Supporting Information for:

Experimental evolution of diverse strains as a method for the determination of biochemical mechanisms of action for novel pyrrolizidinone antibiotics

Kathryn Beabout[†1], Megan D. McCurry[†1], Heer Mehta[†], Akshay A. Shah[‡], Kiran K. Pulukuri[‡], Stephan Rigol[‡], Yanping Wang[‡], K. C. Nicolaou[‡], Yousif Shamoo*[†]

[1]These authors contributed equally to this work.

[†]Department of BioSciences, Rice University, 6100 Main Street, Houston, Texas, 77005, United States

[‡]Department of Chemistry, BioScience Research Collaborative, Rice University, 6100 Main Street, Houston, Texas, 77005, United States

*Corresponding author. E-mail: shamoo@rice.edu

Table of Contents

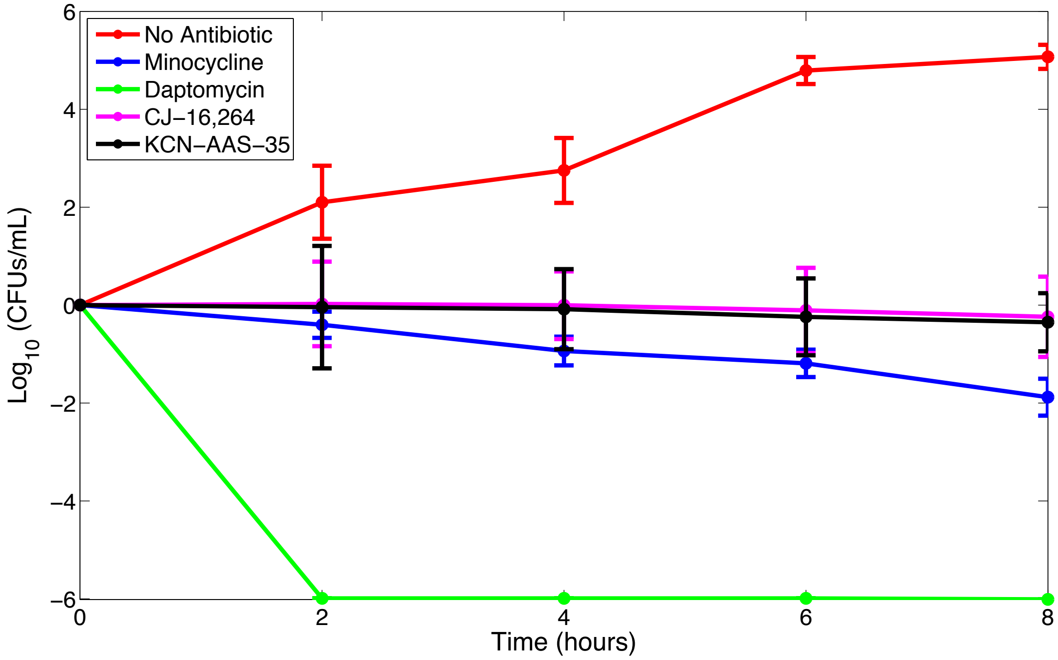

Figure S1. Killing curves for pyrrolizidinone natural product (CJ-16,264) and analog (KCN-AAS-35) against *S. aureus* MRSA131 demonstrate bacteriostatic mechanism of action. We performed time-kill assays using *S. aureus* MRSA131 cultures. Cells were treated with five times the MIC of different antibiotics and the CFUs were determined at 0, 2, 4, 6 and 8 hours after the addition of antibiotic. The assay was setup in triplicate with error bars displaying the standard deviation. Three controls were included, a growth control with no antibiotic (red), a bacteriostatic agent (minocycline, blue), and a bactericidal agent (daptomycin, green). When grown in the presence of KCN-AAS-1 (pink) or the analog KCN-AAS-35 (black) bacteriostatic activity was observed.

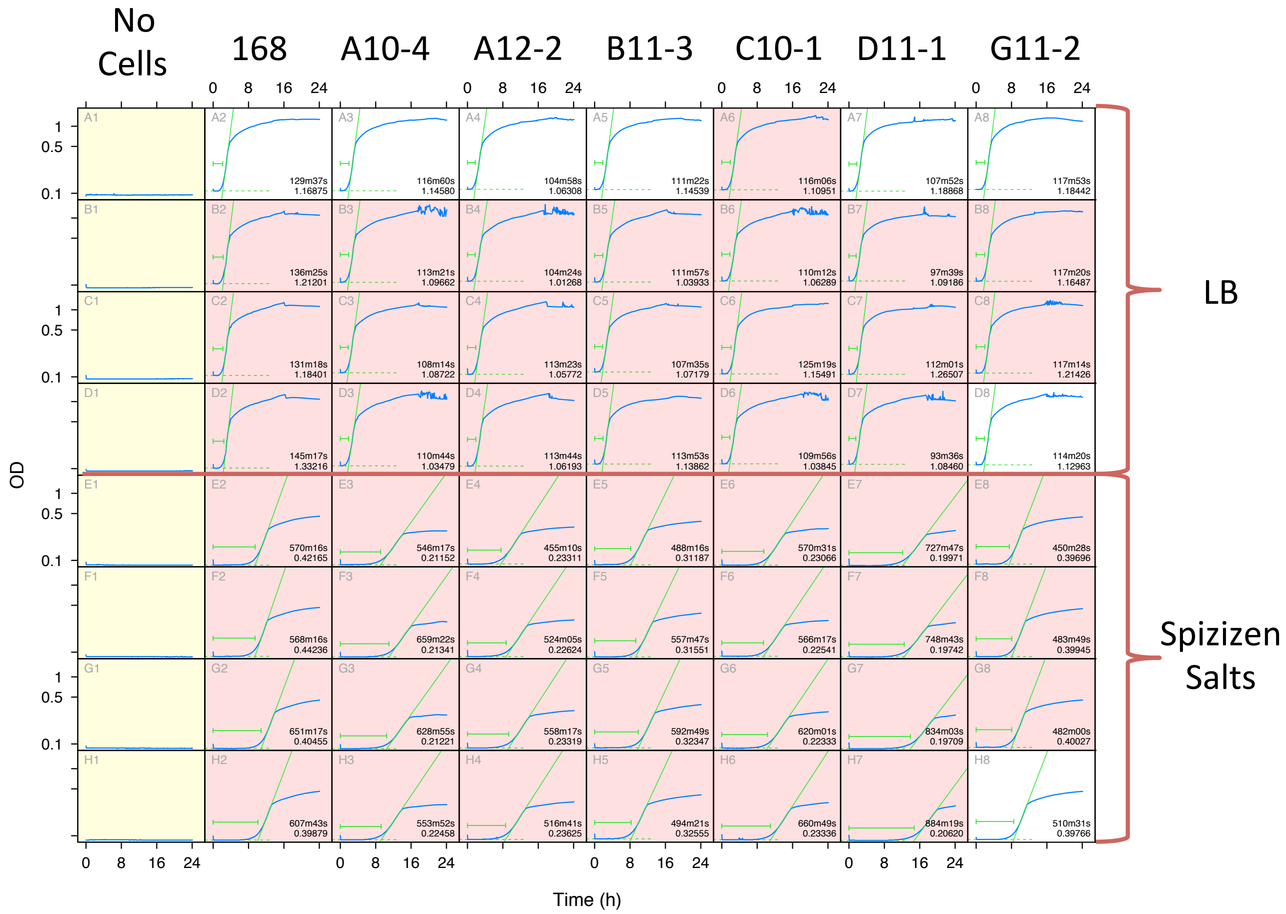

Figure S2: The growth of *B. subtilis* D11-1 and G11-2 was not cysteine-dependent, despite the presence of mutations in cysteine synthetase. The adapted *B. subtilis* D11-1 and G11-2 strains, which harbor mutations in the *cysK* cysteine synthatase gene (Table 2), grew in Spizizen salts minimal media despite the absence of cysteine, demonstrating that the strains are still capable of producing cysteine. *B. subtilis* 168 and the KCN-AAS-35 adapted strains were each cultured overnight in LB media or Spizizen media. The overnight cultures were then diluted 1:100 into the same media and 150 µl of diluted cells was placed into each well of a 96 well plate. The plates were kept at 37°C on a Synergy 2 (BioTek) Gen5 plate reader for 24 hrs and the optical density (OD) at 600 nm was measured every 5 min. The first column is a no growth control with media-lacking cells and the subsequent columns each contain a different strain. The top four rows have wells with four replicates of each strain in LB media, while the bottom four rows have wells with four replicates of each strain in Spizizen salts.

Table S1: Mutations Identified in KCN-AAS-35 adapted clones.

***B. subtilis* 168**

Clone	Chromosomal Position	Mutation	Description
A10-4	490,597	ΔT, Intergenic (+43/+180) (T)17→16	Thiamine pyrophosphate-containing protein YdaP/Hypothetical protein
	660,862	+A, Frame shift: coding(267/1032 nt) (A)6→7	Type-2 restriction enzyme BsuMI component, YdiS
	2,812,132	Δ32 bp (Deletes ribosome binding site & start codon)	Rrf2 family transcriptional regulator, CymR
A12-3	1,232,374	S431L (TCA→TTA)	Oligoendopeptidase F
	2,812,054	S27L (TCA→TTA)	Rrf2 family transcriptional regulator, CymR
B11-3	427,389	Y48C (TAT→TGT)	Sensor histidine kinase
	490,597	ΔT, Intergenic (+43/+180) (T)17→16	Thiamine pyrophosphate-containing protein YdaP/hypothetical protein
	659,999	ΔT, Frame shift: coding(377/942 nt) (T)7→6	Type-2 restriction enzyme BsuMI component, YdiR
	2,812,005	E43D (GAG→GAT)	Rrf2 family transcriptional regulator, CymR
C10-1	660,281	W220* (TGG→TAG)	Type-2 restriction enzyme BsuMI component, YdiR
	2,812,021	S38F (TCC→TTC)	Rrf2 family transcriptional regulator, CymR
D11-1	82,425	ΔC, Frame shift: coding(655/927 nt) (C)5→4	Cysteine synthase, CysK
	657,210	Δ8,753 bp	Methylase, YdiR
G11-2	81,909	R47C (CGT→TGT)	Cysteine synthase, CysK
	659,575	A→G, Intergenic (+1878/-48)	BsuMI modification methylase subunit, YdiP/Type-2 restriction enzyme BsuMI component, YdiR

***S. aureus* MRSA131**

Clone	Contig	Position	Mutation	Description
B11-3	GL638171	68,446	V567V (GTG→GTT)	ABC transporter, ATP-binding protein
	GL638175	100,508	Δ25, Intergenic (-169/+68) (GTTAATATTGCGTATAATTTTCAAA) 2→3	RIP metalloprotease RseP/phosphatidate cytidylyltransferase
	GL638175	282,915	+T, Frame shift: coding(279/663 nt) (T)7→8	TrkA N-terminal domain protein
	GL638177	1	Δ1,166 bp	Deletion of entire contig (Deletion of Repilcation initiation protein)
	GL638181	69,198	+G, Frame shift: coding(66/423 nt)	Transcriptional regulator, Rrf2 family, CymR
	GL638181	69,199	Q23K (CAA→AAA)	Transcriptional regulator, Rrf2 family, CymR
	GL638182	92,282	S68A (TCA→GCA)	Transposase
	GL638182	92,287	S66L (TCA→TTA)	Transposase
	GL638182	92,311	L58P (CTA→CCA)	Transposase

	GL638182	113,164	A13P (GCA→CCA)	HTH-type transcriptional regulator, SarR
	GL638185	1	Δ594 bp	Deletion of entire contig
	GL638192	1	Δ926 bp	Deletion of entire contig (Deletion of Hypothetical membrane protein)
	GL638198	86,041	L379P (CTT→CCT)	Putative Na+/H+ antiporter
C11-2	GL638168	24,639	+A, Frame shift: coding(234/312 nt)(A)6→7	Ribosomal protein L7Ae
	GL638171	43,620	A→G, Intergenic (+1332/+142)	Serine-rich adhesin for platelets/Flavin reductase
	GL638181	69,192	ΔA, Frame shift: coding(60/423 nt) (A)6→5	Transcriptional regulator, Rrf2 family, CymR
	GL638188	9,748	Y397Y (TAT→TAC)	Transposase, IS4 family
	GL638196	25,144	Y71N (TAT→AAT)	Pur operon repressor PurR
	GL638198	88,914	ΔT, Frame shift: coding(40/1830 nt) (T)8→7	Amino acid permease
	GL638198	101,619	A→G, Intergenic (+221/-387)	Amino acid permease family protein/Carboxylesterase
E10-1	GL638164	32,633	N421K (AAT→AAG)	EVE-domain containing protein
	GL638166	3,187	P34L (CCA→CTA)	Hypothetical protein
	GL638171	55,306	+56 bp, Intergenic (+244/+147)	Hypothetical protein/Triacylglycerol lipase
	GL638175	100,508	Δ25, Intergenic (-169/+68) (GTTAATATTGCGTATAATTTTCAAA) 2→3	RIP metalloprotease RseP/phosphatidate cytidylyltransferase
	GL638175	283,123	L24* (TTA→TGA)	TrkA N-terminal domain protein
	GL638181	26,612	+6 bp, Frame shift: coding(62/1263 nt) (CCAAGA)1→2	ATP-dependent Clp protease, ATP-binding subunit, ClpX
	GL638181	66,703	Δ3,901 bp	Deletion of Hypothetical protein; ThIF family protein; Recombination factor protein, RarA; Transcriptional regulator, Rrf2 (CymR); CsbD-like protein; Hypothetical protein; and FMN-dependent luciferase family oxidoreductase
	GL638181	155,387	Δ56 bp, Intergenic (+7/+2)	Biotin/lipoate A/B protein ligase family protein/hypothetical protein
	GL638182	92,282	S68A (TCA→GCA)	Transposase
	GL638182	92,287	S66L (TCA→TTA)	Transposase
	GL638183	131,080	L42I (CTT→ATT)	HTH-type transcriptional regulator, MgrA
	GL638188	9,748	Y397Y (TAT→TAC)	Transposase, IS4 family
	GL638197	50,984	Δ9 bp, In-frame, coding(454-462/1509 nt)	Gram-positive signal peptide protein, YSIRK family
E10-3	GL638164	32,633	N421K (AAT→AAG)	EVE-domain containing protein
	GL638175	100,508	Δ25, Intergenic (-169/+68) (GTTAATATTGCGTATAATTTTCAAA) 2→3	RIP metalloprotease RseP/phosphatidate cytidylyltransferase
	GL638175	180,975	P51P (CCA→CCC)	S4 domain protein
	GL638175	315,526	Q30* (CAG→TAG)	Beta-lactamase

	GL638181	26,612	+6 bp, Frame shift: coding(62/1263 nt) (CCAAGA)1→2	ATP-dependent Clp protease, ATP-binding subunit, ClpX
	GL638181	66,703	Δ3,901 bp	Deletion of Hypothetical protein; ThIF family protein; Recombination factor protein, RarA; Transcriptional regulator, Rrf2 (CymR); CsbD-like protein; Hypothetical protein; and FMN-dependent luciferase family oxidoreductase
	GL638182	92,282	S68A (TCA→GCA)	Transposase
	GL638182	92,287	S66L (TCA→TTA)	Transposase
	GL638182	92,311	L58P (CTA→CCA)	Transposase
	GL638183	131,080	L42I (CTT→ATT)	HTH-type transcriptional regulator MgrA
E11-1	GL638168	25,323	I1076F (ATC→TTC)	DNA-directed RNA polymerase, beta subunit
	GL638175	149,008	G40V (GGT→GTT)	Protein phosphatase 2C
	GL638181	69,124	G→A, Intergenic (-103/-9)	Recombination factor protein RarA/Transcriptional regulator, Rrf2 family, CymR
	GL638183	130,242	Δ3,333 bp	Deletion of CobW/P47K family protein; MgrA; ABC transporter, ATP-binding protein; and ABC transporter, ATP-binding protein
F10-4	GL638164	17,599	G18E (GGA→GAA)	PTS system mannitol-specific EIICB component
	GL638166	2,476	ΔA, Intergenic (-371/+163) (A)9→8	Hypothetical protein/Hypothetical protein
	GL638168	459	L1004S (TTA→TCA)	Serine-aspartate repeat-containing protein D
	GL638168	52,696	ΔT, Frame shift: coding(130/153 nt) (T)7→6	Hypothetical protein
	GL638170	115,158	T→C, Intergenic (-266/-90)	PTS system, glucose-like IIB component/Inosine-uridine preferring nucleoside hydrolase
	GL638171	23,279	V52V (GTG→GTA)	Isochorismatase family protein
	GL638171	60,209	R156R (CGG→CGA)	1-(5-phosphoribosyl)-5-[(5- phosphoribosylamino) methylideneamino]imidazole-4- carboxamide isomerase
	GL638171	62,736	I23I (ATT→ATC)	Aminotransferase, class I/II
	GL638171	125,815	ΔA, Frame shift: coding(582/1335 nt) (A)6→5	YycH protein
	GL638173	22,633	ΔA, Frame shift: coding(124/558 nt)	Probable membrane protein
	GL638175	38,190	+T, Frame shift: coding(156/174 nt) (T)6→7	Hypothetical protein
	GL638175	59,665	+A, Frame shift: coding(246/2619 nt	DNA mismatch repair protein, MutS
	GL638175	100,508	Δ25 bp, Intergenic (-169/+68) (GTTAATATTGCGTATAATTT	RIP metalloprotease RseP/phosphatidate cytidylyltransferase

			TCAAA)2→3	
	GL638175	185,116	H439H (CAT→CAC)	Cell division protein, FtsA
	GL638175	350,096	G313S (GGT→AGT)	Cation transport protein
	GL638175	366,380	S19S (AGT→AGC)	Enoyl-(acyl-carrier-protein) reductase
	GL638177	2	A→G, Intergenic (–/-180)	–/Replication protein
	GL638181	26,612	+6 bp, Frame shift: coding(62/1263 nt) (CCAAGA)1→2	ATP-dependent Clp protease, ATP-binding subunit, ClpX
	GL638181	69,322	G64R (GGA→AGA)	Transcriptional regulator, Rrf2 family, CymR
	GL638181	88,853	+A, Frame shift: coding(567/1362 nt) (A)7→8	Putative acetyl-CoA carboxylase, biotin carboxylase subunit
	GL638181	303,996	A981V (GCA→GTA)	Gram-positive signal peptide protein, YSIRK family
	GL638181	305,699	V1549M (GTG→ATG)	Gram-positive signal peptide protein, YSIRK family
	GL638181	326,965	K8637K (AAA→AAG)	Gram-positive signal peptide protein, YSIRK family
	GL638182	8,643	I250V (ATA→GTA)	ABC transporter, permease protein
	GL638182	28,920	+A, Frame shift: coding(2449/2523 nt) (A)5→6	Phosphatidylglycerol lysyltransferase
	GL638182	34,647	+T, Frame shift: coding(1357/1461 nt) (T)6→7	Amino acid carrier protein
	GL638182	41,612	G→A, Intergenic (+152/-224)	Acyl-phosphate glycerol 3-phosphate acyltransferase/HesB-like protein
	GL638182	92,282	S68A (TCA→GCA)	Transposase
	GL638182	92,287	S66L (TCA→TTA)	Transposase
	GL638182	92,311	L58P (CTA→CCA)	Transposase
	GL638182	180,161	T77A (ACT→GCT)	Alpha-acetolactate decarboxylase
	GL638183	51,763	K27E (AAA→GAA)	Excinuclease ABC, A subunit
	GL638183	86,783	G102G (GGT→GGC)	Amino acid/peptide transporter
	GL638183	244,965	T423T (ACA→ACG)	Transporter, major facilitator family protein
	GL638186	16,294	T→C, Intergenic (+581/+257)	Hypothetical protein/Hypothetical protein
	GL638189	143,611	T→C, Intergenic (+137/-49)	CobB/CobQ-like protein/Hypothetical protein
	GL638189	148,941	ΔA, Frame shift: coding(642/993 nt) (A)8→7	Sensor protein, VraS
	GL638189	149,545	D88D (GAT→GAC)	Response regulator protein, VraR
	GL638189	150,795	G150G (GGT→GGC)	YihY family protein
	GL638191	77,895	Y166H (TAT→CAT)	NAD dependent epimerase/dehydratase family protein
	GL638195	9,096	S198P (TCC→CCC)	Iron chelate uptake ABC transporter, FeCT family, permease protein
	GL638196	37,902	H120R (CAT→CGT)	Orn/Lys/Arg decarboxylase, major domain protein

	GL638197	70,754	N3N (AAC→AAT)	Hypothetical protein
	GL638197	107,409	+A, Frame shift: coding(97/1134 nt) (A)6→7	Low temperature requirement protein, LtrA
	GL638197	136,737	L235L (TTA→CTA)	Oxidoreductase, FAD/FMN-binding protein
	GL638198	51,173	+A, Intergenic (-206/+89) (A)7→8	ABC transporter, substrate-binding protein/addiction module toxin, Txe/YoeB family
	GL638198	88,914	ΔA, Frame shift: coding(40/1830 nt) (T)8→7	Amino acid permease
	GL638198	274,232	W162* (TGG→TAG) C→T	MBL fold hydrolase
	GL638198	276,599	+A, Frame shift: coding(4/1623 nt) (T)6→7	Transporter, betaine/carnitine/choline family
	GL638198	294,575	+A, Intergenic (-82/+34) (A)7→8	Putative esterase/LPXTG-motif cell wall anchor domain protein

***E. faecalis* S613**

Clone	Chromosomal Position	Mutation	Description
A2-1	15,077	W11* (TGG→TAG)	Adenylosuccinate synthetase
	602,198	G141V (GGA→GTA)	Hypothetical protein
	1,680,221	G126G (GGA→GGC)	Substrate-specific component QueT of predicted queuosine-regulated ECF transporter
	2,001,928	L268* (TTA→TAA)	Phage minor structural protein
	2,268,468	ΔA, Frame shift: coding(582/1728 nt)	ABC-type multidrug/protein/lipid transport system, ATPase component
	2,280,609	V305D (GTT→GAT)	ATP synthase beta chain
	2,390,144	D317A (GAC→GCC)	Tellurite resistance protein
	2,418,233	R175H (CGC→CAC)	Unknown protein with CBS domain
	2,506,878	A45V (GCA→GTA)	Transcriptional regulator of fatty acid biosynthesis, FabT
	2,556,951	T110R (ACA→AGA)	Carbonic anhydrase, family 3
A3-1	195,824	D40A (GAT→GCT)	Transcriptional repressor for NAD biosynthesis in gram-positives
	2,280,609	V305D (GTT→GAT)	ATP synthase beta chain
	2,506,878	A45V (GCA→GTA)	Transcriptional regulator of fatty acid biosynthesis, FabT
C3-4	474,856	T→A, Intergenic	Tyrosyl-tRNA synthetase (EC 6.1.1.1)/Decarboxylase, putative
	1,934,327	P519A (CCA→GCA)	Duplicated ATPase component MtsB of energizing module of methionine-regulated ECF transporter
	2,285,730	G188* (GGA→TGA)	ATP synthase F0 sector subunit a
	2,318,363	C→A, Intergenic	SNARE-like domain protein/6-phospho-3-hexuloisomerase
	2,506,892	M40I (ATG→ATT)	Transcriptional regulator of fatty acid biosynthesis, FabT
	2,555,387	G70G (GGG→GGA)	Phosphoglycolate phosphatase
H2-4	105,681	G192E (GGA→GAA)	YheO-like protein, likely involved in signaling

	474,843	+IS204, intergenic	Insertion of transposase IS204
	1,059,412	T96A (ACC→GCC)	Transcriptional repressor of the fructose operon, DeoR family
	1,142,062	R472Q (CGG→CAG)	Hypothetical protein with DNA binding domain and PAS domain. Likely a sugar sensor that regulates sugar usage
	2,285,481	G15C (GGT→TGT)	ATP synthase F0 sector subunit c
H3-2	251,022	T397M (ACG→ATG)	Potassium uptake protein, integral membrane component, KtrB
	718,140	+A, Intergenic	Osmotically activated L-carnitine/choline ABC transporter, ATP-binding protein OpuCA/hypothetical protein with no matches
	741,257	Q282* (CAG→TAG)	Hypothetical protein that may contain family 8 glycosyl hydrolase
	2,282,893	F395C (TTT→TGT)	ATP synthase alpha chain (EC 3.6.3.14)
H3-3	251,022	T397M (ACG→ATG)	Potassium uptake protein, integral membrane component, KtrB
	1,145,169	C→T, Intergenic	Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily F2 (as in PMID19099556)/Acetolactate synthase, catabolic (EC 2.2.1.6)
	1,254,962	P469Q (CCA→CAA)	ABC transporter permease protein
	1,462,227	S121Y (TCT→TAT)	Hypothetical protein with a lysine motif that binds with peptidoglycan layer
	1,478,642	I305I (ATC→ATT)	Chorismate synthase
	2,278,725	ΔT, Frame shift: coding(428/1302 nt) (T)7→6	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
	2,282,893	F395C (TTT→TGT)	ATP synthase alpha chain