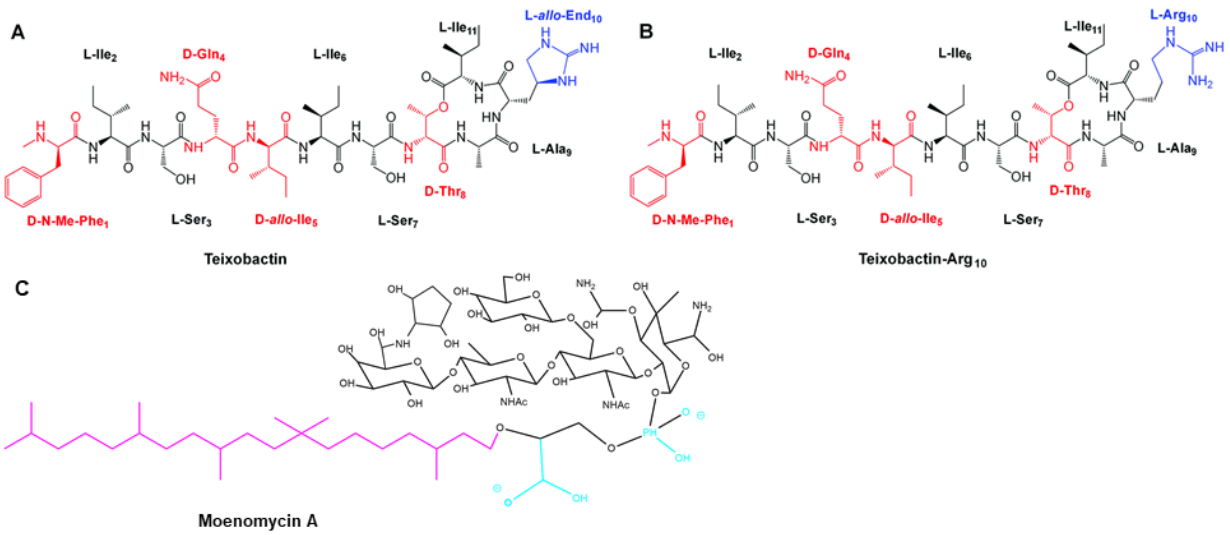
Supplementary Information

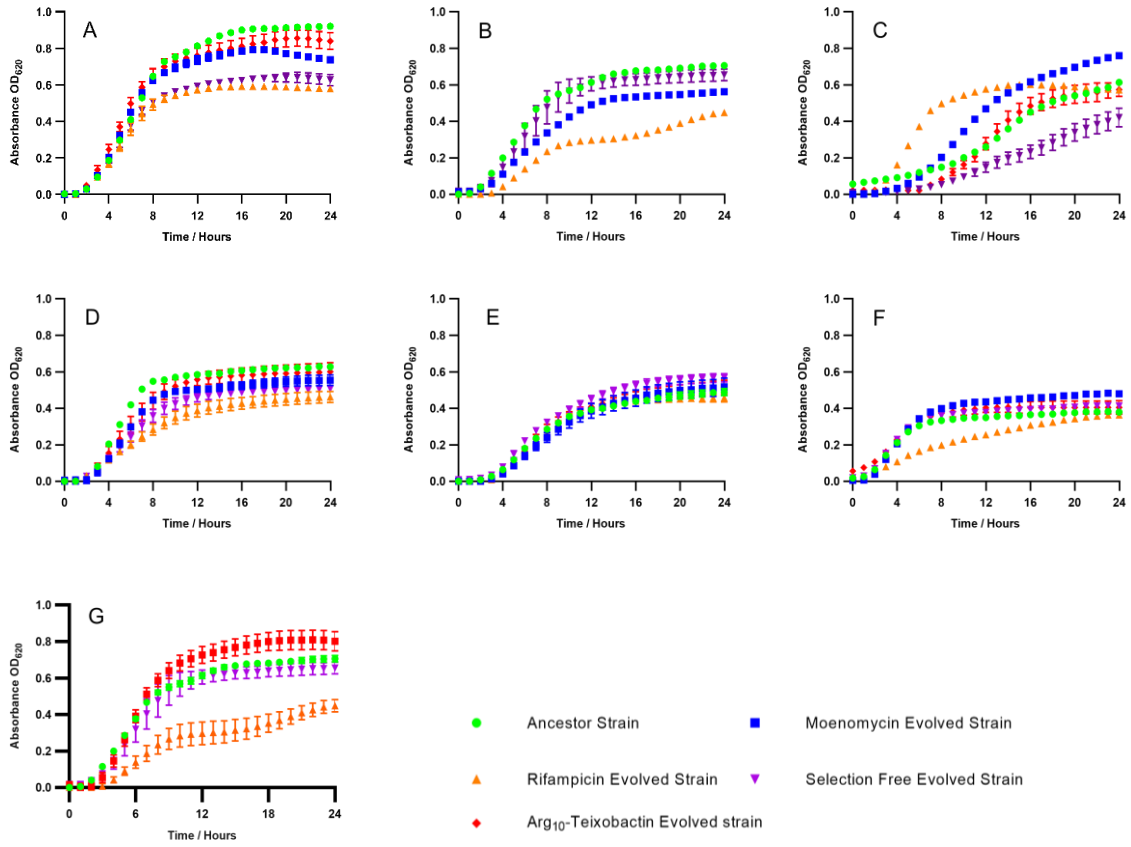


S-figure 1 Structures of (A) teixobactin and (B) arg$_{10}$ teixobactin with the residues numbered 1-11, D amino acids highlighted in red and the structural differences marked in blue. (C) Structure of moenomycin A showing its polar (cyan) and hydrophobic (magenta) regions.

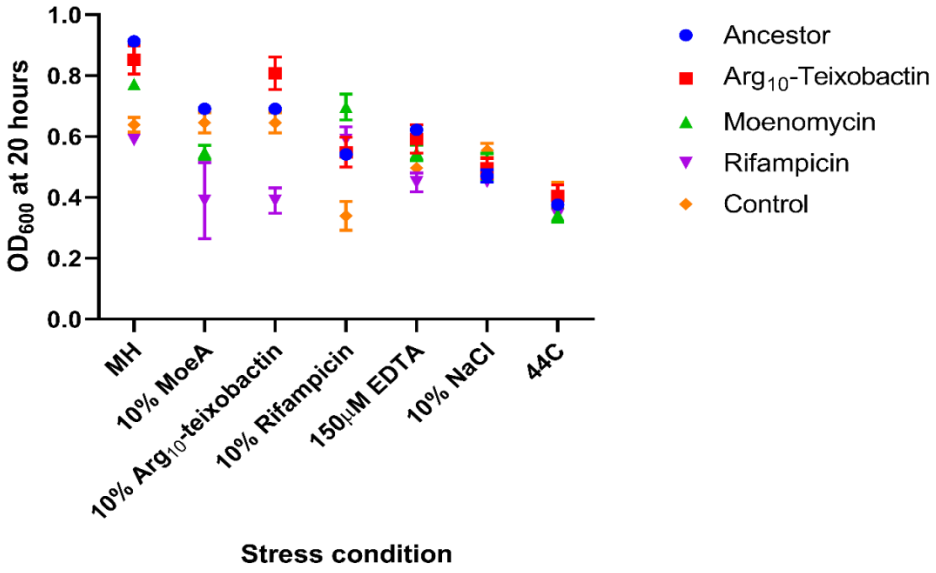
S-table 1 Primers sequences used to amplify a staphylococcal specific region of the 16S rRNA gene, and the mecA gene that conveys methiciliin resistance.

Primer name	Primer sequence (5'-3')
Staph756F	AACTCTGTTATTAGGGAAGAACA
Staph750R	CCACCTTCCTCCGGTTTGTCACC
MecA1	GTAGAAATGACTGAACGTCCGATAA
MecA2	CCAATTCCACATTGTTTCGGTCTAA

S-1 The effect of moenomycin A and arg10-teixobactin evolved resistance on growth rate of *S. aureus*



S-figure 2 The average growth of ancestral, evolved moenomycin A, evolved arg_{10}-teixobactin and evolved rifampicin resistant strains under stresses as an indicator for fitness. The average absorbance at 620nm for each strain generated compared with the starting ancestor strain across a range of conditions at 37°C unless otherwise stated. From top left clockwise: (A) Unaltered MH, (B) MH supplemented with moenomycin A to 10% MIC, (C) MH supplemented with rifampicin to 10% MIC, (D) MH supplemented to 150μM EDTA, (E) MH supplemented to 10% NaCl, (F) MH media at 44°C and (G) MH supplemented with arg_{10}-teixobactin to 10% MIC. N=3, where visible error bars represent SEM.



S-figure 3 The mean maximal population growth of ancestral, evolved moenomycin A, evolved arg_{10}-teixobactin and evolved rifampicin resistant strains under stress conditions after 20 hours, used as an indicator of fitness. The average absorbance at 620nm for each strain generated compared with the starting ancestor strain across a range of conditions at 37°C unless otherwise stated. N=3, where visible error bars represent SEM.

*S-table 2 The mean rates of intrinsic increase (r) per unit of time or growth rates derived from changes in optical density of moenomycin A, arg_{10}- teixobactin and rifampicin resistant strains and selection free cells relative to the ancestral strain (rate of 1) across a range of sub-lethal stress conditions in a base Mueller Hinton media. * indicates significant difference from the ancestral strain determined through one-way ANOVA (P <0.05), with post hoc Tukey tests (P < 0.05). N/A – not tested.*

Strain	37 °C	10% Moe A MIC supplement	10% rifampicin MIC	10% arg_{10}-teixobactin MIC	150mM EDTA	44°C	10% w/v NaCl
Arg_{10}-teixobactin (45days selection)	0.996	N/A	0.122*	1.361*	0.722*	0.941	1.216*
Moenomycin A (45days selection)	0.952	0.773*	3.068*	N/A	0.783*	1.267*	1.124*
Rifampicin (45 days selection)	0.739*	0.643*	6.881*	0.616*	0.417*	0.473*	1.095
Control (45 days without selection)	0.763*	1.095*	0.706	1.049	0.551*	1.072	1.093

S-2.1 Discussion of induced resistance mechanisms

The role and location of common mutations found within moenomycin A resistant strains

Mutations unique to moenomycin A resistant were observed across three key genes, these were acquired across all six replicate strains. Two of these genes, penicillin-binding protein 2 (*pbp2*) and a

bifunctional autolysin (*SagB*), play key roles in cell wall modulation, whilst *purR* is the regulatory domain of cellular Purine metabolism (1).

*Pbp*2, an important component of cell wall synthesis, is a bifunctional enzyme which catalyses the formation of the NAG-NAM β-1-4 carbohydrate and amide linkages within peptidoglycan. Moenomycin A is a well know inhibitor of *pbp2*, preventing the formation of the nascent peptidoglycan chain. Multiple mutations were observed in the active site of *pbp2*. This is consistent with Rebets *et al* who previously described individual active site mutations in two of these positions (Y196 and P234) individually these gave rise to a 25-fold increase in resistance. *SagB* is a bifunctional autolysin which modulates mature peptidoglycan and has a role in cell wall restructuring. *SagB* is a family 73 muramidase, which cleaves the NAG NAM β-1-4 peptidoglycan linkage. The F134L mutation appears to be away from the *SagB* activate site. *purR* is the repressor protein regulating the purine biosynthetic pathway. The I154* mutation causes a truncation of *purR* resulting in the loss of the 5-phospho ribosyl 1-pyrophosphate binding site. This may constitutively induce transcription of the Pur operon. Purine biosynthesis is linked to DNA and ATP metabolism, and its upregulation may play a role in offsetting the cost of resistance. The effect of all mutations working in concert gave rise to ~1000-fold increase in moenomycin resistance across all six strains.

The role and location of common mutations found within arg_{10}-teixobactin resistant strains

Strains with resistance against arg_{10}-teixobactin showed three distinct paths to resistance. Though there was little overlap between these mechanisms, there are common themes of modifications with links to lipid biosynthesis, cell wall modifications and energy metabolism.

Path one: four arg_{10}-teixobactin resistant strains carried identical mutations in Phosphatidylglycerol lysyltransferase (*mprF*), transcriptional regulatory protein (*srrA*), KDP operon transcriptional regulatory protein (*kdpE*) and DNA gyrase subunit A (*gyrA*). The tolerance of these strains was: Strain 1 (8µg/mL), strain 2 (8µg/mL), strain 3 (4µg/mL) and strain 4 (5.3µg/mL). *mprF* has been previously implicated in modifying staphylococcal lysylphosphatidylglycerol to convey a net extracellular positive charge, reducing susceptibility to several cationic antimicrobial peptides(2) . *srrA* and *kdpE* are phospho-relay signal transduction proteins which regulate separate, two-component signalling pathways. These influence a number of downstream processes thought to be immunomodulatory and virulence linked (14, 15). *gyrA* is subunit of DNA gyrase which is responsible for negatively supercoiling DNA(3). Two further mutations appear to increase resistance further, the glutamine methyltransferase (PRM C) present in strains 1, 2 & 4 and protein dltD (*dltD*) present in strains 1 & 2. *prmC* regulates gene expression through methylation of the 'GGQ motif' and is crucial to bacterial protein translation (4). *DltD* involved in D-alanylation of lipoteichoic acid and potentially influencing the charge of the cell wall excluding charged compounds from their active sites (5).

Path two: Strain 6 exhibited the joint highest tolerance to arg_{10}-teixobactin (8µg/mL), harbouring three primary mutation, the putative peptidoglycan glycosyltransferase FtsW (*ftsW)*, Extracellular matrix-binding protein (*ebh1*) and Isocitrate dehydrogenase (*icd*).

FtsW is one of a number of proteins suggested to function as a lipid II flippase and peptidoglycan synthase (6, 7), like *pbp2* it has been speculated to catalyse the incorporation of lipid II into peptidoglycan, though unproven, this may identify a mechanism requiring further study. Lipid II has been shown to be the primary target for teixobactin (figure 3 panel a). *Ebh1*, is a > 400 Kda protein, thought to sequester harmful compounds including antimicrobial peptides (8). Isocitrate dehydrogenase (Icd) is an enzyme found within the TCA cycle. An A317P point mutation was observed within *ICD.* This near active site mutation may lead to a shift in cellular energy metabolism.

Path three: arg_{10}-teixobactin resistant strain 5 acquired moderate resistance (5.3 µg ml^{-1}). Mutations were found within a Tyrosine-protein kinase (YWQ D) and Protoporphyrinogen oxidase (*hemY*). *YwqD* is a close homologue of a regulatory protein for the capsule biogenesis pathway (9). An increase in extracellular capsid production has been previously implicated in intermediary vancomycin resistance, this may carry over into resistance to other cell wall targeting antimicrobials (22). *HemY* is a Protoporphyrinogen oxidase which catalyses the oxidation of haem precursors (10). An M1V mutation resulted in the loss of start codon and subsequent truncation of the protein.

In addition to these identified adaptations, several mutations were identified in two genes with commonality between all 12 sequenced strains. These two genes appear to be immediately next to one another in an ORF encoding hypothetical proteins of no certain function. Such proximity and commonality in mutations suggests these sites are either important adaptations under the experimental conditions, or that they are sites key to the evolution of resistance to these antibiotics

S-2.2 Full discussion of individual mutations

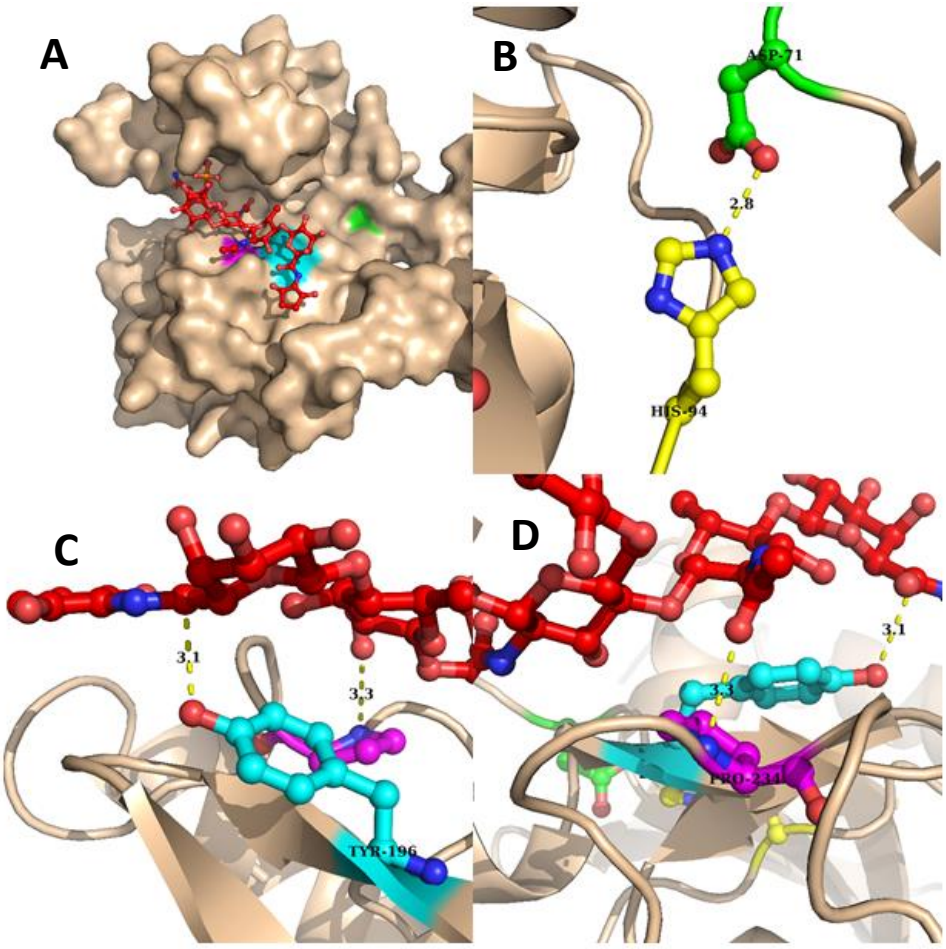
S-2.1 Genes with mutations unique to moenomycin A resistant strains

PurR

The mutation I154fs in the Pur operon repressor protein was consistently present in all 6 evolved strains (MoeA 1-6). It was identified as a frame shift mutation, resulting in a truncated p*urR* repressor protein. The truncation results in loss of the PRPP, 5-phosphoribosyl 1-pyrophosphate binding site. A deletion in this binding region has been shown to abolish repression of transcription of the Pur operon (1). The Pur operon encodes genes involved in purine synthesis, specifically inosine monophosphate, a precursor compound related to AMP2 and DNA production. This mutation is consistent with other PurR mutations which have been previously implicated in vancomycin resistance development (11).

Pbp2

A suite of point mutations were discovered in penicillin-binding protein 2 (*pbp2*0 (D71V, Y196H and P234Q). These were all present in all 6 evolved strains except for strain 5 which lacked the D71V mutation. Pbp2 catalyses the formation of the carbohydrate linkage in peptidoglycan and the glycosyltransferase domain is inhibited by moenomycin A (12). When mapped onto the PBP2 structure (2OLU5) the D71V mutation was found to be remote from the active site (S-figure 4 panel B, green) is likely that this mutation disrupts the hydrogen bonding made between Asp 71 and His 94. It is likely that the D71V mutation results in a conformational change with possible implication on substrate binding. The Y196H and P234Q mutations were found to be within the substrate binding pocket of the enzyme and be within bonding distance of moenomycin A (S-figure 4 panels C and D, cyan and magenta). These mutations are consistent with the findings of Rebets et al (13), who used phage mediated mutagenesis to induce moenomycin A resistance. There two of the same locations with mutations were reported, but with a differing residue at position 196 (Y>D as opposed to this study that obtained Y>H. The P234Q was the same in both studies. Rebets et al showed a 5-fold increase in the minimum inhibitory concentrations (MICs), from 0.5 μg mL−1 to 12.5 μg mL−1. Much higher rates of resistance were observed in this study in strains harbouring all six mutation from 0.125 μg mL−1 to 128 μg mL.



S-figure 4 ***The location and bonding interactions of residue within PBP2 that when mutated give rise to moenomycin A resistance.*** *Panel A. The locations for the resistance mutations in PBP2, D71V (green), Y196H (cyan) and P234Q (magenta) in relation to Moenomycin A (red ball and stick) the surface of PBP2 shown in wheat. (PDB code 2OLU (14)) 98%(91%) by BLAST. Panel B. PBP2 showing secondary structure in cartoon, the hydrogen bonding interaction between Asp71 (green) and His94 (yellow). It is likely be abolished by the D71V mutation. Panel C. PBP2 showing secondary structure in cartoon, the electrostatic interaction between OH group of Tyr196 (cyan) and the CCM/1000 atom of (red ball and stick) is likely to be abolished by the Y196H mutation. Panel D. PBP2 showing secondary structure in cartoon, the hydrogen bonding interaction between the nitrogen of Pro234 (magenta) and OCG /1000 atom of Moenomycin A. It is likely the bonding will be abolished by the P234Q mutation. The figure was drawn using PYMOL and (PDB code :2OLU (14)) which show a high sequence identity to the Mutant PBP2 evolved in this study (98% (91% coverage) by BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi), all distances are shown in angstrom (dashed yellow lines).*

SagB

The bifunctional autolysin SagB is a family 73 (http://www.cazy.org/GH73.html) muramidases which modulates peptidoglycan by the hydrolyses β-1,4-glycosidic linkage between N-acetylglucosaminyl (NAG) and N-acetylmuramyl (NAM) moieties during cell grown and division. *SagB* shares a 37% identity (67% coverage) with another staphylococcal autolysin *S. aureus* autolysin E (*atlE*) (15) , in addition mutations in SagB have been show to effect the thickness of cell wall peptidoglycan in *S. aureus (16)*. The N-terminal portion consists of a transmembrane helix and the catalytic domain is predicted to be outside the cell membrane TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Using Clustal Omega alignments and the *atlE* structural homologue, it is possible to predict the

F134L mutation lies within the A-6 helix part of the L-domain. It is unclear how the F134L mutation may confers moenomycin A resistance, however it is tempting to speculate that F134 may mediate a hitherto undescribed interaction.

LyrA

Two of the moenomycin A resistant strains (strains 3 and 4) have acquired a S107I mutation in the Lysostaphin resistance protein A hypothetical protein (*lyrA*). Lysostaphin endopeptidase which cuts the Gly-Gly bond of peptidoglycan of certain Staphylococci. *LyrA* is a intramembrane metalloprotease which has been shown to confer resistance to Lysostaphin (17). It is unclear how alteration would lead to moenomycin A resistance, with little known about this enzyme, including the lack of solved protein structure.

S-2.2 Genes with mutations unique to arg_{10}-teixobactin resistant strains

MprF Phosphatidylglycerol lysyltransferase

The L826I mutation was found to be present in a Phosphatidylglycerol lysyltransferase Lys-tRNALys–dependent lysyl-phosphatidylglycerol synthase (L-PGS) in arg_{10}-teixobactin resistant strains 1,2,3 and 4. This is an enzyme involved in Lipid homeostasis and has been shown to modify the polar head group of phosphatidylglycerol into the respective aminoacyl-ester of phosphatidylglycerol. Its activity has been previously identified as a widely used strategy to mediate bacterial resistance(18), with this same mutation associated with daptomycin resistance (2). *This enables bacteria to tolerate cationic peptides that are harmful to the integrity of the cell membrane and have been associated with resistance to a number of cationic cationic antimicrobial peptides (2). L-PGS consists of an N-terminal transmembrane flippase domain and a C-terminal catalytic domain. Pfam, Blast and Clustal Omega alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/) revealed a high degree of homology to the C-terminal catalytic domain and the deposited structure derived from B.licheniformis (2) (47% identify by blast). Analysis of this structure 4V36 pdf showed that the corresponding residue in B. licheniformis sequence (L844), was situated away from the ligand binding site in an helix near to the C-terminal end of the protein. It is unclear what effect and the L826I mutation might have on the activity of the enzyme, but it is possible that an alteration to the* polar head group phosphatidylglycerol, may make the membrane less permeable to arg_{10}-teixobactin and so provide a degree of resistance, by exclusion from its target.

Icd

Arg_{10}-teixobactin strain 6 contains an A317P mutation in isocitrate dehydrogenase, this enzyme that catalyses the oxidative decarboxylation of isocitrate to yield α-ketoglutarate and CO_2 with concomitant reduction of $NADP^+$ to NADPH Icd (18)plays a critical role the TCA cycle. The mechanism

through which this may convey resistance to cell wall antimicrobials is unclear, and it may simply be that this is simply to offset the fitness costs of resistance.

SrrA

A G228E mis-sense mutation in the *srrA* regulatory repressor protein. Mutations were found to be present in a*rg$_{10}$-teixobactin resistant* strains 1,2,3 and 4. *SrrAB* belongs to the OmpR/PhoB family of repressor proteins. The role of s*rrA/*B has been attributed to the regulation of energy transduction in response to changes in oxygen availability and has been linked to virulence alterations under microaerobic conditions (19). *Pfam, Blast and clustal omega alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/) revealed a degree of homology to the C-terminal catalytic domain of the deposited structure derived from Mycobacterium tuberculosis,* REGX3 with a 38% identity and 96% coverage (PDB code 20QR(20)). This mutation is found within a conserved GYK motif close to the C-terminal effector domain, which is usually involved in DNA-binding (20).

KdpE

A K187N miss-sense mutation in the Kdp transcriptional regulatory protein *kdpE* was found to be present in a*rg$_{10}$-teixobactin resistant* strains 1,2,3 and 4. This forms part of the two-component system (TCS) *kdpD/kdpE*, extensively studied for its regulatory role in potassium (K^+) transport, has more recently been identified as an adaptive regulator involved in the virulence and intracellular survival of pathogenic bacteria. *KdpE* regulates many downstream genes including virulence factors by directly binding to their promotor(21). *Pfam, Blast and clustal omega alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/) revealed a degree of homology (40% and 96% coverage) to KdpEB domain of the E.coli KdpE* protein (PDB code *4KNY*(21)*),* a member of the OmpR/PhoB family (22). The mutation occurs in a none conserved region of the $KdpE_B$ domain, just outside the highly conserved DNA binding region. It appears to be situated such that it may in-part alter the coordination of a binding helix (E189 to R102 (*4KNY numbering))* which interacts with one of the major grooves of DNA. Thus, this may affect DNA binding and regulation of the gene, changing the expression profiles of downstream virulence factors. Though the role that this has in antimicrobial resistance is unclear, it may have implications in a clinical setting.

FtsW

FtsW is a is a core member of the divisome complex and is essential for septal wall assembly cell wall separation(7, 23, 24). *FtsW* is a peptidoglycan polymerase that is functional only in complex with its cognate penicillin-binding protein (7) . It has been shown that FtsW interacts with PBP1b and lipid II. That PBP1b, *ftsW* and PBP3 co-purify suggests that they form a trimeric complex. We have identified a mutation within *ftsW* in one of the 6 parallel arg_{10}-teixobactin evolved stains (strain 6). Using sequence alignments and the analogous *rodA* structure of *Thermus thermos* (pdb code BAR_6) (25)We can predict the A260Q mutation lies in a highly conserved extracytoplasmic region, falling

between trans-membrane helix 7 and trans membrane helix 8. Teixobactin has been shown to be a potent lipid II and lipid III binder (25). As such we speculate that lipid II, when bound to teixobactin, may interfere with the formation of the divisome complex. Further, that the mutation may lead to the exclusion of the 'lipid II-teixobactin' complex.

GyrA

DNA gyrase (Type IIA topoisomerase) is a DNA modulating enzyme that cuts both strands of the DNA helix simultaneously in order to manage DNA tangles and supercoils (26). The synonymous R655R mutation was present in trains arg_{10}-teixobactin resistant strains 1, 2, 3 and 4. This codon usage went from 3.1% to 4.8% indicating a possible up regulation in the expression of this DNA replication regulation enzyme(27).

PrmC

Arg_{10}-teixobactin strains 1, 2 and 4. Truncation of the protein due to the inclusion of a stop codon Q105stop. (n5)-glutamine Methyltransferase Protein glutamine Posttranslational methylation of release factors on the glutamine residue of a conserved GGQ motif is required for efficient termination of protein synthesis methylation at GGQ sites of protein chain release factors plays a pivotal role in the termination of translation(28). This mutation only allows the expression of the N-terminal putative substrate binding domain.

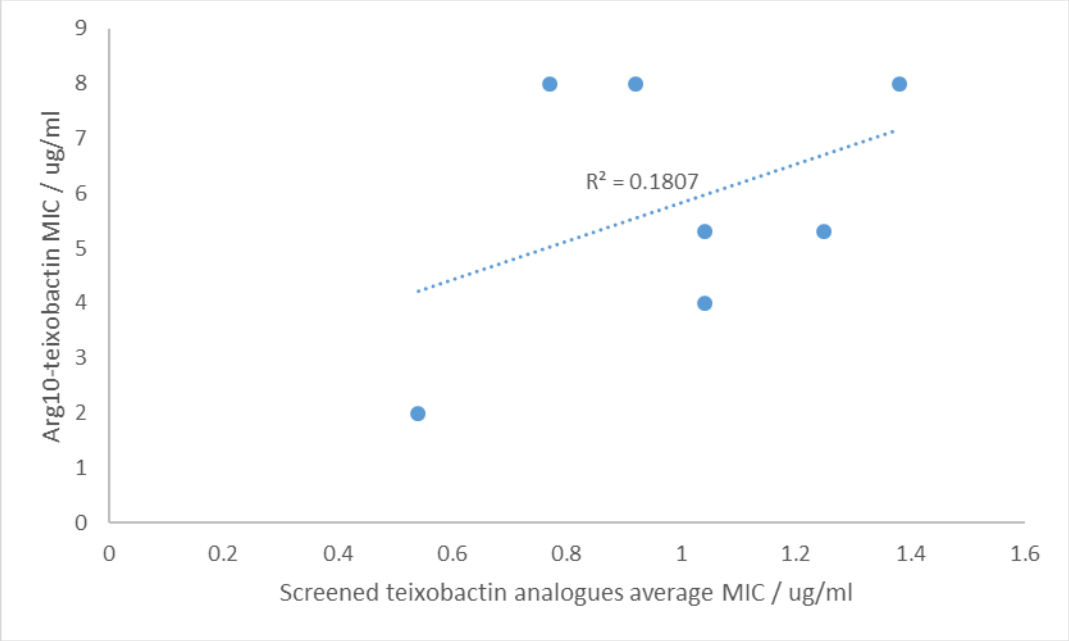
DltD

Teichoic acids are bacterial cell wall compounds which are important for growth, biofilm formation, adhesion and virulence of *S. aureus. DltD* is an D-alanyl-lipoteichoic acid synthetase which anchors outside of the cell wall (5) (see **Error! Reference source not found.** panel e). It catalyses the final transfer of the d-alanine to lipoteichoic acid. Interestingly strains of *S. aureus* which lack this d-alanine decoration show an increased susceptibility to cationic antimicrobial peptides (5). It had been proposed that the reduced D-alanyl content of the cell wall results in an overall increase in negative cell surface charge, thus promoting attraction of the cationic antimicrobial peptides (29) . P*fam, Blast and clustal omega alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/) revealed that the dltD* sequence showed a 25% identity (80% identity) to the D-alanyl-lipoteichoic acid synthetase from *Streptococcus pneumoniae* R6 (PDB code D3BM). According to mapping onto this structure the Q129P mutation, found in arg_{10}-teixobactin resistant strains 1 and 2, is likely to be located on the surface to the protein within a cleft region.

S-3 Cross resistance of arg_{10}-teixobactin resistant strains to teixobactin analogues

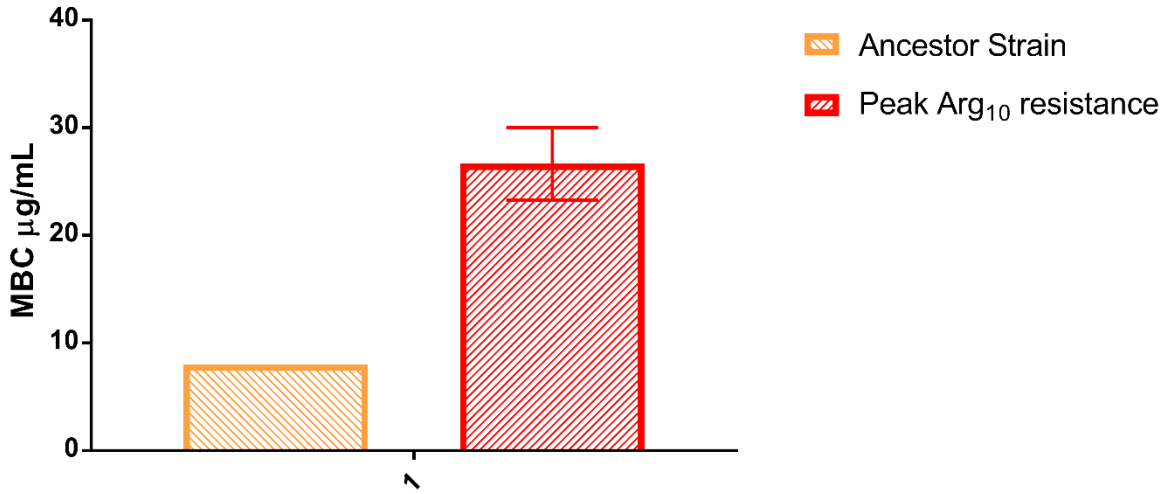
Cell Line	1	2	3	4	5	6	Ancestor
Orn_{10} teixobactin	1	1	2	2	4	4	1
Cha_{10} teixobactin	1	2	2	2	2	2	0.5
Chg_{10} teixobactin	2	1	2	1	2	1	1
Ala_{10} teixobactin	1	1	1	2	2	2	1
Leu_{10} teixobactin	0.5	1	0.5	0.5	0.5	1	0.25
Val_{10} teixobactin	0.5	1	0.5	0.5	0.5	1	0.25
Ile_{10} teixobactin	0.5	0.5	0.5	0.5	0.5	0.5	0.25
Dap_{10} teixobactin	1	1	1	1	1	2	0.5
D-Arg_9-Ile_{10} teixobactin	0.5	0.5	1	1	1	1	0.5
Arg_9-Arg_{10} teixobactin	0.25	0.5	0.5	0.5	0.5	0.5	0.25
D-Arg_4-Arg_{10} teixobactin	0.5	1	0.5	1	0.5	1	0.5
D-Arg_4-Arg_9-Arg_{10} teixobactin	0.5	0.5	1	0.5	0.5	0.5	0.5
Average	0.77	0.92	1.04	1.04	1.25	1.38	0.54
Arg_{10}-teixobactin	8	8	4	5.3	5.3	8	2

S-Table 3 A heat map showing the susceptibility of peak arg_{10}-teixobactin (day 45) resistant strains susceptibility against a panel of teixobactin analogues. Heat mapping was applied independently by teixobactin analogue, comparing the six resistant strains and ancestral strain. Red colouring highlights the lowest MIC, moving to yellow then to green for the highest MICs. All analogues with the same structure as arg_{10}-teixobactin except where stated modifications. Orn: Ornithine, Cha: Cyclohexylalanine, Chg: Cyclohexylglycine, Ala: Alanine, Leu: Leucine, Val: Valine, Ile, Isoleucine, Dap: Diaminopimelic acid, Arg: Arginine.



S-figure 5 The correlation between the susceptibility of each strain against arg_{10}-teixobactin, plotted against that same strains average susceptibility across the panel of screened teixobactin analogues (S-Table 3). The R-squared value was then plotted across the best fitting trendline and is displayed on the graph.

S-4 Changes in arg_{10}-teixobactin minimum bactericidal concentration



S-figure 6 Changes in the minimum bactericidal concentration of arg_{10}-teixobactin against MRSA 33591 before (mean MBC 8.0 µg /lL SEM 0) 0 and after (mean MBC 26.6 µg /lL SEM 3.4) induction of resistance. N=3, error bar indicates SEM.

S-table 4 Physiological tests used selective to confirm the identity of 45 day evolved and ancestral S. aureus MRSA. E. faecium, S. epidermis and K. pneumoniae strains were included to act as internal controls.

Strain	**Mannitol salt agar Growth** (X/✓)	**MacConkeys agar Growth** (X/✓)	**Eosin methylene blue agar Growth** (X/✓)	**Coagulase** (X/✓)	**Catalase** (X/✓)
Ancestor ATCC 33591	✓	X	X	✓	✓
Moenomycin A X 6 replicates	✓	X	X	✓	✓
Rifampicin X 6 replicates	✓	X	X	✓	✓
Control selection free X 6 replicates	✓	X	X	✓	✓
E. faecium DSM 17050	X	X	X	X	X
S. epidermis DSMZ 28319	X	X	X	X	✓
K. pneumoniae ATCC 700603	X	✓	✓	X	X

S-5 Mutation profiles of resistance

S-table 5 The mutation profile of arg_{10}-teixobactin resistant cells, showing the distribution of mutations across mutations that appear to correlate with 'key' mutations conveying resistance. Ticks represent the presence of the mutations, crosses the absence of change from the wild-type.

Strain	Resistance after 45 days / µg/mL	Phosphatidylglycerol lysyltransferase **Leu826Ile**	Transcriptional regulatory protein SrrA **Gly228Gln**	KDP operon transcriptional regulatory protein KdpE **Lys187Asn**	DNA gyrase subunit A **Arg655Arg**	Release factor glutamine methyltransferase **Gln105***	Protein dltD **Gln129Pro**	putative peptidoglycan glycosyltransferase FtsW **Ala260Thr**	Isocitrate dehydrogenase [NADP] **Ala317Pro**	Tyrosine-protein kinase YwqD **Asp79Asn**	Protoporphyrinogen oxidase **Start-loss**
Ancestor	2	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
1	8	✓	✓	✓	✓	✓	✓	X	X	X	X
2	8	✓	✓	✓	✓	✓	✓	X	X	X	X
3	4	✓	✓	✓	✓	X	X	X	X	X	X
4	5.3	✓	✓	✓	✓	✓	X	X	X	X	X
5	5.3	X	X	X	X	X	X	X	X	✓	✓
6	8	X	X	X	X	X	X	✓	✓	X	X

S-table 6 The mutation profile of moenomycin A resistant cells, showing the distribution of mutations across mutations that appear to correlate with 'key' mutations conveying resistance. Ticks represent the presence of the mutations, crosses the absence of change from the wild-type.

	Target protein / SNP					
Strain	Resistance after 45 days / µg/mL	Pur operon repressor **Ile154fs**	Penicillin-binding protein 1A/1B **N71V**	Penicillin-binding protein 1A/1B **Tyr196His**	Penicillin-binding protein 1A/1B **Pro234Gln**	Bifunctional autolysin **Phe134Leu**
Ancestor	0.125	✓	✓	✓	✓	✓
1	>128	✓	✓	✓	✓	✓
2	>128	✓	✓	✓	✓	✓
3	>128	✓	✓	✓	✓	✓
4	>128	✓	✓	✓	✓	✓
5	>128	✓	**X**	✓	✓	✓
6	>128	✓	✓	✓	✓	✓

S-6.1 All mutations arising in arg_{10}-teixobactin resistant *S. aureus* strains

S-table 7 A list of all detected polymorphisms across all six replicate strains with evolved resistance to arg_{10}-teixobactin.

Product	Gene	Location	Reference	Alternate	SNP effect	SNP shortened	Strains
Phosphatidylglyce-rol lysyltransferase	mprF	01285	C	A	missense_variant c.2476C>A p.Leu826Ile	SNP Leu826Ile	1, 2, 3, 4
SrrA regulatory repressor protein	srrA	01415	C	T	missense_variant c.683G>A p.Gly228Glu	SNP Gly228Glu	1, 2, 3, 4
KDP operon transcriptional regulatory protein KdpE	kdpE	02037	C	A	missense_variant c.561G>T p.Lys187Asn	SNP Lys187Asn	1, 2, 3, 4
DNA gyrase A	gyrA	02622	C	A	synonymous_variant c.1965C>A p.Arg655Arg	syn Arg655Arg	1, 2, 3, 4
Glutamine Methyltransferase	prmC	02001	C	T	stop_gained c.313C>T p.Gln105*	stop codon Gln105*	1, 2, 4
Protein dltD	dltD	00857	A	C	missense_variant c.386A>C p.Gln129Pro	SNP Gln129Pro	1, 2
hypothetical protein		01693	G	A	synonymous_variant c.762G>A p.Pro254Pro	syn Pro254Pro	1, 2, 4
hypothetical protein		01693	A	G	missense_variant c.106A>G p.Asn36Asp	SNP Asn36Asp	1,4
hypothetical protein		01693	G	T	missense_variant c.524G>T p.Arg175Ile	SNP Arg175Ile	1, 4
hypothetical protein		01693	A	ACTGAT	frameshift_variant c.633_637dupTGATC p.His213fs	frameshiftHis213fs	1, 4
hypothetical protein		01693	T	A	synonymous_variant c.777T>A p.Thr259Thr	syn Thr259Thr	1, 4
hypothetical protein		01693	T	C	synonymous_variant c.783T>C p.Ile261Ile	syn Ile261Ile	1, 4
hypothetical protein		01693	T	A	missense_variant c.139T>A p.Phe47Ile	SNP Phe47Ile	2, 6
hypothetical protein		01693	T	C	synonymous_variant c.186T>C p.Ile62Ile	syn Ile62Ile	2, 6
hypothetical protein		01693	C	T	synonymous_variant c.192C>T p.Phe64Phe	syn Phe64Phe	2, 6

hypothetical protein		01693	CTTATT GATAAT AATTCA ATTT	C	conservative_inframe_deletion c.226_246delAATAATTCAATTTTTATTGAT p.Asn76_Asp82del	inframe_del Asn76_Asp82del	2, 6
hypothetical protein		01693	A	G	synonymous_variant c.276A>G p.Arg92Arg	syn Arg92Arg	2, 6
hypothetical protein		01693	A	G	missense_variant c.461A>G p.Asp154Gly	SNP Asp154Gly	2, 6
hypothetical protein		01693	TGTG	AGTA	missense_variant c.490_493delTGTGinsAGTA p.CysAla164SerThr	SNP CysAla164SerThr	2, 6
hypothetical protein		01693	AGAACTG	GAACTA	frameshift_variant&missense_variant c.523_529delAGAACTGinsGAACTA p.Arg175fs	frameshift& snpArg175fs	2, 6
hypothetical protein		01693	G	T	missense_variant c.549G>T p.Lys183Asn	SNP Lys183Asn	2, 6
hypothetical protein		01693	T	C	synonymous_variant c.621T>C p.Ser207Ser	syn Ser207Ser	2, 6
hypothetical protein		01693	G	A	synonymous_variant c.762G>A p.Pro254Pro	syn Pro254Pro	2, 6
hypothetical protein		01694	A	G	missense_variant c.86A>G p.Asp29Gly	SNP Asp29Gly	1
hypothetical protein		01694	G	A	missense_variant c.127G>A p.Asp43Asn	SNP Asp43Asn	1
hypothetical protein		01694	C	T	synonymous_variant c.228C>T p.Cys76Cys	syn Cys76Cys	1
hypothetical protein		01694	G	T	missense_variant c.241G>T p.Ala81Ser	SNP Ala81Ser	1
hypothetical protein		01694	GCAA	ACAT	missense_variant c.255_258delGCAAinsACAT p.Gln86His	SNP Gln86His	1
hypothetical protein		01694	A	G	synonymous_variant c.279A>G p.Ser93Ser	syn Ser93Ser	1
hypothetical protein		01694	A	G	missense_variant c.86A>G p.Asp29Gly	SNP Asp29Gly	6
hypothetical protein		01694	G	A	missense_variant c.127G>A p.Asp43Asn	SNP Asp43Asn	6

hypothetical protein		01694	C	T	synonymous_variant c.228C>T p.Cys76Cys	syn Cys76Cys	6
hypothetical protein		01694	G	T	missense_variant c.241G>T p.Ala81Ser	NP Ala81Ser	6
hypothetical protein		01694	GCAA	ACAT	missense_variant c.255_258delGCAAinsACAT p.Gln86His	SNP Gln86His	6
hypothetical protein		01694	A	G	synonymous_variant c.279A>G p.Ser93Ser	syn Ser93Ser	6
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrD	00547	C	T	synonymous_variant c.3651C>T p.Asp1217Asp	syn Asp1217Asp	4
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrD	00547	A	G	synonymous_variant c.3672A>G p.Ser1224Ser	syn Ser1224Ser	4
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrD	00547	C	T	synonymous_variant c.3732C>T p.Ser1244Ser	syn Ser1244Ser	4
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrD	00547	G	A	synonymous_variant c.3738G>A p.Ser1246Ser	syn Ser1246Ser	4
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrD	00547	TAGTGAT	CAGCGAC	synonymous_variant c.3765_3771delTAGTGATinsCAGCGAC p.1258	syn TAGTGATinsCAGCGAC	4
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrD	00547	T	C	synonymous_variant c.3783T>C p.Asp1261Asp	syn Asp1261Asp	4
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrE	00548	C	T	synonymous_variant c.2895C>T p.Asp965Asp	syn Asp965Asp	6
cell wall-anchored adhesion (Serine-aspartate repeat)	sdrE	00548	C	T	synonymous_variant c.2901C>T p.Asp967Asp	syn Asp967Asp	6
cell wall-anchored adhesins,	sdrE	00548	T	C	synonymous_variant c.2907T>C p.Asp969Asp	syn Asp969Asp	6

cell wall-anchored adhesins,	sdrE	00548	G	A	synonymous_variant c.2928G>A p.Ser976Ser	syn Ser976Ser	6
cell wall-anchored adhesins,	sdrE	00548	T	C	synonymous_variant c.2967T>C p.Asp989Asp	syn Asp989Asp	6
cell wall-anchored adhesins,	sdrE	00548	CGAC	TGAT	synonymous_variant c.3060_3063delCGACinsTGAT p.1022	syn del CGACinsTGAT	6
cell wall-anchored adhesins,	sdrE	00548	G	A	synonymous_variant c.3084G>A p.Ser1028Ser	syn Ser1028Ser	6
cell wall-anchored adhesins,	clfA	00782	C	T	synonymous_variant c.2280C>T p.Asp760Asp	syn Asp760Asp	6
Putative peptidoglycan glycosyltransferase	ftsW	01033	G	A	missense_variant c.778G>A p.Ala260Thr	SNP Ala260Thr	6
Extracellular matrix-binding protein	ebh_1	01359	A	T	missense_variant c.20006T>A p.Val6669Glu	SNP Val6669Glu	6
Isocitrate dehydrogenase	icd	01603	C	G	missense_variant c.949G>C p.Ala317Pro	SNP Ala317Pro	6
Tyrosine-protein kinase YwqD	ywqD_1	92459	G	A	missense_variant c.235G>A p.Asp79Asn	SNP Asp79Asn	5
hypothetical protein		106990	A	G	missense_variant c.106A>G p.Asn36Asp	SNP Asn36Asp	5
hypothetical protein		107408	G	T	missense_variant c.524G>T p.Arg175Ile	SNP Arg175Ile	5
hypothetical protein		107515	A	ACTGAT	frameshift_variant c.633_637dupTGATC p.His213fs	frameshift	5
Protoporphyrinogen oxidase	hemY	29534	T	C	start_lost c.2T>C p.Val1	Loss of start codon	5
hypothetical protein		29165	CTTTGAAATGTCAAATATACAATCTTTATTTGTTTTCGTATTTAATATAGATATT	C	disruptive_inframe_deletion c.174_227delAATATCTATATTAAATACGAAAACAAATAAAGATTGTATATTTGACATTTCAAA p.Ile59_Lys76del	Disruptive in-frame deletion	5

S-6.2 All mutations arising in moenomycin A resistant *S. aureus* strains

S-table 8 A list of all detected polymorphisms across all six replicate strains with evolved resistance to moenomycin A

Product	Gene	Location	Reference	Alternate	SNP effect	SNP shortened	Strains
Pur operon repressor	purR	00438	AT	A	frameshift_variant c.460delA p.Ile154fs	Del Ile154fs	1, 2, 3, 4, 5, 6
Pur operon repressor	purR	00438	CA	C	intergenic_region n.25448delA	Intergenic del 25448delA	1, 2, 3, 4, 5, 6
Penicillin-binding protein 1A/1B	ponA	01374	A	T	missense_variant c.212A>T p.Asp71Val	SNP Asp71Val	1, 2, 3, 4, 6
Penicillin-binding protein 1A/1B	ponA	01374	T	C	missense_variant c.586T>C p.Tyr196His	SNP Tyr196His	1, 2, 3, 4, 5, 6
Penicillin-binding protein 1A/1B	ponA	01374	C	A	missense_variant c.701C>A p.Pro234Gln	SNP Pro234Gln	1, 2, 3, 4, 5, 6
Bifunctional autolysin	atl_2	01671	A	G	missense_variant c.400T>C p.Phe134Leu	SNP Phe134Leu	1, 2, 3, 4, 5, 6
hypothetical protein		01693	T	A	missense_variant c.139T>A p.Phe47Ile	SNP Phe47Ile	1, 2, 5, 6
hypothetical protein		01693	T	C	synonymous_variant c.186T>C p.Ile62Ile	Syn Ile62Ile	1, 2, 5, 6
hypothetical protein		01693	C	T	synonymous_variant c.192C>T p.Phe64Phe	Syn Phe64Phe	1, 2, 5, 6
hypothetical protein		01693	CTTATT GATAAT AATTCAATTT	C	conservative_inframe_deletion c.226_246delAATAATTCAATTTTTATTGAT p.Asn76_Asp82del	Inframe del Asp82del	1, 2, 5, 6
hypothetical protein		01693	A	G	synonymous_variant c.276A>G p.Arg92Arg	Syn Arg92Arg	1, 2, 5, 6
hypothetical protein		01693	A	G	missense_variant c.461A>G p.Asp154Gly	SNP Asp154Gly	1, 2, 5
hypothetical protein		01693	TGTG	AGTA	missense_variant c.490_493delTGTGinsAGTA p.CysAla164SerThr	SNP CysAla164SerThr	1, 2, 5

hypothetical protein		01693	AGAACTG	GAACTA	frameshift_variant&missense_variant c.523_529delAGAACTGinsGAACTA p.Arg175fs	Frameshift/SNP Arg175fs	1, 2, 5
hypothetical protein		01693	G	T	missense_variant c.549G>T p.Lys183Asn	SNP Lys183Asn	1, 2, 5
hypothetical protein		01693	T	C	synonymous_variant c.621T>C p.Ser207Ser	Syn Ser207Ser	1, 2, 5
hypothetical protein		01693	G	A	synonymous_variant c.762G>A p.Pro254Pro	Syn Pro254Pro	1, 2, 5
hypothetical protein		01694	C	T	synonymous_variant c.228C>T p.Cys76Cys	Syn Csy76Cys	1, 2
hypothetical protein		01694	G	T	missense_variant c.241G>T p.Ala81Ser	SNP Ala91Ser	1, 2
hypothetical protein		01694	GCAA	ACAT	missense_variant c.255_258delGCAAinsACAT p.Gln86His	SNP Gln86His	1, 2
hypothetical protein		01694	A	G	synonymous_variant c.279A>G p.Ser93Ser	Syn Ser93Ser	1, 2
Lysostaphin resistance protein A	LyrA	02220	G	T	missense_variant c.320G>T p.Ser107Ile	SNP Ser107Ile	3, 4
hypothetical protein		00702	G	A	missense_variant c.143T>G p.Ile48Ser	Ile48Ser	5
hypothetical protein		01682	C	T	missense_variant c.434G>A p.Ser145Asn	SNP Ser145Asn	2
Stress response protein NhaX	nhaX	01614	A	T	missense_variant c.10A>T p.Asn4Tyr	Asn4Tyr	5
hypothetical protein		01671	C	T	missense_variant c.434G>A p.Ser145Asn	Ser145Asn	4
hypothetical protein		01716	A	G	synonymous_variant c.861T>C p.Ile287Ile	Ile287Ile	5
hypothetical protein		01693	T	C	synonymous_variant c.783T>C p.Ile261Ile	Syn Ile261Ile	2
hypothetical protein		01694	C	T	synonymous_variant c.6C>T p.Ala2Ala	Syn Ala2Ala	3
Staphylococcal secretory antigen ssaA2	ssaA2_2	02184	GCAATGCAAGT	G	frameshift_variant c.564_573delCAATGCAAGT p.Asn189fs	Frameshift Asn189fs	2

Clumping factor B		02529	ATCT	GTCC	synonymous_variant c.1908_1911delAGATinsGGAC p.638	Syn AGAT > GGAC	5
Clumping factor B		02529	A	G	synonymous_variant c.1902T>C p.Ser634Ser	Syn Ser634Ser	5
Protein McrC	mcrC	00021	T	A	missense_variant c.1013T>A p.Ile338Lys	Syn Ile338Lys	5
hypothetical protein		00469	C	G	synonymous_variant c.51G>C p.Ala17Ala	Syn Ala17Ala	6
Serine-aspartate repeat-containing protein I	sdrI	00546	G	A	synonymous_variant c.2310G>A p.Ser770Ser	Syn Ser770Ser	5
Serine-aspartate repeat-containing protein I	sdrI	00546	A	G	synonymous_variant c.2328A>G p.Ser776Ser	Syn Ser776Ser	5
Serine-aspartate repeat-containing protein I	sdrI	00546	T	C	synonymous_variant c.2349T>C p.Asp783Asp	Ser Asp783Asp	5
Serine-aspartate repeat-containing protein I	sdrI	00546	C	T	synonymous_variant c.2421C>T p.Asp807Asp	Syn Asp807Asp	5
Serine-aspartate repeat-containing protein I	sdrI	00546	T	C	synonymous_variant c.2448T>C p.Ser816Ser	Syn Ser816Ser	5
Serine-aspartate repeat-containing protein I	sdrI	00546	TTCAGAC	CTCGGAT	synonymous_variant c.2457_2463delTTCAGACinsCTCGGAT p.822	Syn TTCAGAC > CTCGGAT	5
Serine-aspartate repeat-containing protein I	sdrI	00546	C	T	synonymous_variant c.2493C>T p.Asp831Asp	Syn Asp831Asp	5
Serine-aspartate repeat-containing protein D	sdrD	00547	TTCAGAC	CTCTGAT	synonymous_variant c.3561_3567delTTCAGACinsCTCTGAT p.1190	Syn TTCAGAC > CTCTGAT	5
Serine-aspartate repeat-containing protein E	sdrE	00548	C	T	synonymous_variant c.3171C>T p.Asp1057Asp	Syn Asp1057Asp	5
Serine-aspartate repeat-containing protein E	sdrE	00548	C	T	synonymous_variant c.3177C>T p.Asp1059Asp	Syn Asp1059Asp	5
Serine-aspartate repeat-containing protein E	sdrE	00548	TGATTCAGAT	AGAC	missense_variant&conservative_inframe_deletion c.3186_3195delTGATTCAGATinsAGAC p.Ser1062_Ser1064delinsArg	Complex	5

Serine-aspartate repeat-containing protein E	sdrE	00548	CAGCGAC	TAGTGAT	synonymous_variant c.3201_3207delCAGCGACinsTAGTGAT p.1070	Syn CAGCGAC > TAGTGAT	5
Serine-aspartate repeat-containing protein E	sdrE	00548	G	A	synonymous_variant c.3234G>A p.Ser1078Ser	Syn Ser1078Ser	5

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