Supporting information

Antibiotic combinations that enable one-step, targeted mutagenesis of chromosomal genes

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mNeonGFP DNA sequence (5`→ 3`)

ATGGTGAGTAAAGGTGAGGAGGATAACATGGCTTCTTTACCTGCGACTCATGAATTACACATTTTTGGATCAATTAACGGCGT

TGATTTTGACATGGTGGGACAAGGCACGGGCAACCCAAACGATGGCTATGAAGAGTTGAACTTGAAGTCTACGAAGGGTGACT

TACAGTTTTCACCATGGATCTTAGTCCCGCATATTGGATATGGTTTCCACCAATACTTGCCGTACCCGGACGGTATGTCTCCAT

TTCAAGCAGCTATGGTCGACGGAAGTGGCTACCAGGTCCATAGAACAATGCAGTTTGAGGATGGCGCAAGTTTGACAGTTAAC

TACAGATACACATACGAGGGAAGTCACATCAAGGGAGAAGCTCAGGTAAAGGGTACAGGCTTTCCGGCGGATGGTCCAGTCAT

GACAAACAGTTTAACGGCAGCGGATTGGTGTCGTTCAAAAAAAACATATCCAAATGATAAGACTATCATCTCTACATTCAAAT

GGTCTTACACAACTGGAAACGGTAAAAGATATCGATCAACGGCGCGAACAACGTACACATTCGCTAAGCCGATGGCGGCGAACT

ATTTGAAGAACCAACCGATGTATGTGTTTAGAAAAACGGAATTGAAGCACAGTAAGACGGAGTTAAACTTTAAAGAGTGGCAG

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Supplementary Figures

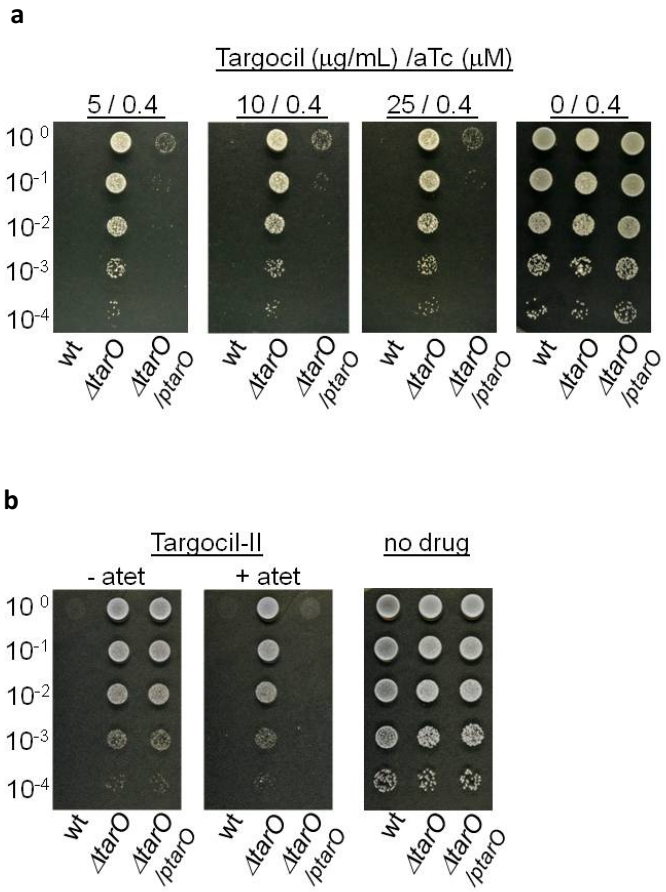


Figure S1. *S. aureus ΔtarO* is resistant to targocil and targocil-II. (**a**) *ΔtarO*, the complementation strain *ΔtarO*/*ptarO*, and wildtype (wt) were tested against targocil (5, 10, or 25 μg/mL) in the presence of 0.4 μM anhydrotetracycline (aTc) to induce *ptarO* expression. The phenotype is complementable. (**b**) Susceptibility of *ΔtarO*, *ΔtarO*/*ptarO*, and wildtype were also tested against 10 μg/mL targocil-II in the presence or absence of aTc.

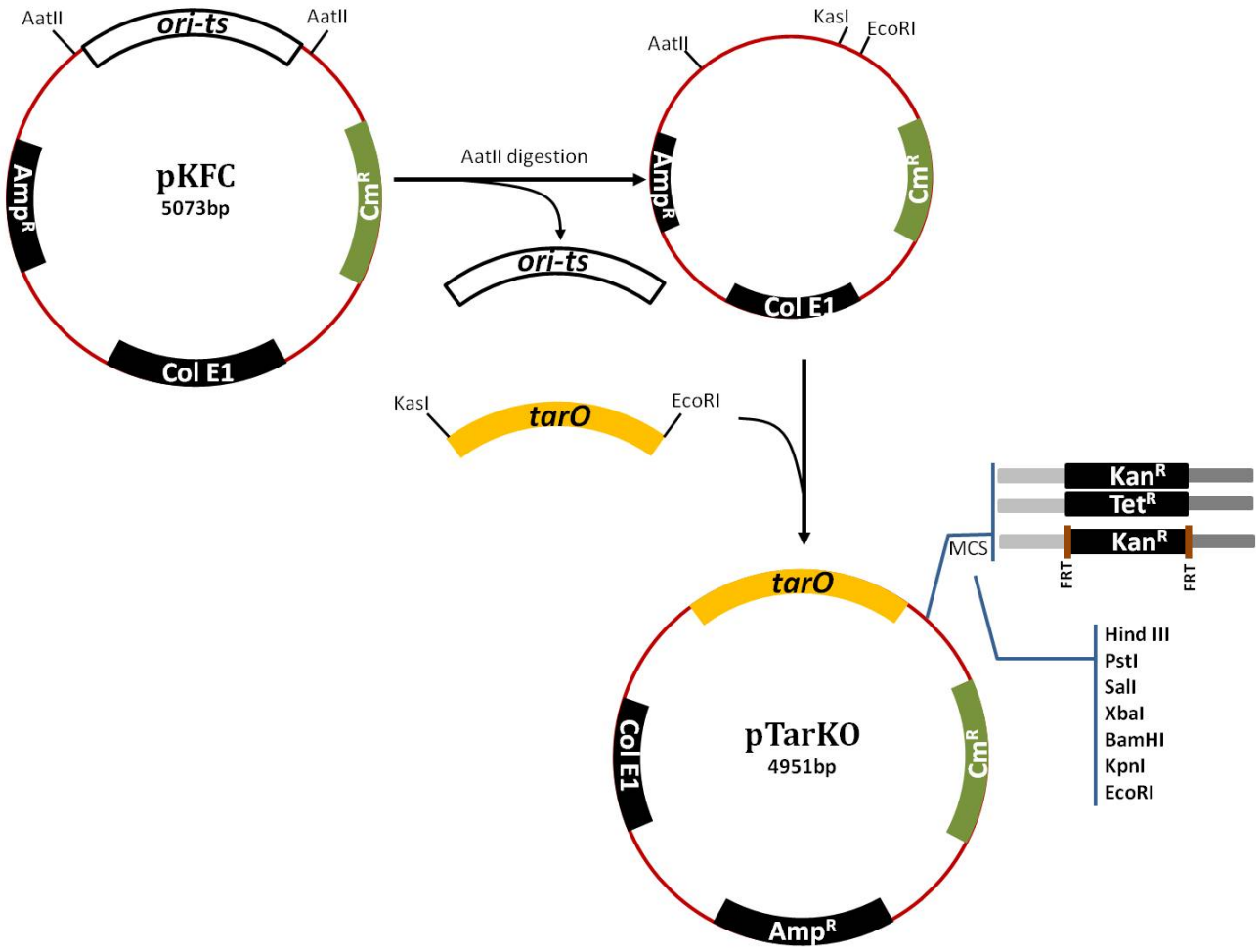


Figure S2. Construction and map of pTarKO plasmid. The temperature sensitive replication origin (*ori-ts*) for *S. aureus* found in the pKFC vector was removed and the *tarO* gene sequence with its native promoter was added. pTarKO contains the following features: ColE1 (replication origin for *E. coli*), Cm^R (chloramphenicol acetyltransferase), Amp^R (β-lactamase), and a multiple cloning site (MCS). Antibiotic resistant markers for pTarKO: Kan^R (aminoglycoside 3'-phosphotransferase) and tet^R (tetM, tetracycline resistance gene). Unique restriction sites are listed.

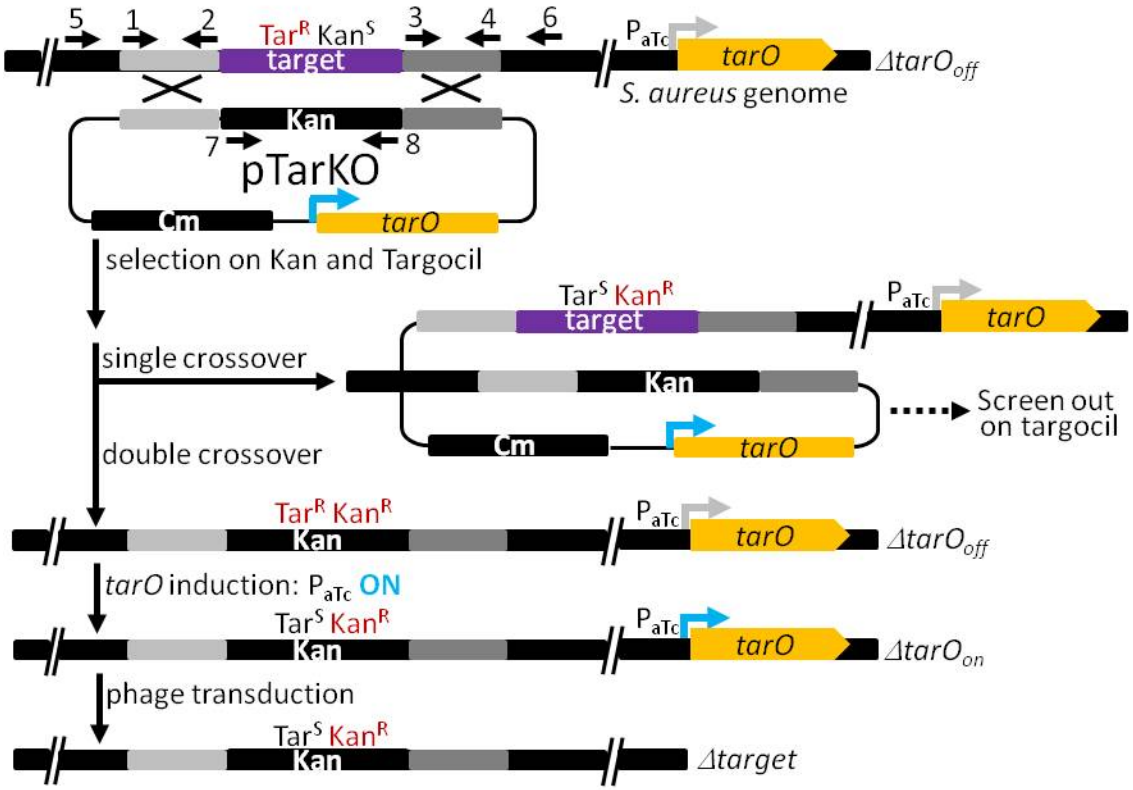


Figure S3. Schematic of allelic exchange with the pTarKO targeting plasmid in *S. aureus*. Recombineering plasmid isolated from DH10B *E. coli* (NEB 10-beta) is irradiated with UV and electroporated into $tarO_{off}$ *S. aureus*, and transformants are selected on plates containing both targocil (or targocil-II) and kanamycin. Mutants with a single crossover are screened out, and only mutants with a double crossover are selected from the double selection. A confirmed double-crossover mutant is grown in the presence of aTc to induce *tarO* for wall teichoic acid production, and the marked modified locus is transduced to other *S. aureus* strains by phage transduction. Primers for homologous arms, antibiotic marker, and confirmation of gene modification are shown as numbers: 1,AA; 2,AB; 3,BA; 4,BB; 5,CA; 6,CB; 7,KanR-F; 8,KanR-R.

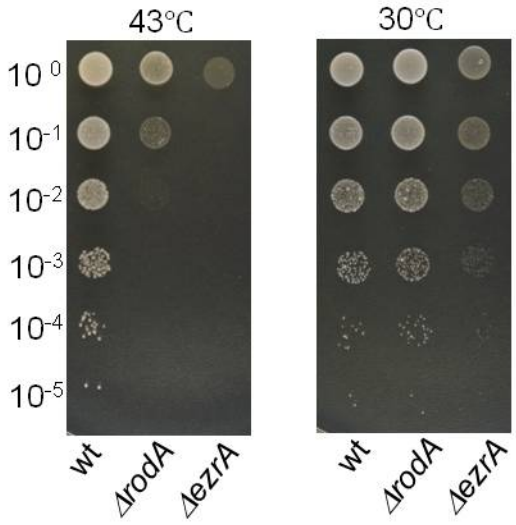


Figure S4. Both *ΔrodA* and *ΔezrA* show severe growth defects at the elevated temperature of 43℃. 5 µL of each dilution of *ΔrodA* RN4220 and *ΔezrA* RN4220 grown to mid-log phase was spotted on TSA agar plate and incubated at 43°C or 30°C.

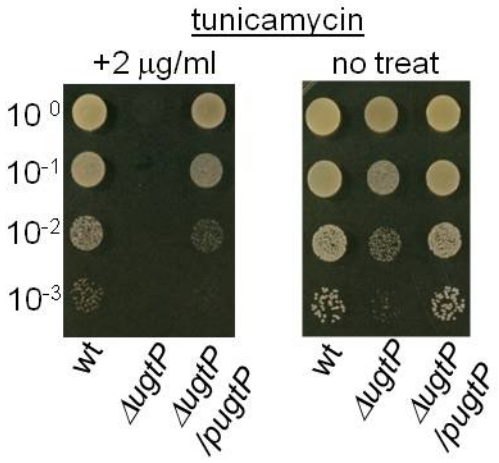


Figure S5. UgtP is synthetically lethal with inhibition of wall teichoic acid biosynthesis. Tunicamycin inhibits TarO, the first step of the WTA pathway in *S. aureus*, and prevents growth of a *ΔugtP* mutant**.** Susceptibility of *ΔugtP*, *ΔugtP*/*pugtP*, and wildtype strains were tested against 2 μg/mL tunicamycin. The phenotype is complementable.

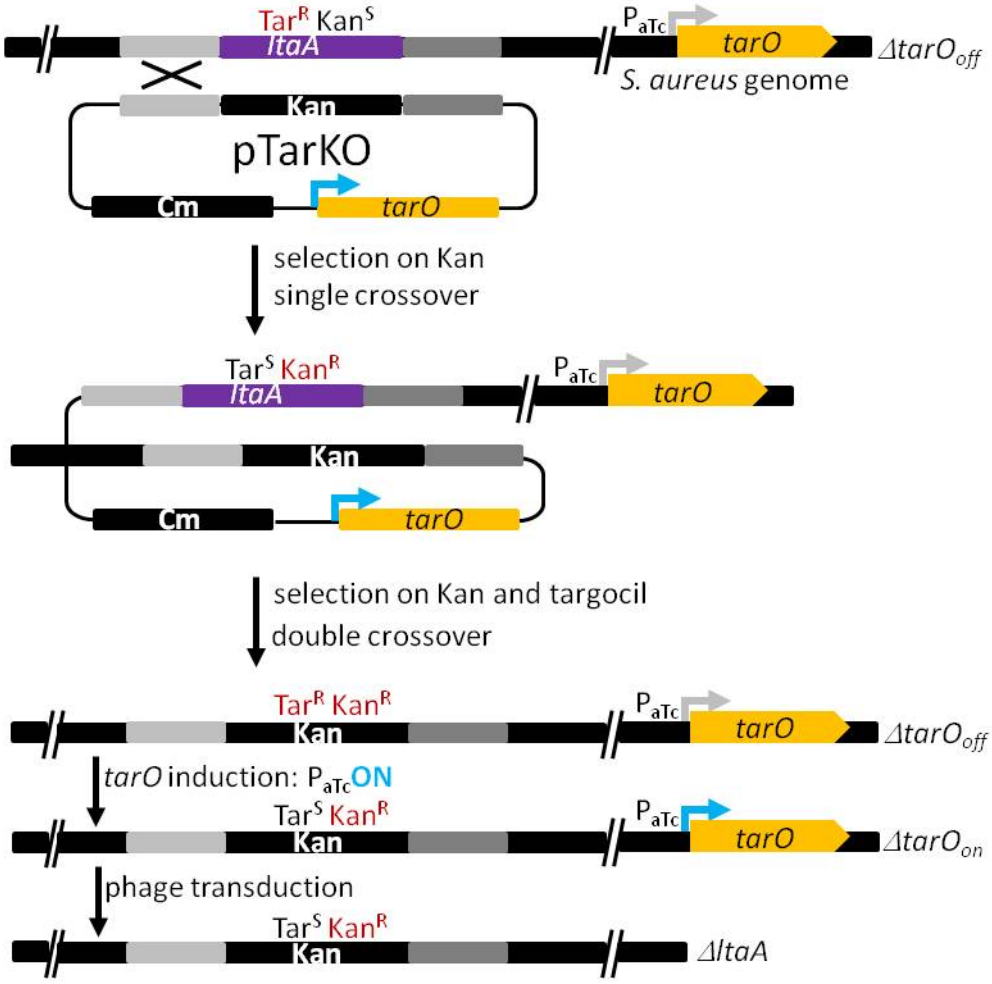


Figure S6. Schematic of the two-step process to delete *ltaA* using pTarKO. A UV-irradiated targeting plasmid was electroporated into *tarO*$_{off}$ *S. aureus* and transformants are selected on plates containing only kanamycin to obtain mutants with a single crossover. A confirmed single crossover mutant is grown to high density and plated on both kanamycin and targocil to select for double crossover mutants.

a

b

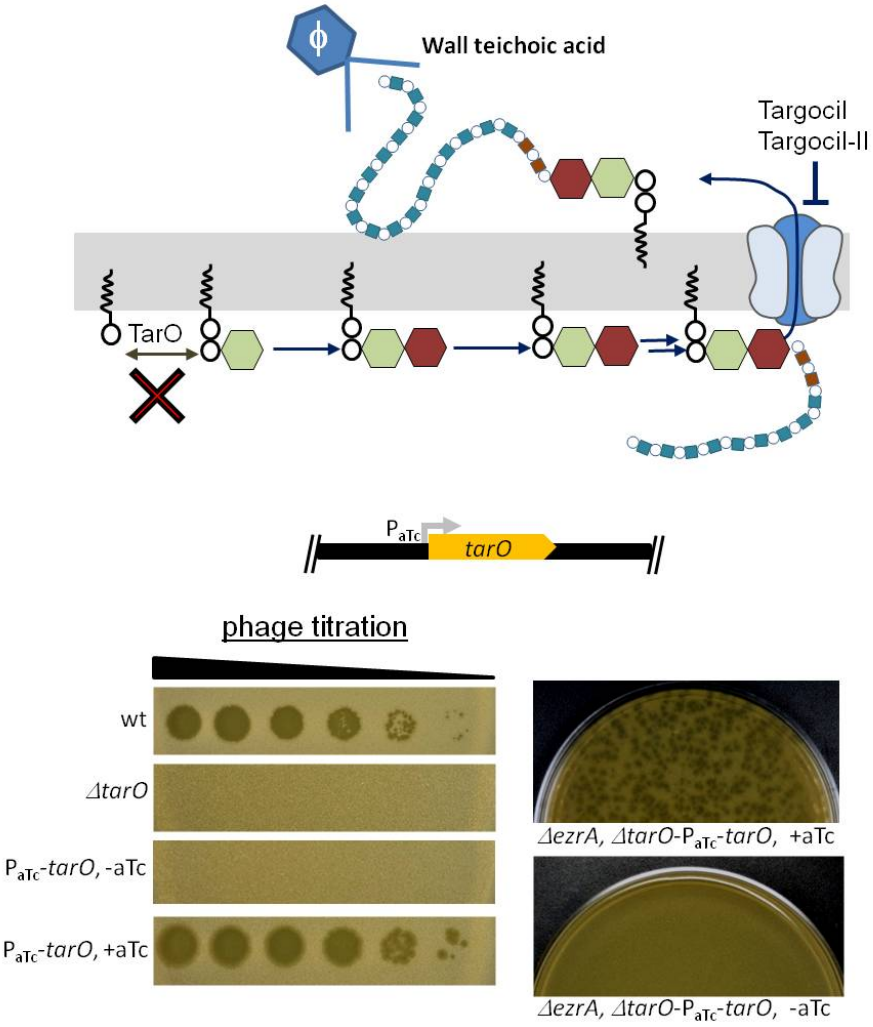
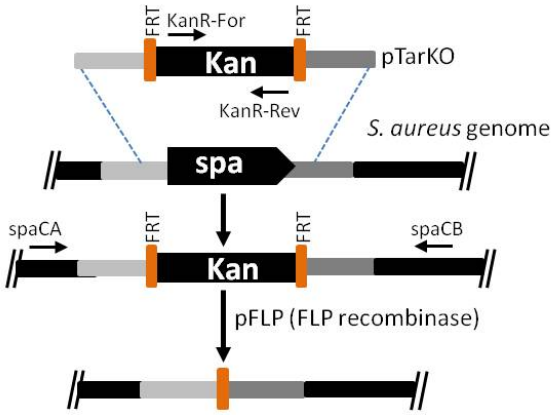
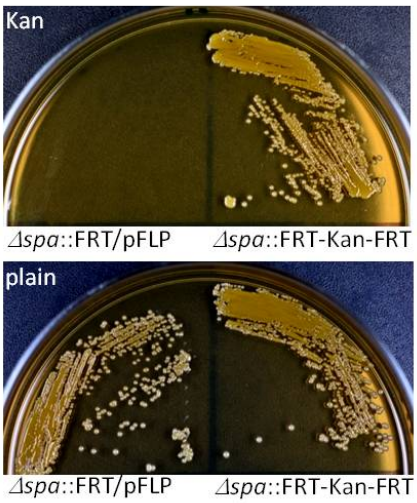


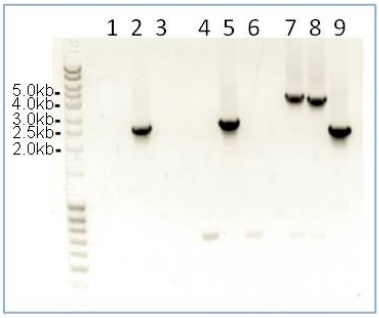
Figure S7. Construction of an inducible *tarO* strain. (a) Staphylococcal phage Φ11 FRT cannot form plaques on *ΔtarO* cells since wall teichoic acids serve as surface receptors for phage attachment prior to infection. Labels are the same as shown in **Figure 2**. **(b)** In the presence of aTc, which induces *tarO* expression for WTA production, the phage generated plaques on bacterial lawns of *S. aureus.* Phage lysate was prepared from the plaques and used for phage transduction. As an example, *ΔezrA ΔtarO*-ptarO was mixed with phage Φ11 in the presence or absence of 0.4 μM aTc.

a **b**

c







lane	strain	primer
1	wildtype	
2	*Δspa*, Kan^R	spaCA, Kan^R-Rev
3	*Δspa*, markerless	
4	wildtype	
5	*Δspa*, Kan^R	spaCB, Kan^R-For
6	*Δspa*, markerless	
7	wildtype	
8	*Δspa*, Kan^R	spaCA, spaCB
9	*Δspa*, markerless	

Figure S8. Markerless deletion of *spa* using FRT/FLP recombinase. (a) Schematic of the strategy to delete *spa* using pTarKO and to remove the antibiotic marker. **(b)** Phenotypic validation of *Δspa*::FRT-Kan-FRT and *Δspa*::FRT in the presence or absence of kanamycin. **(c)** PCR validation of allelic exchange of *spa* and removal of the kanamycin cassette.

a

b

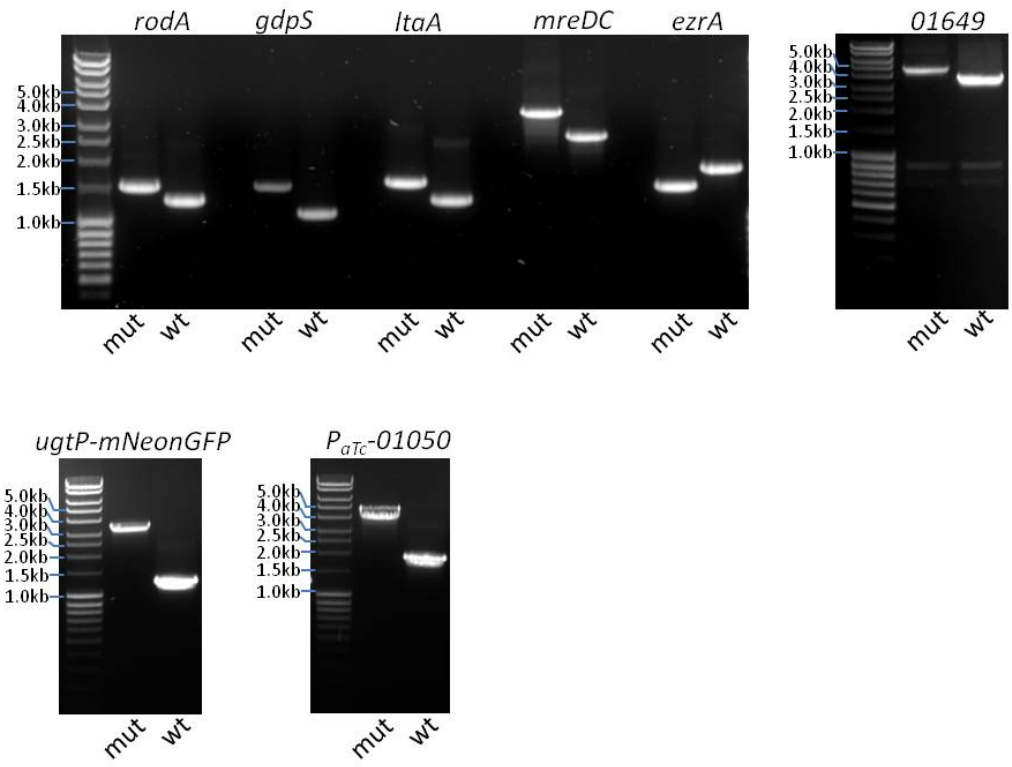
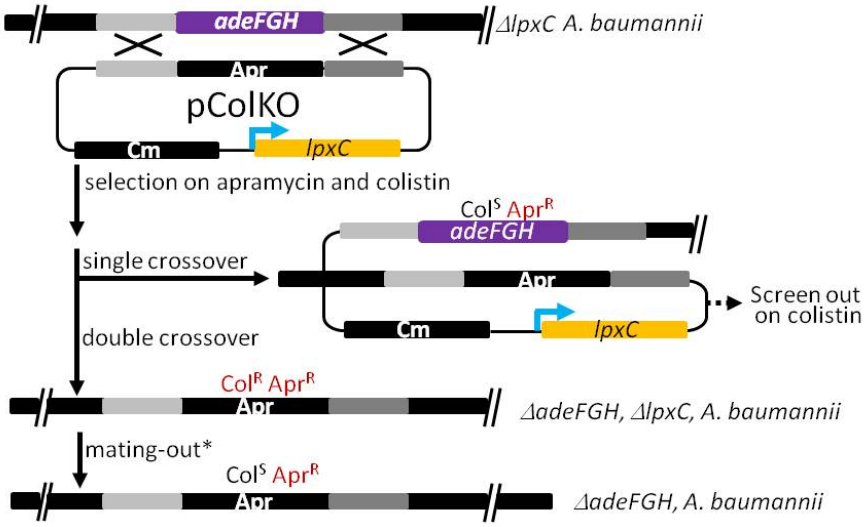


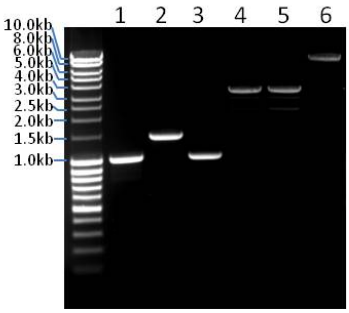
Figure S9. PCR validation of allelic exchange of target loci. (a) Deletions of *rodA, gdpS, ltaA, mreDC, ezrA, and SAOUHSC_01649* in *S. aureus* were confirmed by PCR with primers CA and CB. **(b)** A C-terminal mNeonGFP fusion to UgtP and the exchange of the native promoter of *SAOUHSC_01050* for an aTc-inducible promoter were confirmed by PCR with primers CA and CB.

a



*Selected on rifampin 0.04 μg/mL and apramycin 100 μg/mL
(rifampin MIC : wildtype 1 μg/mL, *ΔlpxC* <0.01 μg/mL)

b



lane	strain	primer
1	*ΔadeFGH*	
2	*ΔlpxC, ΔadeFGH*	lpxCA, lpxCB
3	wildtype	
4	*ΔadeFGH*	
5	*ΔlpxC, ΔadeFGH*	adeFGH-CA,
6	wildtype	adeFGH-CB

Figure S10. Allelic exchange of the *adeFGH locus* with pColKO-*adeFGH* in *A. baumannii*. (a) A targeting plasmid isolated from *E. coli* (NEB 10-beta) is irradiated with UV and electroporated into the pass-*ΔlpxC A. baumannii* strain, and transformants are selected on colistin and apramycin. Mutants with a single crossover are screened out, and only mutants with a double crossover are selected from the double selection. To transfer the marked deletion into wildtype *A. baumannii*, a mating-out assay was performed using the *ΔadeFGH ΔlpxC* strain (donor) and wildtype (recipient). **(b)** Mutations transferred to wildtype were confirmed by PCR using primers CA and CB.

Table S1. Strains and Plasmids

Strain	Description	Source
S. aureus		
RN4220	wild type	-
HG003	wild type	-
ΔtarO RN4220	Tar^R	Ref. 22
$\Delta tarO_{on/off}$	*ΔtarO* pTP63-tarO RN4220	This study
$\Delta rodA,\Delta tarO_{on/off}$	Kan^R, Cm^R, Tar^R	This study
ΔrodA RN4220	Kan^R	This study
ΔrodA HG003	Kan^R	This study
$\Delta ltaA,\Delta tarO_{on/off}$	Kan^R, Cm^R, Tar^R	This study
ΔltaA RN4220	Kan^R	This study
ΔltaA HG003	Kan^R	This study
ΔltaA MW2	Kan^R	This study
ΔmreDC,ΔtarO RN4220	tet^R, Tar^R	This study
ΔmreDC RN4220	tet^R	This study
ΔmreDC HG003	tet^R	This study
$\Delta ezrA,\Delta tarO_{on/off}$	Kan^R, Cm^R, Tar^R	This study
ΔezrA RN4220	Kan^R	This study
ΔezrA HG003	Kan^R	This study
ΔezrA USA300	Kan^R	This study
ΔezrA Newman	Kan^R	This study
$\Delta gdpS,\Delta tarO_{on/off}$	Kan^R, Cm^R, Tar^R	This study
ΔgdpS RN4220	Kan^R	This study
ΔgdpS HG003	Kan^R	This study
ΔgdpS USA300	Kan^R	This study
$\Delta spa,\ \Delta tarO_{on/off}$	Kan^R, Cm^R, Tar^R	This study
Δspa HG003	Kan^R	This study
Δspa HG003	unmarked	This study
Δ01649,ΔtarO RN4220	tet^R, Tar^R	This study
ugtP-mNeonGFP,$\Delta tarO_{on/off}$	Kan^R, Cm^R, Tar^R	This study
ugtP- mNeonGFP RN4220	Kan^R	This study
P_{aTc}-01050*,ΔtarO* RN4220	Kan^R, Tar^R	This study
P_{aTc}-01050 RN4220	Kan^R	This study
P_{aTc}-01050 HG003	Kan^R	This study
ΔugtP RN4220	Kan^R	Ref. 30
ΔugtP /p*ugtP*	Kan^R, Em^R	This study
ΔltaA /p*ltaA*	Kan^R, Em^R	This study
A. baumannii		
ATCC19606		Ref. 43
ΔlpxC ATCC19606	Kan^R	Ref. 43
ΔlpxC ATCC19606-pass	Kan^R *dapD* (DNA:CDS756: G→A, protein: none) *WP_031949781*(DNA: CDS1165:insertion(A), protein: frame shift) *baeS* (DNA:CDS307:C→T, protein: substitution: A→T)	This study
ΔlpxC, ΔadeFGH ATCC19606-pass	Kan^R, $apramycin^R$	This study
ΔadeFGH ATCC19606	$apramycin^R$	This study
ΔlpxC ATCC19606-pass/p*lpxC*	Kan^R	This study
E. coli		
NEB 10-beta (DH10B™ derivative)		NEB

Continued Table S1. Strains and Plasmids		
plasmid	Description	Source
pKFC	Cm^R , ts-Ori-replication	Ref. 5
pFLP	FLP recombinase	Ref. 15
pTM204*attLint*	tet^R	Ref. 15
p*ugtP*	pLOW-*ugtP-ltaA*	This study
p*ltaA*	pLOW-*ugtP*	This study
pTarKO		This study
pTP63	Cm^R , integrative plasmid	Ref. 30
pTP63-P_{aTc}-*tarO*	Cm^R , integrative plasmid	This study
pTarKO-*rodA*-Kan^R	*rodA* targeting vector	This study
pTarKO-*ltaA*- Kan^R	*ltaA* targeting vector	This study
pTarKO-*mreDC*-tet^R	*mreDC* targeting vector	This study
pTarKO-*spa*-Kan^R	*spa* targeting vector	This study
pTarKO-*ezrA*-Kan^R	*ezrA* targeting vector	This study
pTarKO-*gdpS*-Kan^R	*gdpS* targeting vector	This study
pTarKO-*ugtP*-gfp-Kan^R	*ugtP* targeting vector	This study
pTarKO-*01050*-P_{aTc}- Kan^R	*01050* targeting vector	This study
pTarKO-*01649*-tet^R	*01649* targeting vector	This study
pKFC-*rodA*-Kan^R	*rodA* targeting vector	This study
pColKO		This study
pColKO-*adeFGH*-Apr^R	*adeFGH* targeting vector	This study
pWH1266		Ref. 43
p*lpxC*	pWH1266-*lpxC*	This study

Table S2. Primers

Primers	Sequence (5`→ 3`) restriction site in red	Gene
tarO-F-KasI	ATA**GGCGCC**GTCTGAATCGACTCCTTTAATTGACCAC	*tarO*
tarO-R-EcoRI	AC**GAATTC**CCCTATTCCTCTTTATGAGATGACTTACG	*tarO*
tarO-rbs-tetF-KpnI	AGA**GGTACC**ATATCGATGAAGGTGAATAAATGGTT	*tarO*
tarO-R-EcoRI	ACA**GAATTC**TTTCATTCCCTATTCCTCTTTATGAGAT	*tarO*
kan-F	GCGAACCATTTGAGGTGATAGGTAAGATTAT	*kan R*
kan-R	TCCTAGGTACTAAAACAATTCATCCAGTAA	*kan R*
rodA-AA-BamHI	TTAA**GGATCC**TTCTATAACAAGCTTACGGTCAA	*rodA*
rodA-AB	CTTACCTATCACCTCAAATGGTTCGCCGGCTGTTGACGAGATGAATA	*rodA*
rodA-BA	TTACTGGATGAATTGTTTTAGTACCTAGGACGATATGTCGATTTATACCATC	*rodA*
rodA-BB-SalI	GATT**GTCGAC**GCACATCATTCTGAACAAATAAA	*rodA*
rodA-CA	GGATAGTCATTTCCTAAAACTGCTACTTC	*rodA*
rodA-CB	AGTATAGTAAGGAATGTAAATGAAGGAGTGA	*rodA*
ltaA-AA-BamHI	TTAA**GGATCC**TACAAAGGGTTTTATTACAGCCGCC	*ltaA*
ltaA-AB	ATAATCTTACCTATCACCTCAAATGGTTCGCGGATATACATGCCTCTCGCAAATTC	*ltaA*
ltaA-BA	TTACTGGATGAATTGTTTTAGTACCTAGGATTTAAACAATACATTTTATTTCTCGGCA	*ltaA*
ltaA-BB-SalI	GATT**GTCGAC**AACAAATGGTAAGCGTGCCGAT	*ltaA*
ltaA-CA	CGTCATTGAGCACGATTTATTTATG	*ltaA*
ltaA-CB	AAAAAGTATTAGATAAGCTAAATCAATGTGC	*ltaA*
gdpS-AA-SalI	ATT**GTCGAC**ATCCAATCATTAAAGCCCCACTATC	*gdpS*
gdpS-AB	ATCTTACCTATCACCTCAAATGGTTCGCAATAAATAGATTCCAGCGACTATAACAGA	*gdpS*
gdpS-BA	TTACTGGATGAATTGTTTTAGTACCTAGGAAAAAATCAAGGGCGAAACAAAGTAA	*gdpS*
gdpS-BB-BamHI	CCG**AAGCTT**AATAGCATGCTTTAACAGTCCTTCCTTA	*gdpS*
gdpS-CA	AATACAAATTATCCCATACAGCTATGCT	*gdpS*
gdpS-CB	TTGAAAATGATAGAGAAAAGTACTGTTGATA	*gdpS*
mreDC-AA-BamHI	TTAA**GGATCC**ATCATGATTAAGGCTGAAGACT	*mreDC*
mreDC-AB	TTACCTATCACCTCAAATGGTTCGCCACCCAGAACACCTCTATTATG	*mreDC*
mreDC-BA	TATGTTCAATAAAATAACTTAGAAGATGCAATAGTTGAGTAGTTA	*mreDC*
mreDC-BB-SalI	GATT**GTCGAC**ACCACGACCTACATTTTC	*mreDC*
mreDC-CA	CAATGCTACAAACCTCCTAATACGC	*mreDC*
mreDC-CB	CCGTCGATTTTAGCGAATAATG	*mreDC*
tetM-F	CAACCCAAATCTCGCAATTTGAG	*tetR*
tetM-R	CTAAGTTATTTTATTGAACATATATCTTACTT	*tetR*
spa-AA-BamHI	CCCGG**GGATCC**AGATTTATGTTATAAACAATCGGATTTAGTACAGCATA	*spa*
spa-AB	TACTTTCTAGAGAATAGGAACTTCATTAATACCCCCTGTATGTATTTGTAAAGTCATC	*spa*
spa-BA	TATTCTCTAGAAAGTATAGGAACTTCAAACAAACAATACACAACGATAGATATCATTTTATCC	*spa*
spa-BB-SalI	TGCAG**GTCGAC**TTAGTATGGAGTGCACCATTCTTCAAAAAATTATTC	*spa*
spa-CA	GTCAAGCCTGAAGTCGATATGACTATAA	*spa*
spa-CB	ATCACTAGCAACAATGGTGGTGTTAGC	*spa*
kan-FRT-F	TATTCTCTAGAAAGTATAGGAACTTCGCGAACCATTTGAGGTGATAGGTAAGATTAT	*kanR*
kan-FRT-R	TACTTTCTAGAGAATAGGAACTTCTCCTAGGTACTAAAACAATTCATCCAGTAA	*kanR*
Kan For	GCGAACCATTTGAGGTGATAGGTAAGATTAT	*kanR*
Kan Rev	TCCTAGGTACTAAAACAATTCATCCAGTAA	*kanR*
ezrA-AA-BamHI	TTAA**GGATCC**AACCTAATGAATAAACAACAGCGTCTAAA	*ezrA*
ezrA-AB	ATCTTACCTATCACCTCAAATGGTTCGCTATATAACACCATATGCTTCTCCTCCTAAT	*ezrA*
ezrA-BA	GGATGAATTGTTTTAGTACCTAGGACAGGTGTTACTAAACATATTGAAGAAGAAGTTAT	*ezrA*
ezrA-BB-SalI	ATT**GTCGAC**AAAGTTTTCATTAGCTATCTTCATCGC	*ezrA*
ezrA-CA	TGGTTAACTAATTGACGTGCTTGAC	*ezrA*
ezrA-CB	TGACGGACGTCATTATTTAACTCAGT	*ezrA*

Continued Table S2. Primers

Primers	Sequence (5`→3`), restriction site in red	Gene
01649-AA-BamHI	CCCGG**GGATCC**AGCGCATGGTTGTAAAATTTTAACTTTGCA	*01649*
01649-AB	CAACTCAAATTGCGAGATTTGGGTTGTTAGTCCTCCACTATGCTGCTTGATA	*01649*
01649-BA	GATAGATAAAGTAAGATATATGTTCAATAAAATAACTTAGATGAGTCGAAAAATAAATA ACTTTTATGATGTACAAC	*01649*
01649-BB-SalI	TGCAG**GTCGAC**CCTAGAACGATATATTTCGGATTACTTGTA	*01649*
01649-CA	CAGGTGGAGACTTAGGTAGAGATG	*01649*
01649-CN	CAATTTCAGTTTCAAATTGAGCAGGTGC	*01649*
ugtP-gfp-AB	AGCTCCACCAGCGCTACCACCACCTTTAACGAAGAATCTTGCATATAAAGG	*ugtP*
ugtP-gfp-BA	TAATCTTACCTATCACCTCAAATGGTTCGCTTACGCTAATCATAAAAATTTCATTTTAA	*ugtP*
ugtP-gfp-BB-HindIII	GGC**AAGCTT**CCTATCATTGAGCCGAATCCTTG	*ugtP*
ugtP-gfp-CA	ATGGTTACTCAAAATAAAAAGATATTGATTA	*ugtP*
ugtP-gfp-CB	TTACTTAGCTTTTTCTCTATTTACTATAAAGT	*ugtP*
ugtP-F-SalI	AAGTT**GTCGAC**CAAACTAACGGAGGGTGGCT	*ugtP*
ugtP-R-BamHI	GTTTTAA**GGATCC**TTATTTAACGAAGAATCTTGCATATA	*ugtP*
mNeon-GFP-F	ATGGTGAGTAAAGGTGAGGAGGATAACA	*GFP*
mNeon -GFP-R	TTATTTATACAACTCATCCATTCCCATA	*GFP*
mNeon -GFP-Km-R	TTACTGGATGAATTGTTTTAGTACCTAGGATTATTTATACAACTCATCCATTCCCATA	*GFP*
01050-AA-SalI	ATT**GTCGAC**GAATATACAGTTCAA AAATTGAAAAGAGA	*01050*
01050-AB	TACTGGATGAATTGTTTTAGTACCTAGGACGTTACAACTCCTTATAGTACTTATCCC	*01050*
01050-tetR-rbs-BA	GTATGATGGTACCATATCGATGAAGGTGAATAAATGACTGGAGAACAATTTACTCAAATT	*01050*
01050-BB-HindIII	TTAT**AAGCTT**TACGTTTTTCTTTTTTCTTAGC	*01050*
01050-CA	CGGTACACATTATGATGTTGCTTTTC	*01050*
01050-CB	GTTGTAAATTTGACGTCGCTCTTC	*01050*
P_{aTc}-Km-F	ATAATCTTACCTATCACCTCAAATGGTTCGCCCGTGAAGTTACCATCACGGAAA	*aTc promoter*
P_{aTc}-rbs-R	TTATTCACCTTCATCGATATGGTACCATCATACTCTATCAATGATAGAGAGCT	*aTc promoter*
Apr-F	TTTGCAAGCAGCAGATTACGC	*Apr* ^{R}
Apr-R	CGTCATCTCGTTCTCCGCTCAT	*Apr* ^{R}
KFC-F	AAGTTGGGTAACGCCAGGGTTTTC	
KFC-R	ATTGTGAGCGGATAACAATTTCACACA	
lpxC-prom-F-SalI	TTTAGCGTACAATCTATTGAAAGGCA	*lpxC*
lpxC-R-HindIII	TTATGTCACACTCACGTATGGAATTG	*lpxC*
lpxC-prom-F-KasI	TTTAGCGTACAATCTATTGAAAGGCA	*lpxC*
lpxC-R-EcoRI	TTATGTCACACTCACGTATGGAATTG	*lpxC*
adeFGH-AA-SalI	GGC**GTCGAC**GAAGATAAACTGCTGAAATCGGC	*adeFGH*
adeFGH-AB	GCGTAATCTGCTGCTTGCAAACGGGAAAATGACATGAGGTGCT	*adeFGH*
adeFGH-BA	ATGAGCGGAGAACGAGATGACGAGGGGGCGGTTGGAGTAGTTA	*adeFGH*
adeFGH-BB-KpnI	TAG**GGTACC**GCTCACAAGCTAACTCTGCTGTTTAT	*adeFGH*
adeFGH-CA	AGTTCAGCGACCCAATCTACAAAC	*adeFGH*
adeFGH-CB	AGAACTTTTTGGTGCAGATTACGC	*adeFGH*

Table S3. Synthetic lethal with *ΔtarO*

SAOUHSC	Gene name	Source
00965		Ref. 30, 31
00618		Ref. 30
00718		Ref. 30
00665	*graR*	Ref. 30, 31
02611	*lyrA*	Ref. 30, 31
00668	*vraG*	Ref. 30, 31
00948		Ref. 30
00953*	*ugtP*	Ref. 30
00667	*vraF*	Ref. 30, 31
01187	*stk1*	Ref. 30
00869	*dltA*	Ref. 30, 31
00870	*dltB*	Ref. 30, 31
00871	*dltC*	Ref. 30, 31
00872	*dltD*	Ref. 30, 31
00952*	*ltaA*	This study

* manipulated in this study