Supplementary text

Materials and methods:

Construction of *gdpP* deletion mutant and its complemented strains: To create *gdpP* deletion strains, up- and down-stream regions of *gdpP* were PCR amplified with the primers *gdpP*-P1 & P2 and *gdpP*-P3 & P4, respectively. The full-length *gdpP* gene was PCR amplified using splice overlap extension PCR and was cloned into *pKOR1*. The resultant plasmid was sequence confirmed and transformed to the target strains. Allelic replacement of *gdpP* was carried out as previously described (1). The deletion mutation was confirmed by PCR and through analytical sequencing.

To create the constitutively expressing *gdpP* in pTX_{Δ}, full-length *gdpP* gene was PCR amplified from the genomic DNA of strains as mentioned in three different fragments using PCR primers *gdpP*-1 & 2, 3 & 4 and 5 & 6. The full-length *gdpP* was obtained by performing splice overlap extension PCR with the PCR fragments that were obtained in the previous step. The fragmented PCR amplification of the full-length *gdpP* was necessary to remove additional BamH1 & Mlu1 internal cutting sites, which were the restriction enzymes used to clone the full-length *gdpP* into the constitutively expressing vector pTX_{Δ}. The resultant plasmid was sequence verified for sequence accuracy of the cloned *gdpP*s.

*gdpP*s from the selected clinical isolates, which had missense mutation/s **(Table S4)** were amplified and cloned into the pTX_{Δ} plasmid. Additional PCR primers *gdpP*-7 - *gdpP*-10 were used to clone *gdpP* of the clinical strains mentioned below to remove additional BamH1 & Mlu1 internal cutting sites. *gdpP*-7 and *gdpP*-8 was used repair a third Mlu1 in *gdpP* from strain 10. Primer *gdpP*-9 was used for strain 5 instead of primer *gdpP*-3. Primer *gdpP*-10 was used for

strain 4 instead of primer *gdpP*-5. The plasmids were first transformed into RN4220 followed by transduction into SF8300 *ΔgdpP*.

Tolerance assay: Bacterial tolerance assay was carried out as previously described (2). Briefly, 1x 10^6 bacteria from overnight cultures were inoculated in 10 ml TSB with the indicated drug. Bacteria were incubated at 37°C in a shaking incubator and plated at 0, 3 and 6 h for enumeration of CFUs.

Competition assay: Bacterial inoculum (SF8300^{tet} and SF8300 Δ*gdpP* strains) was prepared by centrifugation of 10 ml bacterial culture that was grown in TSB media for 24 h followed by wash with PBS containing 10% glycerol. The bacterial pellet was re-suspended in PBS containing 10% glycerol, aliquoted in 1ml batches and stored at -80ºC. Bacterial titers were determined by plating serial dilutions of bacteria from 2 different tubes of stored bacteria. For competition assay, 2X 10^6 bacteria (1:1 mixture of the 2 strains mentioned above) were inoculated in 10 ml TSB. The following conditions were tested 1) No drug and 2) nafcillin treatment (4, 16 and 32 µg/ml). Bacterial CFU were determined by plating bacteria on TSA plates at 0 and 24 h post inoculation. Enumeration of respective number of SF8300^{tet} and SF8300 Δ*gdpP* strains were determined by replica plating the bacterial colonies onto TSA and TSA containing tetracycline (3 µg/ml).

Western Blotting: Overnight cultures of bacteria were subcultured into 50mL flasks containing TSB such that the initial OD_{600} of the flasks were 0.1. The flasks were cultured for 2 h and 2 µg/ml nafcillin was added. Bacterial cells were harvested following 2 h incubation of antibiotic addition. The collected cells were washed with Phosphate Buffered Saline (PBS), resuspended in lysis

buffer (50mM Tris pH 7.5, 150mM NaCl, 2mM EDTA, 1mM Sodium Pyrophosphate, 1X CompleteMini protease inhibitor cocktail (Roche)) and were mechanically lysed using the FastPrep FP120 (Thermo Savant). Following centrifugation, the supernatant was collected and cell membrane fractions were isolated by performing ultracentrifugation (66000g, Sorvall WX Ultra 80 Centrifuge, Thermo Fisher Scientific). After resuspending the pellet with lysis buffer, protein estimation was carried out using Pierce BCA Protein Assay kit (Thermo Fisher). Western blot assay was carried out with Rabbit polyclonal anti-PBP2a antibody (RayBiotech) at a 1:1000 dilution for overnight incubation and Goat anti-Rabbit secondary antibody (Azure Biosystems) (1:10000) for 1 h.

Bocillin assay: As previously described (3), overnight cultures of the selected *S. aureus* strains were diluted in TSB such that the OD_{600} value was 0.1. Cells were grown at 37°C, 180 rpm until OD_{600} of 1 after which 50 mL of the culture was collected for centrifugation at 2451 g (3500 rpm) to obtain cell pellets. The pellets were dried and resuspended in 500 µL PBS and were then mechanically lysed using the FastPrep FP120 (Thermo Savant). Following centrifugation, the supernatant was collected and cell membrane fractions were isolated by performing ultracentrifugation (66000g, Sorvall WX Ultra 80 Centrifuge, Thermo Fisher Scientific). The obtained pellets were resuspended in 100 µL PBS and protein estimation was carried out using Pierce BCA Protein Assay kit (Thermo Fisher). The membrane fraction was then incubated with 10 µM Bocillin-Fl (Thermo Fisher) for 30 minutes at 35°C. The reaction was stopped by boiling with sample buffer for 10 minutes following which 20 µg of protein was loaded onto a 10% SDS-polyacrylamide gel and electrophoresis was carried out at 80V. The bands were visualized using the Typhoon 9410 imager (Amersham/GE Healthcare).

Electron microscopy: Transmission Electron Microscopy (TEM) was carried out as described previously (4). Briefly, overnight bacterial cultures were collected and fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (Electron Microscopy Sciences). Following post-fixation with 1% osmium tetroxide reduced with 0.8% potassium ferrocyanide, the cell pellets were treated with 1% tannic acid and stained with uranyl acetate replacement (UAR). Samples were dehydrated with a graded ethanol series, followed by dehydration with acetone and embedded in Embed 812 resin. Thin sections were cut with a Leica EM UC6 ultramicrotome (Leica), and stained with 1% UAR and Reynold's lead citrate prior to viewing at 120 kV on a Tecnai BT Spirit transmission electron microscope (FEI). Digital images were acquired with a Hamamatsu side mount digital camera system (Advanced Microscopy Techniques).

For scanning electron microscopy, cell suspensions were adsorbed on silicon chips and fixed with 2.5% glutaraldehyde in 0.1m sodium cacodylate buffer. Similar to the TEM specimen processing, the cells were post-fixed with 1% reduced osmium tetroxide and dehydrated in graded ethanol series. The specimen was dried using a critical point dryer (Bal-Tech AG), sputter coated with iridium (South Bay Technology, Inc.) and imaged on a Hitachi SU8000 scanning electron microscope (Hitachi High Technologies).

Sequencing: Fidelity of all the mutants and plasmid constructs were validated through Sanger sequencing (Eurofins Genomics, USA).

References

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112 oxacillin and/or cefoxitin resistance not linked to the presence of mec genes. Antimicrob
113 Agents Chemother 62.

114

115 **Supplementary tables and figures**

116 **Table S1a: List of wild-type and passaged strains used in this study**

Strains	Notes	*pbp4* promoter	PBP4	GdpP
COLnex	*mecA* excised COLn (3)	-	-	-
CRB	COLnex passaged in Ceftobiprole (3)	36bp insertion	E183A; F241R	N182K
CmTc	COLnex passaged in Ceftaroline (5)	A>C at -399bp upstream of *pbp4* start codon	T201A; F241L	H443Y
SF8300ex	*mecA* and blaZ excised SF8300 (5)	-	-	-
SRB	SF8300ex passaged in Ceftobiprole (5)	-	E183V; F241R	T509A
SRT	SF8300ex passaged in Ceftaroline (5)	"A" deletion at -378bp upstream and 11bp deletion at 300bp upstream of pbp4 start codon	N138K; H270L	X306
SF8300	Community associated MRSA	-	-	-
MW2	Community associated MRSA	-	-	-
COLn	Hospital associated MRSA	-	-	-
N315	Hospital associated MRSA	-	-	-
RN4220	Laboratory *S. aureus* strain	-	-	-

117

Table S1b: List of strains created in this study

Strains created in this study	Notes
SF8300 Δ*gdpP*	*gdpP* deleted SF8300 strain
SF8300ex Δ*gdpP*	*gdpP* deleted SF8300ex strain
MW2 Δ*gdpP*	*gdpP* deleted MW2 strain
COLn Δ*gdpP*	*gdpP* deleted COLn strain
N315 Δ*gdpP*	*gdpP* deleted N315 strain
SF8300 [E]	SF8300 with empty constitutively expression vector pTX_{Δ}
SF8300 Δ*gdpP* [E]	SF8300 Δ*gdpP* with empty constitutively expression vector pTX_{Δ}
SF8300 Δ*gdpP* [*gdpP*]	SF8300 Δ*gdpP* with pTX_{Δ} expressing gdpP from SF8300
MW2 [E]	MW2 with empty constitutively expression vector pTX_{Δ}
MW2 Δ*gdpP* [E]	MW2 Δ*gdpP* with empty constitutively expression vector pTX_{Δ}
MW2 Δ*gdpP* [*gdpP*]	MW2 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from SF8300
SF8300 Δ*gdpP* [CRB]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from CRB
SF8300 Δ*gdpP* [CmTc]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from CmTc
SF8300 Δ*gdpP* [SRB]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from SRB
SF8300 Δ*gdpP* [SRT]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from SRT
SF8300 Δ*gdpP* [strain 1]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 1
SF8300 Δ*gdpP* [strain 2]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 2
SF8300 Δ*gdpP* [strain 3]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 3
SF8300 Δ*gdpP* [strain 4]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 4
SF8300 Δ*gdpP* [strain 5]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 5
SF8300 Δ*gdpP* [strain 6]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 6
SF8300 Δ*gdpP* [strain 7]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 7
SF8300 Δ*gdpP* [strain 8]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 8
SF8300 Δ*gdpP* [strain 9]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 9
SF8300 Δ*gdpP* [strain 10]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 10
SF8300 Δ*gdpP* [strain 11]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 11
SF8300 Δ*gdpP* [strain 12]	SF8300 Δ*gdpP* with pTX_{Δ} expressing *gdpP* from strain 12
$SF8300^{tet}$	Tetracycline resistant SF8300

Table S2: List of plasmids used in this study

Plasmids used	Notes
pTX_{Δ}	Empty plasmid for constitutive expression (6)
pTX_{Δ} + *gdpP* [SF8300]	Constitutively expressed *gdpP* from SF8300
pTX_{Δ} + *gdpP* [CRB]	Constitutively expressed *gdpP* from CRB
pTX_{Δ} + *gdpP* [CmTc]	Constitutively expressed *gdpP* from CmTc
pTX_{Δ}+ *gdpP* [SRB]	Constitutively expressed *gdpP* from SRB
pTX_{Δ} + *gdpP* (strain 1)	Constitutively expressed *gdpP* from strain 1
pTX_{Δ} + *gdpP* (strain 2)	Constitutively expressed *gdpP* from strain 2
pTX_{Δ} + *gdpP* (strain 3)	Constitutively expressed *gdpP* from strain 3
pTX_{Δ} + *gdpP* (strain 4)	Constitutively expressed *gdpP* from strain 4
pTX_{Δ} + *gdpP* (strain 5)	Constitutively expressed *gdpP* from strain 5
pTX_{Δ} + *gdpP* (strain 6)	Constitutively expressed *gdpP* from strain 6
pTX_{Δ} + *gdpP* (strain 7)	Constitutively expressed *gdpP* from strain 7
pTX_{Δ} + *gdpP* (strain 8)	Constitutively expressed *gdpP* from strain 8
pTX_{Δ} + *gdpP* (strain 9)	Constitutively expressed *gdpP* from strain 9
pTX_{Δ} + *gdpP* (strain 10)	Constitutively expressed *gdpP* from strain 10
pTX_{Δ} + *gdpP* (strain 11)	Constitutively expressed *gdpP* from strain 11
pTX_{Δ} + *gdpP* (strain 12)	Constitutively expressed *gdpP* from strain 12
pKOR1	pKOR1 deletion construct for *gdpP* gene (6)
pLL29	Chromosomal integrative plasmid (7)

Table S3: List of primers used in this study

Primer	Sequence (5'- 3')
	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTTAATTTTCATT
gdpP-P1	AAAGAGGTTAAAATAATAGCTATAGTTAAAAATATGG
gdpP-P2	TATTCCACCTCTATTCACTTTTTAGAATTATTTTTCATGATTCG
	AAGTGAATAGAGGTGGAATAATGAAAGTAATTTTTACACAAGA
gdpP-P3	TGTTAAAGGTAAAGGTAAAAAAGG
	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCAGCTGTTTC
gdpP-P4	ATACACTTGTCCTAAGACGTCTCGAATGTCTTTAAAGC
gdpP-1	AAAGGATCCTAAAAAGTGAATAGAGGTGGAATAATG
gdpP-2	ACTAATGACACGTGTTACCATTGAGTTGATTTCC
gdpP-3	ATGGTAACACGTGTCATTAGTCGATGGGCAACTG
gdpP-4	TCGTTCATCACACGTCGTAATGTTGGATCAATG
gdpP-5	AACATTACGACGTGTGATGAACGAAATAGATAAAAAGC
gdpP-6	TAAACGCGTCTTTCATGCATCTTCACTCCTACTTAATTG
gdpP-7	ACGTTTAACGCGACTTGAATCAACAGTGATGTATG
gdpP-8	TGTTGATTCAAGTCGCGTTAAACGTTGTTCTG
gdpP-9	ATGGTAACACGTGTCATTAGTCGATGGTCAACTGAG
gdpP-10	AACATTACGACGTGTGATGAACGAAATTGATAAAAAGTC

Table S4: List of the clinical isolates used for measurement of CDA abundance, and their associated *gdpP* mutations in comparison to GdpP of SF8300 (USA300HOU_0014). (8)

Strains	Corresponding strain annotation as per Argudín *et al* (8)	*gdpP* associated mutations
1	004	V496E
2	007	F54L
3	009	P312L
4	013	D105N, P392S, A601E
5	019	A210S
6	021	Q642X
7	022	R504X
8	024	D105N, P392S, V496X
9	025	E486K
10	027	V609D
11	028	T317I
12	032	I263N

Table S5: Mutations in *gdpP* and *pbp4* among SRT passaged cultures

Passaged stocks	Ceftaroline concentration (μg/ml)	*gdpP*	*Ppbp4+pbp4*
Day 3 clone 1	0.25	X515	-
Day 3 clone 2	0.25	X402	-
Day 3 clone 3	0.25	X238	-
Day 4 clone 1	0.5	X306	-
Day 4 clone 2	0.5	X306	-
Day 4 clone 3	0.5	X306	-

X = missense mutation

Table S6: MIC (µg/ml) of β-lactam drugs

Strains	NAF	CTX	FOX	CFZ	CPT
MW2	16	64	32	64	0.5
MW2 Δ*gdpP*	32	> 256	64	128	1
SF8300	16	128	16	16	0.5
SF8300^{tet}	16	128	16	8	0.5

NAF- nafcillin; CTX- ceftriaxone; FOX- cefoxitin; CFZ- cefazoline; CPT- ceftaroline

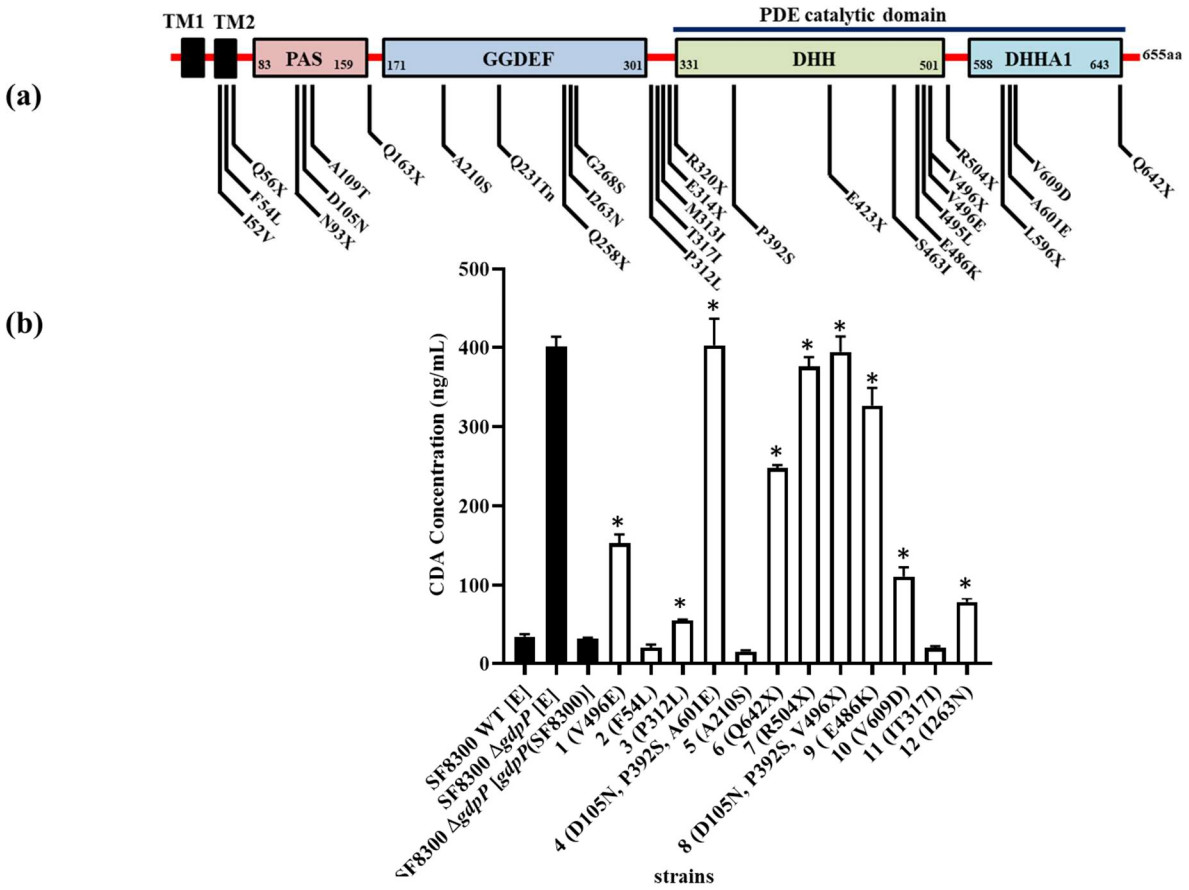
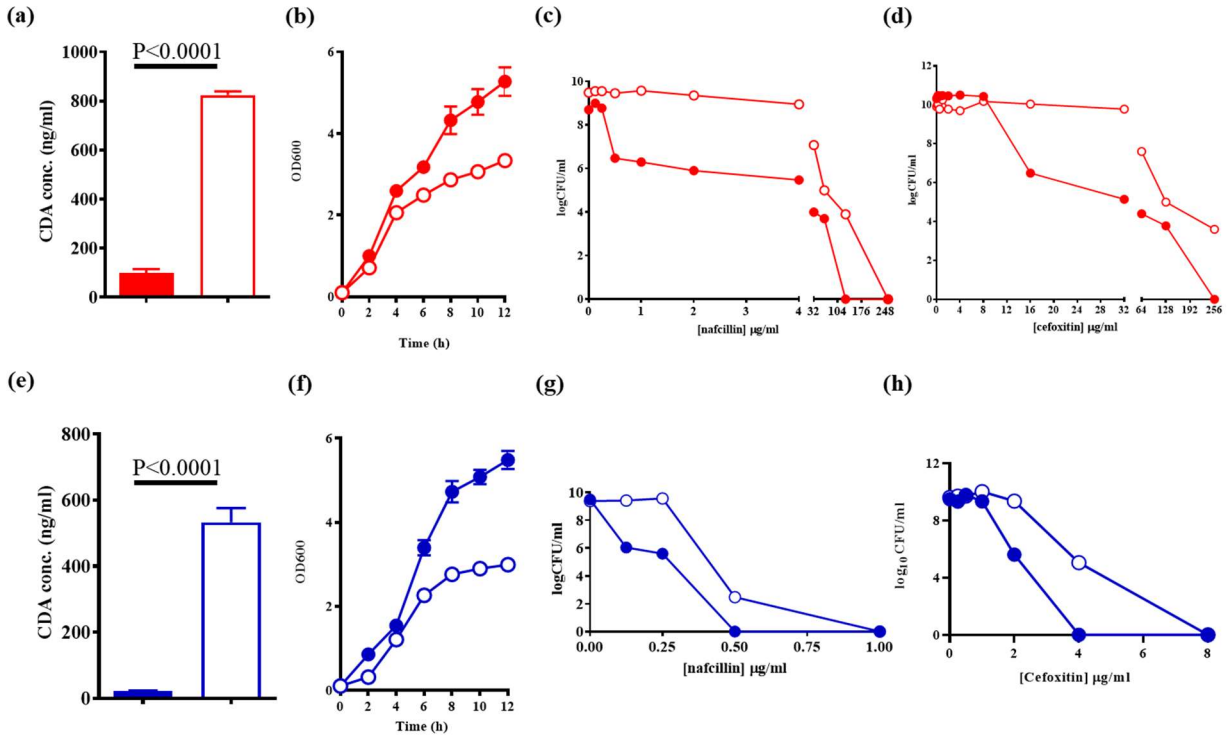


Figure S1: Clinical strains of *S. aureus* containing *gdpP* associated mutations.

(a) Schematic diagram showing all different domains of GdpP and mutations detected among clinical isolates.

(b) Analysis of CDA levels of SF3000 *ΔgdpP* cells containing *gdpP* from clinically isolated strains. Only *gdpP* with missense mutation/s (see Table S4) of clinical strains were analyzed. The X-axis contains the clinical strains (strains 1 through 12) having different mutations associated in the *gdpP* gene. The Y-axis is the CDA concentration. Strains having an asterisk had significantly higher concentrations of CDA compared to SF8300 WT [E]. P value for these strains < 0.05. [E] represents complementation with an empty vector.



143

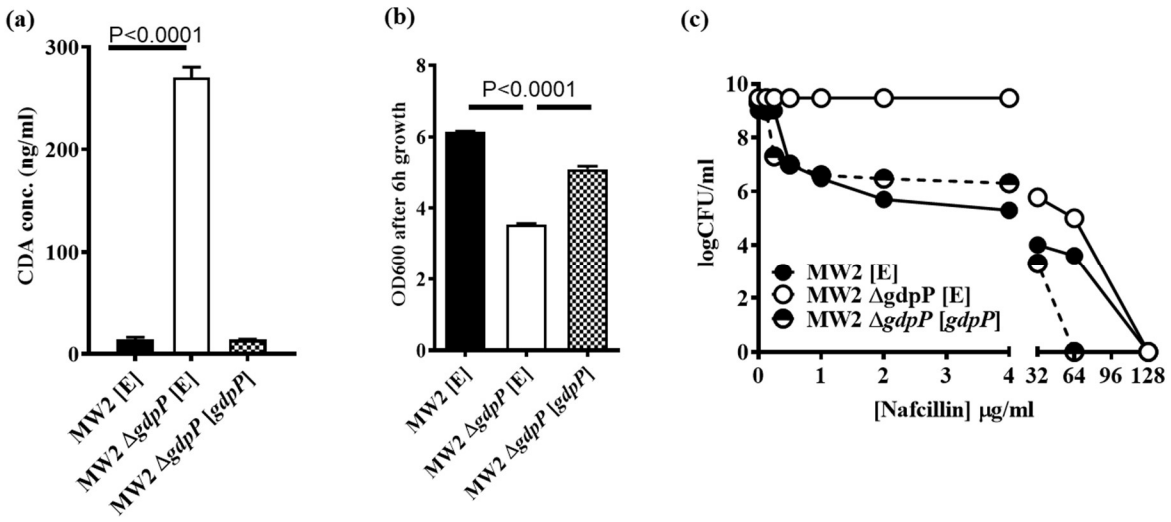
144 **Figure S2: GdpP phenotypes are not dependent on *mecA*.**

145 **(a)** & **(e)** CDA concentrations in the cytosol of SF8300 (closed red) and SF8300ex (closed blue)
146 and their isogenic Δ*gdpP* (open symbols) strains respectively.

147 **(b)** & **(f)** Growth curve of SF8300 and SF8300ex wild-type strains (closed red and closed blue
148 circles, respectively) and their isogenic Δ*gdpP* strains (open red and open blue circles,
149 respectively) in TSB media.

150 **(c)** & **(g)** Population analysis of SF8300 and SF8300ex wild-type strains (closed red and closed
151 blue circles, respectively) and their isogenic Δ*gdpP* strains (open red and open blue circles,
152 respectively) in nafcillin.

153 **(d)** & **(h)** Population analysis of SF8300 and SF8300ex wild-type strains (closed red and closed
154 blue circles, respectively) and their isogenic Δ*gdpP* strains (open red and open blue circles,
155 respectively) in cefoxitin.



156

157 **Figure S3: Complementation with *gdpP* restored wild-type phenotypes in MW2 strains.**

158 **(a)** CDA levels in the cytosol: Deletion of *gdpP* caused a significant increase in the cytosolic CDA

159 concentration (P value for wild-type (MW2 [E]) and deletion mutant (MW2 Δ*gdpP* [E]) < 0.0001)

160 **(b)** Growth pattern in TSB media: The deletion of *gdpP* in MW2 Δ*gdpP* [E] caused a growth

161 defect when compared to the wild-type strain containing an empty vector, MW2 [E]. This growth

162 defect was abolished when complemented with wild-type *gdpP* in strain MW2 Δ*gdpP* [*gdpP*].

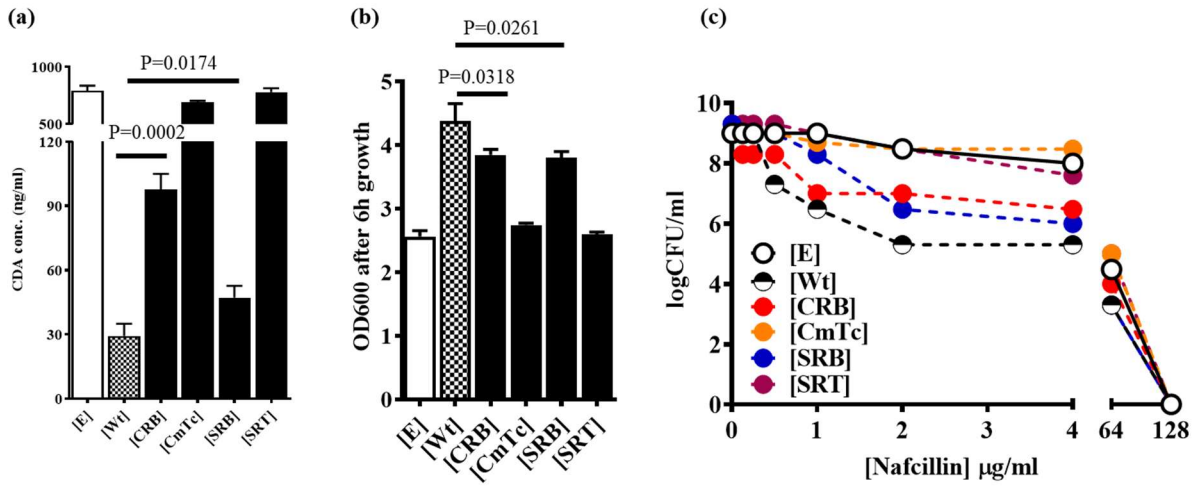
163 **(c)** Population analysis with nafcillin: The deletion of *gdpP* in strain MW2 Δ*gdp*P [E] (open

164 circles) enabled the cells to survive a nafcillin challenge, when compared to wild-type MW2 [E]

165 (completely filled circles) and the complemented strain MW2 Δ*gdpP* [*gdpP*] (half-filled circles).

166 [E] represents complementation with an empty vector.

167



168

169 **Figure S4: CDA drives Δ*gdpP* associated phenotypes.**

170 **(a)** Cytosolic measurements of CDA of SF8300 Δ*gdpP* strain complemented with either and empty
171 vector [E], wild-type *gdpP* and mutant *gdpP*s obtained from passaged strains showed that
172 complementation with *gdpP* from CRB, CmTc, SRB and SRT caused significantly increased
173 levels of CDA when compared to wild-type *gdpP*.

174 **(b)** Measurement of OD600 after 6 hours of bacterial culture in TSB showed that when SF8300
175 Δ*gdpP* was complemented with *gdpP* from strains such as CRB, CmTc, SRB and SRT, there was
176 a growth defect when compared to complementation with wild-type *gdpP*.

177 **(c)** Population analysis with nafcillin showed that when SF8300 Δ*gdpP* was complemented with
178 *gdpP* from strains such as CRB, CmTc, SRB and SRT, the cells had increased survival when
179 compared to when complemented with *gdpP*.

180 [E] represents complementation of SF8300 Δ*gdpP* with an empty vector.

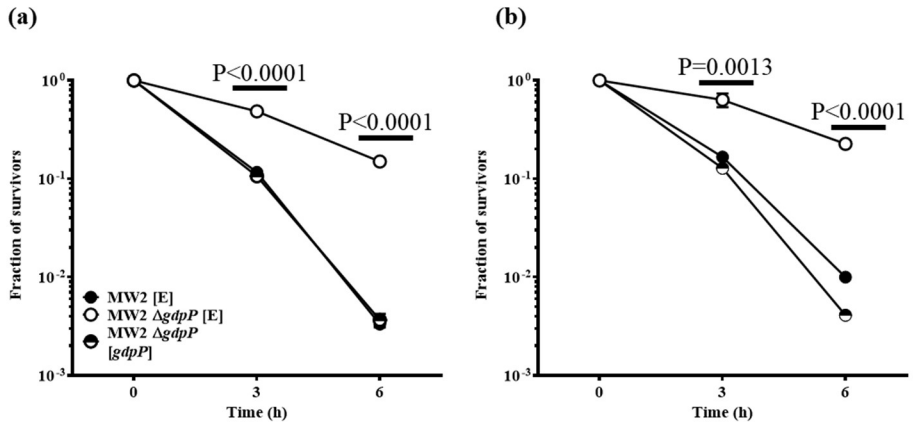


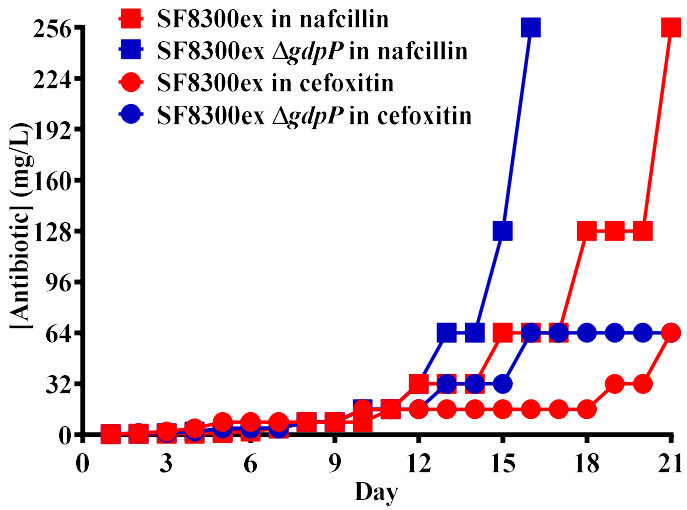
Figure S5: Deletion of *gdpP* leads to β-lactam tolerance in MW2 strains.

β-lactam tolerance assay carried out in:

(a) nafcillin (128 µg/ml)

(b) cefoxitin (128 µg/ml)

Showed that in the strain MW2 Δ*gdpP* [E] (open circles) had a significant increase in the fraction of survivors, and thus, in tolerance when compared to the an empty vector MW2 [E] (filled circles) and a complemented strain MW2 Δ*gdpP* [*gdpP*] (half-filled circles). [E] refers to complementation with an empty vector which serves as a control.



190

191 **Figure S6: Passaging of SF8300ex and its Δ*gdpP* strains in nafcillin and cefoxitin.**

192 SF8300ex Δ*gdpP* was able to develop resistance faster than SF8300ex Wt, when passaged in

193 nafcillin as well as in cefoxitin.

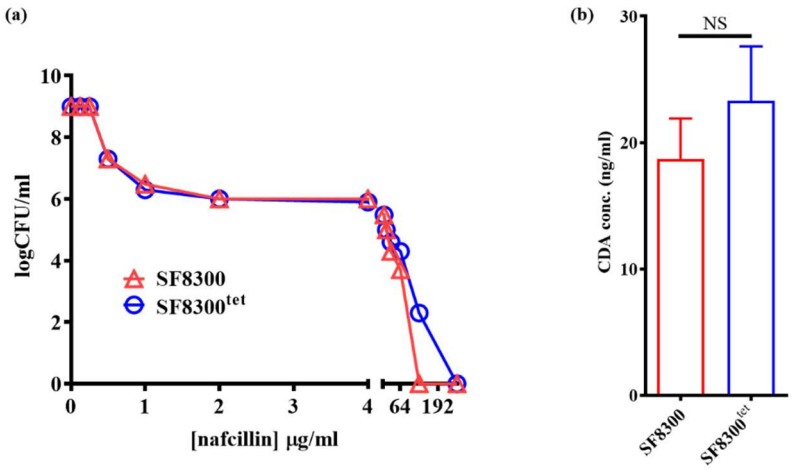


Figure S7: Loss of *gdpP* function could lead to β-lactam treatment failure.

(a) Population analysis with nacfillin showed that there was no difference in β-lactam resistance phenotypes for SF8300 and the tetracycline resistant SF8300 strain, SF8300^{tet}.

(b) Measurement of CDA concentration in the bacterial cytosol showed that there was no significant difference in cytosolic CDA levels between SF8300 and the tetracycline resistant SF8300 strain, SF8300^{tet}.

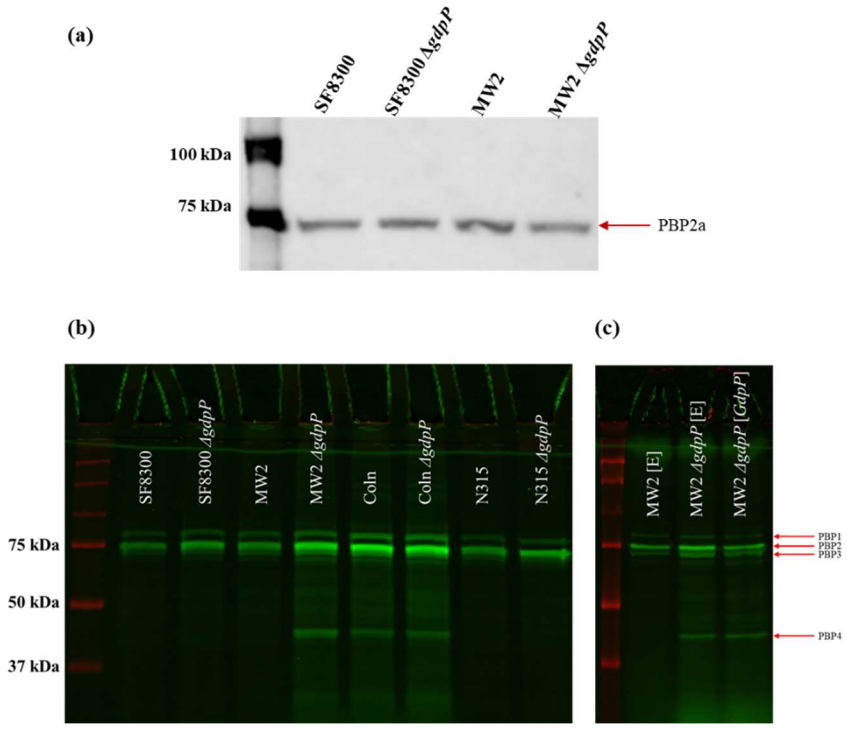
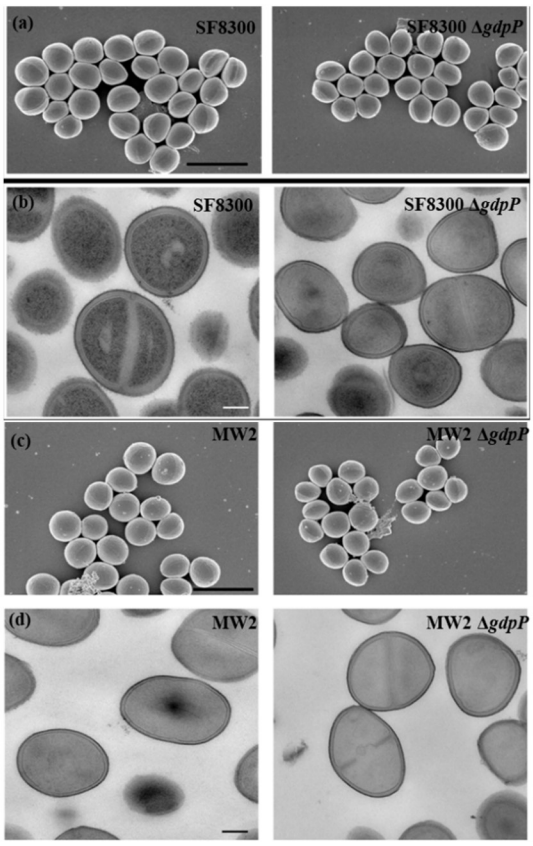


Figure S8: Deletion of *gdpP* did not have an effect on the expression of PBPs.

(a) Western blots of SF8300, MW2 and their isogenic Δ*gdpP* strains showed that there was no difference in the expression of PBP2a among the selected strains.

(b) Bocillin assay showing PBPs in SF8300, MW2, COLn, N315 and their isogenic Δ*gdpP* strains showed that there was no difference in the expression of Penicillin Binding Proteins 1-4 among the Wt and Δ*gdpP* strain pairs. **(c)** Bocillin assay showing PBPs in MW2, MW2 Δ*gdpP* complemented with an empty vector [E] and MW2 Δ*gdpP* complemented with *gdpP* suggested that the difference in expression of PBP4 in MW2 and MW2 Δ*gdpP* was potentially due to a secondary site mutation and not due to the deletion of *gdpP*, as the complementation of *gdpP* did not cause any alterations on PBP4 levels.



212

213 **Figure S9 (a) to (d): Transmission electron microscopic (TEM) and Scanning electron**

214 **microscopic (SEM) images of SF8300, MW2 and their isogenic Δ*gdpP* strains** showed that

215 there was no apparent difference in cell morphologies between Wt and Δ*gdpP* strain pairs**.**