
		4 (BsubB)	S168C, G201D
		5 (BsubB)	S168C, G201D
		6	S168C, G201D, A348T, C350S
		7	S168C, G201D, A348T, C350S
		8	S168C, G201D, A348T, C350S
		9	S168C, G201D, A348T, C350S
		10	E16A, K155I
	150	1 (BsubF)	Q89R, G201D
		2 (BsubF)	Q89R, G201D
		3 (BsubK)	S168C, G201D, A348T
		4	K38R, S168C, G201D, R340S
		5	K141I, S168C, G201D, V280I
		6	N35S, S117T, S168C, G201D, Q342H
		7	Q89R, I149N, F228L, A348T, C350S
Round #2	100	1	T163M, S168C, G201D, F323L
		2	E51D, I128M, A348T, C350S, R358C
		3	R127H, S168C, D320N, K330I, A348T, C350S
		4	G201D, G351S
	150	1	S168C, S169F, A187V, G201D
		2	A43T, D164N, Q222L, A348T, C350S
		3	A43T, S168C, H194Y, G201D, A234G, A348TÇ350S
		4	E69K, T121M, I128M, S168C, T266S, Q342R
	175	1	C126S, S168C, V259I, R316S, K327N, A348T, C350S
		2	I128N, F188L, D195N, S208Y, N236D, V280I, A348T, C350S
		3	E98G, K155R, F228L, A348T, C350S
		4	I128M, S168C, G201D, P245T, D345G, A348T, C350S

Round	Concentration of tiamulin (μg/mL)	Clone number	Mutations
Round #2	150	1 (BsubB)	S168C, G201D
		2	T121M, S168C, N193I, G201D, I239T
		3	A83T, S168C, G201D, P214A, F323L, T337I, G351S
		4	A134T, S168C, F179V, G201D, S271R, V280I, N335S
		5	L186F, C350S
		6	N35T, I128N, T131A, V167I, G176C, K190N, N193K, I120F, F228L, A348T, C350S
		7	M137V
		8	F179V, S210R
		9	E47K, T78S, I92V, G201D, I246L, F272V, P310H, T337S, A348T, C350S
		10	E69G, K85I, S117T, L137P, S168C, K196T, G201D, N247D, V309M
		11	L189M, P214Q, F228L, I239V, N307K, D347Y, A348T, C350S
		12	I28F, I128N, S168C, A127T, V207I, G275R, R316H, A324S
Round #3	175	1 (BsubB)	S168C, G201D
		2	T121A, G139D, S168C, G201D, D254E, G333E, E361D
		3	V84A, S168C, G201D, Q222L, Q223E, D254E, R269C, P305S
		4	S168C, G201D, F272I
		5	A2T, V84A, I128M, F228L, S241G, N281Y
		6	D26Y, A83T, F227V, D345V, A348T, C350S
		7	Q30H, L96V, D195N, G201D, T206M, E341G
		8	P245T, A348T, C350S
		9	E113K, N116S, C119R, G201D, K215I
		10	F23I, G36E, F46I, K81R, I128M, C130S, A249E
		11	L25F, I128M, A159V, S168C, G201D, V280I, E288D, A348T, C350S
	200	1	S168C, G201D, L293Y
		2	E69G, K155N, S168C, G201D, K251R
		3	P39R, S168C, G201D, P318T
		4	R166L, S168C, G201D
		5	T136M, T328A, A348T, C350S
		6	T15I, N35T, G201D, L353F
		7	Q30H, L96V, I128M, D195N, G201D, E341G
		8	N5S, K38R, R127H, I128M, K155N, S168C, P235G, E257K, A348T, C350S
		9 (BsubB)	S168C, G201D
		10	S168C, T337S, A348T, C350S
		11	S88P, Q89R, G91D, S117T, V170I, V171L, G278R

Supplementary Table S2.

Mutation	% of clones with specific mutation Round #1	% of clones with specific mutation Round #2	% of clones with specific mutation Round #3	% of clones with specific mutation Cumulative
Q89R	12 %	0 %	4 %	6 %
S169C	54 %	50 %	62 %	57 %
G201D	62 %	36 %	69 %	60 %
A348T	39 %	57 %	31 %	39 %

Supplementary Table S3.

Strain	Plasmid	MIC (μg/mL)				
		TIA	**VIR M_1**	**CLI**	**CHL**	**TMP**
BW25113	pZA_empty	350-400	300	150-200	8	0.5
	pZA_WT Bsub_RlmN	350-400	300	150	8	0.25-0.5
	pZA_BsubB	500-550	400	200	8	0.25-0.5
	pZA_BsubF	500-550	400	150	8	0.25-0.5
	pZA_BsubK	500-550	400	200	8	0.25-0.5
	pZA_Saureus_Cfr	800-1000	800-1000	350-400	16	0.5
BW25113/ *ΔrlmN*	pZA_empty	400-450	400	200-250	8	0.5
	pZA_WT Bsub_RlmN	250-300	300	200	8	0.25-0.5
	pZA_BsubB	400-450	400	200-250	8	0.25-0.5
	pZA_BsubF	400-450	400	200-250	8	0.25-0.5
	pZA_BsubK	400-450	400	200-250	8	0.25-0.5
	pZA_Saureus_Cfr	1200	>1200	350-400	16	0.25-0.5

Supplementary Table S4.

Resistance methyltransferase gene	**Methylated nucleotides in *E. coli* numbering**	**Position of methylation**	**Methylation[a]**	**Target of the resistance gene**	**Antibiotic resistance phenotype**
aviRA	G2535	N1	+	23S rRNA	Avilamycin
aviRb	U2479	2'-O-ribose	+	23S rRNA	Avilamycin
cfr	A2503	C8	+	23S rRNA	$PhLOPS_A$[b] Hygromycin A A201A
emtA	G2470	N1	+	23S rRNA	Evernimicin Avilamycin
ermC[d] *ermAM*[d]	A2058	N6,N6	+	23S rRNA	$MILS_B$[c]
ermN[d] *(tlrD)*	A2058	N6	+	23S rRNA	Lincosamides
rlmA (tlrB)	G748	N1	+	23S rRNA	Tylosin
tsr	A1067	2'-O-ribose	+	23S rRNA	Thiostrepton
armA, rmtA-rmtH	G1405	N7	+	16S rRNA	Kanamycin Gentamicin
npmA	A1408	N1	+	16S rRNA	Neomycin
rlmN	A2503	C2	-	23S rRNA	Tiamulin Virginiamycin M_1
rrma	G745	N1	-	23S rRNA	Viomycin
tlyA	C1920 C1409	2'-O-ribose	-	23S rRNA 16S rRNA	Capreomycin Viomycin
ksgA	A1518 & A1519	N6,N6	-	16S rRNA	Kasugamycin
rsmG	G527	N7	-	16S rRNA	Streptomycin