Title

Loss of GdpP Function in Staphylococcus aureus Leads to β-Lactam Tolerance and Enhanced Evolution of β-Lactam Resistance

Permalink

https://escholarship.org/uc/item/8bf5c6sx

Journal

Antimicrobial Agents and Chemotherapy, 66(2)

ISSN

0066-4804

Authors

Poon, Raymond
Basuino, Li
Satishkumar, Nidhi
et al.

Publication Date

2022-02-15

DOI

10.1128/aac.01431-21

Peer reviewed

Loss of GdpP Function in *Staphylococcus aureus* Leads to *β*-Lactam Tolerance and Enhanced Evolution of *β*-Lactam Resistance

Raymond Poon,[a,b] Li Basuino,[c,h] Nidhi Satishkumar,[a,b] Aditi Chatterjee,[a,b*] Nagaraja Mukkayyan,[a,b] Emma Buggeln,[c,h] Liusheng Huang,[d] Vinod Nair,[e] Maria A. Argudín,[f] Sandip K. Datta,[g§] Henry F. Chambers,[c,h] Som S. Chatterjee[a,b]

aDepartment of Microbial Pathogenesis, School of Dentistry, University of Maryland, Baltimore, Maryland, USA

bInstitute of Marine and Environmental Technology, Baltimore, Maryland, USA

cDivision of HIV/AIDS, Infectious Diseases and Global Health, Department of Medicine, University of California, San Francisco, San Francisco, California, USA

dDrug Research Unit, Department of Clinical Pharmacy, University of California, San Francisco, San Francisco, California, USA

eMicroscopy Unit, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

fNational Reference Centre for Staphylococcus aureus, Department of Microbiology, Laboratoire Hospitalier Universitaire de Bruxelles Universitair Laboratorium Brussel (LHUB-ULB), Université Libre de Bruxelles, Brussels, Belgium

gBacterial Pathogenesis Unit, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

hSan Francisco General Hospital, San Francisco, California, USA

Raymond Poon and Li Basuino contributed equally to this work.

ABSTRACT Infections caused by *Staphylococcus aureus* are a leading cause of mortality. Treating infections caused by *S. aureus* is difficult due to resistance against most traditional antibiotics, including *β*-lactams. We previously reported the presence of mutations in *gdpP* among *S. aureus* strains that were obtained by serial passaging in *β*-lactam drugs. Similar mutations have recently been reported in natural *S. aureus* isolates that are either nonsusceptible or resistant to *β*-lactam antibiotics. *gdpP* codes for a phosphodiesterase that cleaves cyclic-di-AMP (CDA), a newly discovered second messenger. In this study, we sought to identify the role of *gdpP* in *β*-lactam resistance in *S. aureus*. Our results showed that *gdpP*-associated mutations caused loss of phosphodiesterase function, leading to increased CDA accumulation in the bacterial cytosol. Deletion of *gdpP* led to an enhanced ability of the bacteria to withstand a *β*-lactam challenge (2 to 3 log increase in bacterial CFU) by promoting tolerance without enhancing MICs of *β*-lactam antibiotics. Our results demonstrated that increased drug tolerance due to loss of GdpP function can provide a selective advantage in acquisition of high-level *β*-lactam resistance. Loss of GdpP function thus increases tolerance to *β*-lactams that can lead to its therapy failure and can permit *β*-lactam resistance to occur more readily.

KEYWORDS GdpP, *Staphylococcus aureus*, beta-lactams, cyclic-di-AMP, tolerance

Staphylococcus aureus is an important pathogen that causes widespread nosocomial and community-associated infections in humans (1, 2). Along with possessing a vast array of virulence factors, *S. aureus* also harbors the ability to display antibiotic resistance and antibiotic tolerance that enable bacteria to evade the action of antibiotics used for treatment (3). While resistance is typically characterized by an increase in the MIC for a target antibiotic, tolerance is a phenomenon where the survival of cells in the presence of antibiotics is prolonged, without causing a change in drug MIC (4). *β*-lactams are an important class of antibiotics that are often the first choice of treatment for *S. aureus* infections, primarily due to their high safety and efficacy. Over the past

several decades, β-lactam resistance among contemporary clinical strains of *S. aureus* has called for the use of second-line agents that are often less safe and effective (5).

β-Lactam resistance in *S. aureus* is classically mediated by penicillin binding protein 2a (PBP2a) and β-lactamase, which are encoded by *mecA* and *blaZ* respectively (6). While they have been well investigated, there is increasing evidence of the prevalence of nonclassical mechanisms of β-lactam resistance (7–9). Our previous studies that were targeted toward identifying nonclassical mechanisms of resistance detected missense and nonsense mutations in *gdpP* among every resistant passaged strain (10). Recent clinical surveillance studies also detected similar *gdpP* associated mutations among several β-lactam-nonsusceptible isolates, reiterating their clinical relevance (7, 11). GdpP (GGDEF domain protein containing phosphodiesterase [PDE]) contains two N-terminal transmembrane helices followed by a degenerated PAS domain (12), which acts as an internal sensor of oxygen and redox potential (13). This is followed by the degenerated GGDEF domain that is normally associated with cyclic-di-GMP cyclase (14) along with the DHH-DHHA1 domains that have phosphodiesterase activity, which gives GdpP the ability to cleave cyclic-di-AMP (CDA) (12). CDA is a newly discovered second messenger in bacteria and archaea that is synthesized from two molecules of ATP by the action of diadenylate cyclase (encoded by *dacA*) (12, 15). CDA has been deemed essential for bacterial cells and is implicated in a variety of cellular processes, such as ion homeostasis, cell size, and cell wall stress response among others in *S. aureus* (12, 16–18). GdpP, through its phosphodiesterase function, helps maintain the intracellular levels of CDA through its cleavage. In this study, we focused on determining the role of *gdpP*-associated mutations in surviving the antibacterial action of β-lactam drugs. Our results suggest that *gdpP*-associated mutations detected among laboratory-passaged and clinically isolated β-lactam-resistant or -nonsusceptible strains of *S. aureus* were loss-of-function mutations that led to enhanced CDA concentrations in bacterial cytosol. To the best of our knowledge, this study for the first time showed that *gdpP* mutations detected among clinical strains could lead to increased concentrations of CDA due to its loss of function. Enhanced CDA concentrations facilitated bacterial survival against β-lactam antibiotics by promoting drug tolerance but did not alter drug MICs. β-lactam drug tolerance achieved through loss of GdpP function in *S. aureus* can lead to its therapy failure. Our findings further indicate that GdpP loss-of-function mutations arise very early during the evolution of β-lactam resistance and provide new insights into how attaining drug tolerance can promote faster evolution of β-lactam resistance in *S. aureus*.

RESULTS

Mutations associated with *gdpP* cause loss of phosphodiesterase activity in laboratory-generated β-lactam-resistant passaged strains and clinical isolates. Every resistant passaged strain previously obtained through passaging of COLnex and SF8300ex (lacking *mecA* and *blaZ*, the classical mediators of β-lactam resistance in wild-type COLn and SF8300 strains, respectively) had a missense or nonsense mutation in *gdpP* that led to an amino acid alteration or a premature truncation of GdpP, respectively (see Table S1a in the supplemental material; Fig. 1a). The nonsense mutations were such that they ablated the DHH-DHHA1 domains of GdpP, which mediate phosphodiesterase (PDE) activity to degrade CDA (Fig. 1a) (12). Recent clinical surveillance studies have also identified similar *gdpP*-associated mutations among β-lactam nonsusceptible *S. aureus* strains, further implicating their role in β-lactam resistance (see Table S4 and Fig. S1a in the supplemental material) (7, 11).

We hypothesized that the accumulated mutations of *gdpP* caused attenuated PDE function (Table S1a; Fig. 1a), leading to elevated CDA concentrations in passaged strains. To make this determination, we measured the CDA abundance of the resistant passaged strains and compared them to their susceptible parents (Table S1a). We evaluated a representative strain that had a nonsense mutation in *gdpP* (X306) that resulted in a premature truncation before the DHH-DHHA1 domains (SRT with X306) (Table S1a; Fig. 1a) and all of the strains that accumulated missense mutations in our
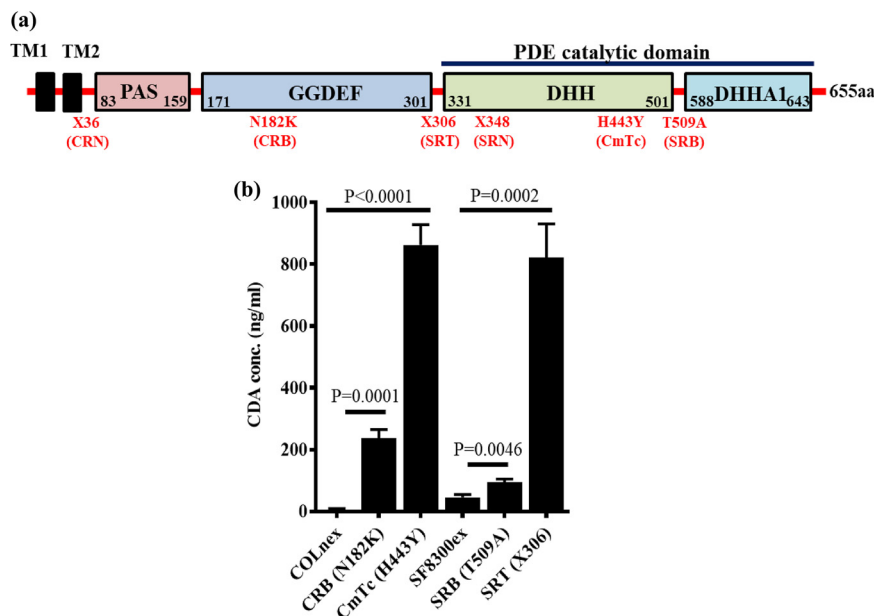
FIG 1 Passaged strains accumulated loss-of-GdpP-function mutations. (a) Schematic diagram showing different domains of GdpP and mutations detected among passaged resistant strains. (b) Cyclic-di-AMP concentrations in the cytosol of passaged strains in comparison to their isogenic susceptible parental strains COLnex and SF8300ex. Concentrations of CDA were significantly higher in CRB and CmTc, respectively, compared to that of the wild-type, COLnex. Similarly, CDA concentrations were higher in SRB and SRT, respectively, compared to that of the wild-type, SF8300ex.

passaging study (CRB with N182K, CmTc with H443Y, and SRB with T509A) (Fig. 1a; see also Table S1a). We detected that every strain, including those having a missense mutation, displayed elevated levels of CDA compared to their isogenic parents, COLnex and SF8300ex (Fig. 1b). SRT had ∼10-fold increased level of CDA. Notably, *gdpP* with the H443Y mutation (in the passaged strain CmTc) that substituted the 2nd histidine residue of the DHH residues with a tyrosine also yielded similarly high levels of CDA in the bacterial cytosol. Two other *gdpP* mutants, CRB (N182K) and SRB (T509A), had significantly elevated CDA amounts compared to their isogenic wild-type strains (Fig. 1b). These results indicated that the accumulated mutations in *gdpP* altered GdpP phosphodiesterase activity that ranged from partial attenuation to complete loss of function. Similarly, the majority of *gdpP* missense mutations identified through a recent clinical surveillance study were GdpP loss-of-function mutations (Table S4 and Fig. S1a and b). Of the 12 clinical strains analyzed for cytosolic CDA concentrations (strains 1 through 12), 9 were seen to have significantly increased CDA levels (Fig. S1b).

Loss of GdpP function leads to a growth defect and enhanced ability to withstand β-lactam challenge but does not change MIC against β-lactam antibiotics. To determine the phenotypes associated with loss of GdpP function, *gdpP* was deleted from *S. aureus* SF8300, a clinical isolate that belongs to the USA300 background strain and from its isogenic *mecA-* and *blaZ*-excised variant, SF8300ex. This approach allowed us to evaluate the role of GdpP in isogenic background strains both with and without the classical mediators of β-lactam resistance. Deletion of *gdpP* caused increased accumulation of CDA in the bacterial cytosol and produced a growth defect compared to the isogenic wild-type strains (Fig. 2a and b). To assess if the observed Δ*gdpP*-related phenotypes were indeed due to loss of *gdpP*, we complemented the Δ*gdpP* mutants with constitutively expressing wild-type *gdpP*. We also transformed the wild-type and Δ*gdpP* mutant strains with an empty vector as control strains. *gdpP* complementation in Δ*gdpP* mutants restored their wild-type phenotypes (Fig. 2). Results of population analysis revealed that the Δ*gdpP* strains had at least a 2 to 3 log fold increase in the proportion of bacterial cells compared to their isogenic wild-type strains, indicating a heightened survival to β-lactam drug treatment (Fig. 2c). This also denoted that the
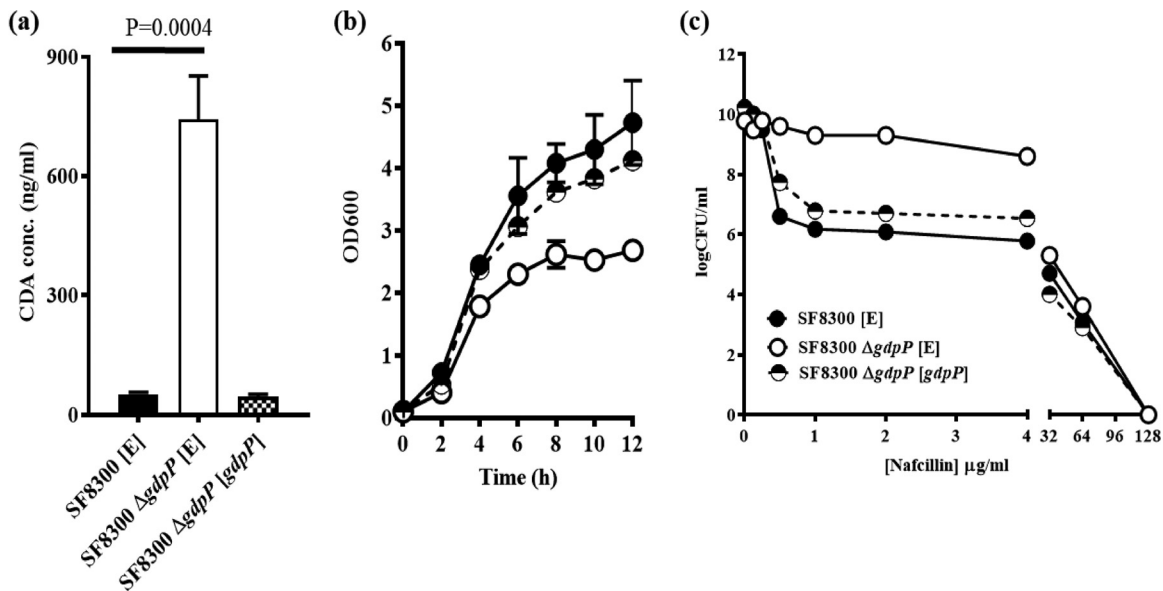
FIG 2 Complementation with *gdpP* restored wild-type phenotypes in SF8300 strains. (a) CDA levels in the cytosol. Deletion of *gdpP* (SF8300 Δ*gdpP* [E]) caused a significant increase in the cytosolic CDA concentration compared to that of the wild-type (SF8300 [E]) and complemented (SF8300 Δ*gdpP* [*gdpP*]) strains. (b) Growth pattern in TSB medium. The deletion of *gdpP* in strain SF8300 (SF8300 Δ*gdpP* [E]) (open circles) caused a growth defect compared to the wild-type strain containing an empty vector, SF8300 [E] (completely filled circles). This growth defect was abolished when complemented with wild-type *gdpP* in strain SF8300 Δ*gdpP* [*gdpP*] (half-filled circles). (c) Population analysis with nafcillin. The deletion of *gdpP* in strain SF8300 Δ*gdpP* [E] (open circles) enabled the cells to survive a nafcillin challenge compared to wild-type SF8300 [E] (completely filled circles) and the complemented strain SF8300 Δ*gdpP* [*gdpP*] (half-filled circles).

observed phenotypes were attributed to *gdpP*. Since both *mecA*- positive and -negative strains (i.e., SF8300 and SF830ex, respectively) displayed a similar pattern of β-lactam resilience, it indicated that *mecA* (the major mediator of β-lactam resistance in *S. aureus*) likely did not play any important role in mediating the phenotypes that the Δ*gdpP* strains displayed (see Fig. S2 in the supplemental material). In addition to the SF8300 Δ*gdpP* strain, we included another clinically relevant and epidemic-associated strain of *S. aureus*, MW2 (a member of the USA400 background), to confirm these findings (see Fig. S3 in the supplemental material). These results reiterated the notion that GdpP played an important role in surviving β-lactam challenge in *S. aureus*.

To further our understanding of the role of GdpP, we focused our study on *mecA*-positive strains, which cause the majority of complicated and hard-to-treat *S. aureus* infections (5). To determine if CDA abundance alone was the factor driving the observed Δ*gdpP*-associated β-lactam-resilient phenotypes, the Δ*gdpP* mutant of SF8300 was complemented with *gdpP* obtained from the passaged strains, namely, CRB, CmTc, SRB, and SRT. An empty vector (E) and *gdpP* from SF8300 (Wt) were used as controls (Table S1a). The resultant isogenic strains displayed varying amounts of CDA (see Fig. S4a in the supplemental material). Growth assays (Fig. S4b) and population assays (Fig. S4c) showed a high correlation between CDA concentrations, bacterial growth attenuation, and increased proportion of resistant populations.

Since the results of the population analysis showed a very similar endpoint of killing of the isogenic strains (Fig. S4c), we sought to determine if *gdpP* loss-of-function-mediated enhanced β-lactam survival actually involved an MIC change. Results of the MIC assays did not show a substantial difference in MIC values between the strains (Table 1). Thus, deletion of *gdpP* did not produce a resistant phenotype when measured through an MIC assay.

Loss of GdpP function leads to increased tolerance to β-lactams. Since deletion of *gdpP* allowed increased bacterial survival without a significant increase in the MIC values in the presence of β-lactams, we determined if the Δ*gdpP* strains mediated

TABLE 1 MICs for strains used in this study

| Strains	MIC (µg/mL) for:[a]				
	NAF	CRO	FOX	CFZ	CPT
SF8300	32	>256	128	128	1
SF8300 ΔgdpP	32	>256	128	128	1

[a]NAF, nafcillin; CRO, ceftriaxone; FOX, cefoxitin; CFZ, cefazolin; CPT, ceftaroline.

β-lactam tolerance. We performed tolerance assays using two representative β-lactam antibiotics (nafcillin and cefoxitin). The ΔgdpP strains in SF8300 (Fig. 3) and MW2 (see Fig. S5 in the supplemental material) backgrounds showed a classical (19) tolerance response, displaying an increased survival over time compared to its isogenic wild-type strain following treatment of antibiotics. Complementation with gdpP in the ΔgdpP strains restored their wild-type phenotype. These results led to the suggestion that ΔgdpP strains were able to survive a β-lactam challenge via drug tolerance.

GdpP loss-of-function mutations accumulate early in the process of β-lactam resistance and lead to faster evolution of β-lactam resistance. As mentioned above, the reported mutations in gdpP among resistant strains were obtained through serial passaging of the susceptible parental strains in the presence of drugs that took several weeks to accomplish (20). While whole-genome sequencing enabled the identification of mutated genes, our study design did not inform about the specific stage of the passage at which the mutations were acquired. To determine this, we sequenced pbp4 and gdpP in one of the passaged strains, SRT (21), for which bacterial stock cultures from each day of passaging were available. SRT was obtained by passaging the susceptible SF8300ex (mecA and blaZ excised) strain in increasing concentrations of ceftaroline for 22 days. At day 22, SRT was able to grow in bacterial medium containing 256 µg/mL ceftaroline (21). The purified SRT clone that was used for subsequent studies showed high-level resistance to β-lactam drugs and had a pbp4 promoter and gene mutations in addition to having the X306 mutation in GdpP (Fig. 1a) (10). Bacteria from the daily stock cultures of SRT passage were streaked onto TSA plates, and three colonies from each day of passage were randomly chosen for sequencing of the gdpP and pbp4 promoter and gene. We detected that SRT acquired its gdpP-associated X306 mutation as early as on day 4 of passaging, during which bacteria were grown in media containing 0.5 µg/mL of ceftaroline. On day 4, SRT did not have any pbp4-related mutations, suggesting that gdpP-associated mutations were acquired
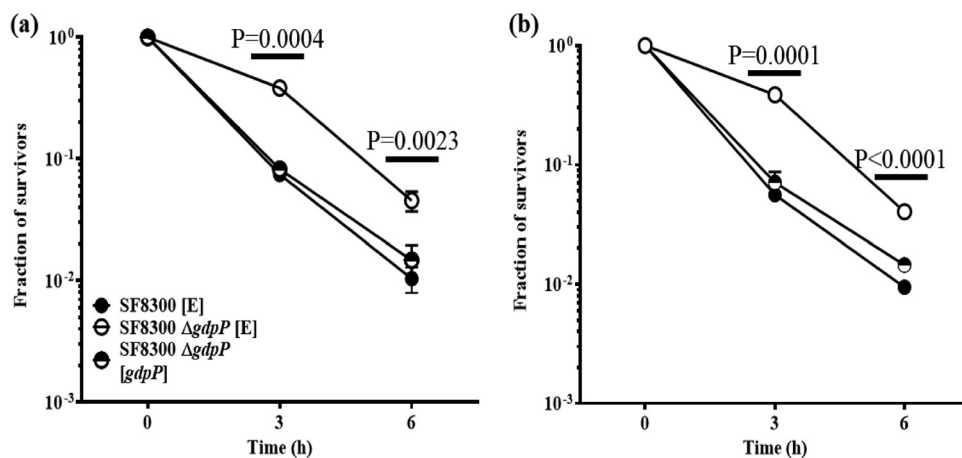
FIG 3 Deletion of gdpP leads to β-lactam tolerance in SF8300 strains. β-Lactam tolerance assay carried out with nafcillin (128 µg/mL) (a) and cefoxitin (256 µg/mL) (b). The deletion of gdpP in the strain SF8300 ΔgdpP [E] (open circles) had a significant increase in the fraction of survivors and, thus, in tolerance when compared to that of the empty vector SF8300 [E] (filled circles) and the complemented strain SF8300 ΔgdpP [gdpP] (half-filled circles). P values were determined for SF8300 [E] versus SF8300 ΔgdpP [E].
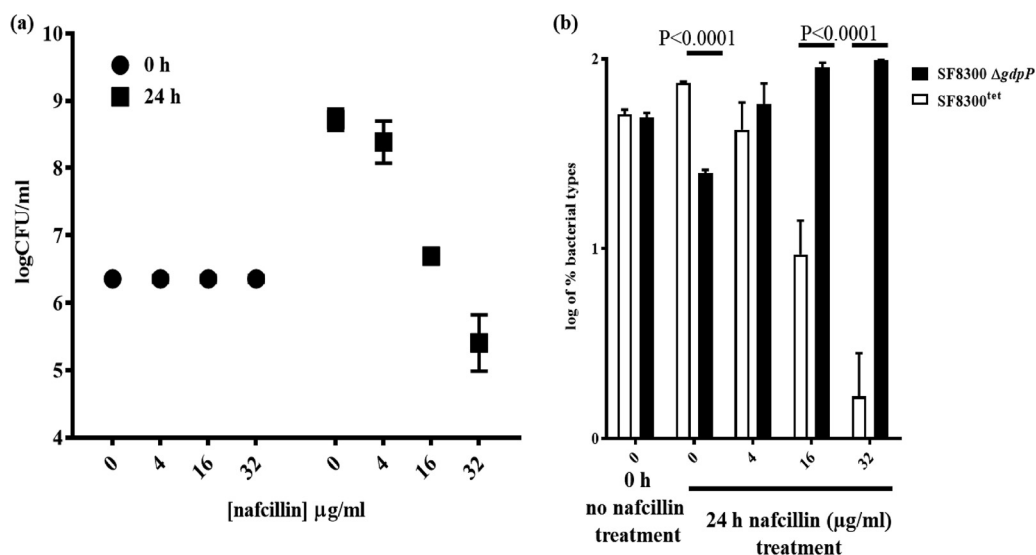
FIG 4 Deletion of *gdpP* leads to β-lactam resistance. (a) Survival of 1:1 mixed cocultures of SF8300ᵗᵉᵗ and SF8300 Δ*gdpP* strains upon treatment with nafcillin resulted in decreasing survival of bacterial CFU with increasing concentrations of nafcillin. (b) Fraction of SF8300ᵗᵉᵗ and SF8300 Δ*gdpP* strains in the mixture showed that after 24 h of nafcillin treatment, SF8300 Δ*gdpP* strains had significantly increased survival than SF8300ᵗᵉᵗ.

very early during the evolution of resistance in SRT. Of note, we also detected *gdpP* mutations among passaged strains obtained in day 3, which were different from the X306 detected on day 4 (see Table S5 in the supplemental material).

To determine if tolerance mediated by loss of GdpP function could facilitate evolution of β-lactam resistance, we performed passaging of the susceptible SF8300ex and its isogenic Δ*gdpP* strains in two β-lactam antibiotics (nafcillin and cefoxitin). Results of this study revealed that the Δ*gdpP* strain was able to develop resistance faster compared to its isogenic SF8300ex strain (see Fig. S6 in the supplemental material). Thus, GdpP-mediated tolerance can lead to faster evolution of resistance.

Loss of GdpP function could lead to β-lactam treatment failure. Since the therapeutic outcome of β-lactam tolerance due to loss of GdpP function is unknown, we created a tetracycline-resistant SF8300 (SF8300ᵗᵉᵗ) strain by integrating *pLL29* (single copy chromosomal integration vector) in its genome (22). The resultant SF8300ᵗᵉᵗ strain displayed no difference in β-lactam resistance phenotypes when measured through MIC assays (see Table S6 in the supplemental material) or population analysis (see Fig. S7a in the supplemental material). There was also no change in CDA amounts in the bacterial cytosol (Fig. S7b) when compared to its isogenic wild-type strain, SF8300. To test the impact of Δ*gdpP*-induced tolerance on β-lactam treatment outcome, SF8300ᵗᵉᵗ was mixed with the SF8300 Δ*gdpP* strain in a 1:1 ratio and was treated with different nafcillin concentrations. The resultant bacterial mixture was incubated for 24 h, upon which surviving bacterial CFU were enumerated by plating them onto Trypticase soy agar (TSA) plates. The bacterial titers of this assay at 24 h showed a stepwise decrease of total surviving bacteria in a nafcillin concentration-dependent manner (Fig. 4a). To determine the respective abundance of bacterial strains in the mixture, the surviving bacteria were selected onto TSA and TSAᵗᵉᵗ (TSA containing tetracycline) plates. The results of this analysis showed a preponderance of the SF8300 Δ*gdpP* strain (i.e., tetracycline-sensitive colonies) in the mixture (Fig. 4b). Furthermore, the Δ*gdpP* strain outcompeted the wild-type bacteria in a nafcillin concentration-dependent manner as well. These results suggested that the Δ*gdpP* strain could survive a β-lactam challenge better than its isogenic wild-type strain. The results of these experiments also revealed that at 24 h following incubation, the fraction of Δ*gdpP* strain was slightly attenuated compared to that of the wild-type strain without any antibiotic treatment, probably

due to the inherent growth defect that the ΔgdpP strain displayed. This suggested that tolerance mediated by gdpP could indeed lead to β-lactam nonsusceptibility or therapy failure.

DISCUSSION

Previous work performed in Gram-positive firmicutes, such as *Bacillus subtilis* (23), *Listeria monocytogenes* (24), and *S. aureus* (12, 25) among others, has shown a broad role of CDA in controlling bacterial physiology, including osmoregulation, peptidoglycan metabolism, biofilm formation, and virulence and has also drawn associations between CDA levels to β-lactam resistance of bacteria. While reduction of CDA concentration (through inhibition of its synthesis machinery) in bacterial cells has been shown to sensitize bacteria to β-lactams, increased amount of CDA (via inhibition of its degradation machinery) produces resistance to β-lactam drugs (12). The mechanism(s) through which CDA modulates drug sensitivity/resistance, however, remains currently unknown.

A high-level association in detection of mutations in gdpP (the machinery that degrades CDA) among laboratory-generated and natural strains of *S. aureus* that were nonsusceptible or resistant to β-lactam drugs prompted us to determine their role in surviving β-lactam drug challenge (7, 20). Our results showed that the gdpP mutations, by virtue of their loss of function, enabled increased intracellular CDA concentration in *S. aureus* (Fig. 1b; see also Fig. S1b in the supplemental material), which in turn provided a significant fitness advantage in surviving β-lactam challenge in a manner that was dependent on CDA concentration. Experiments carried out to decipher an enhanced understanding of CDA's role enabling better survival to a drug challenge indicated that increased CDA concentrations promoted β-lactam tolerance without altering drug resistance when measured through classical means, such as MIC or population analysis (Table 1; Fig. 2c). Although population analysis results showed homoresistance characteristics of bacterial strains with high CDA concentrations (Fig. 2c; see also Fig. S3c in the supplemental material), they indicated an almost identical endpoint of bacterial killing to that of wild-type bacteria. Thus, the term "resistance" in previous studies that associated CDA levels with drug resistance might have been used loosely. Antibiotic tolerance has recently emerged as an important factor that is distinct from traditional resistance, which reduces drug efficacy by producing slower killing of bacteria upon chemotherapeutic challenge (4, 19, 26). Indeed, our results show that acquisition of β-lactam tolerance through GdpP loss of function could lead to β-lactam therapy failure due to slower killing, thereby complicating treatment outcome (Fig. 3 and 4). These results are in alignment with previous studies that show that GdpP has a role in tolerance (27).

Recent studies have reported that increased *mecA* expression can enable *S. aureus* strains to display a β-lactam homoresistance phenotype (25), similar to that observed among strains with elevated CDA concentrations. Western blotting analysis did not indicate altered PBP2a expression among isogenic Wt and ΔgdpP strains used in this study (see Fig. S8a in the supplemental material). The results of Bocillin assay further showed unaltered expression of housekeeping PBPs (PBP1 through 4), suggesting that they were also likely not involved in GdpP-mediated β-lactam tolerance (Fig. S8b). We created two additional isogenic sets of strains (COLn and N315) to verify these results since the MW2 ΔgdpP strain showed slight overexpression of PBP4 in the Bocillin assay. Complementation with gdpP did not alter the PBP4 expression of the MW2 ΔgdpP strain (Fig. S8c), suggesting that the elevated PBP4 level is likely due to secondary site mutation(s) in this strain. Appearance of similar homoresistance phenotypes displayed by SF8300 and SF8300ex strains (see Fig. S2 in the supplemental material) along with detection of loss of GdpP function mutations among natural strains (7, 11) (see Table S4 and Fig. S1b in the supplemental material) further supported the notion that GdpP-mediated effects are most likely carried out through mechanisms that are independent of bacterial PBPs. Strains containing truncated gdpP that were not susceptible to penicillins

were also isolated from human and animal origin in recent studies (28, 29), further validating the clinical relevance of GdpP-associated effects of drug nonsusceptibility.

Appearance of GdpP loss-of-function mutation among one of the resistance-passaged strain, SRT, showed that its acquisition preceded mutations that enabled antibiotic resistance indicating that tolerance precedes resistance during evolution of antibiotic resistance (see Table S5 in the supplemental material). It also indicated that antibiotic tolerance is an independent biological phenomenon to that of resistance, which potentially employs separate mechanism(s) to aid antibiotic survival (19). This is likely the reason why GdpP loss-of-function mutations could be identified in natural strains that are nonsusceptible but not resistant to β-lactam drugs (7).

The mechanism through which GdpP loss of function enables β-lactam tolerance is unknown. Slower growth rate of the strains with elevated CDA levels does not seem to cause β-lactam tolerance, as both diminished and elevated CDA levels, which oppositely affects drug tolerance, reportedly inhibit bacterial growth (17). Thus, slower growth rate likely precludes β-lactam tolerance. Apart from a slightly smaller cell size, results of electron microscopic studies did not show much difference in bacterial gross cell morphologies, including that of the cell wall among the isogenic Wt and $\Delta gdpP$ strain pairs used in our study (see Fig. S9 in the supplemental material). This ruled out the possibility that an altered cell wall structure is the contributing factor for β-lactam tolerance, although further studies are required in order to determine this with more certainty. Previous studies have shown that CDA acts as a vital gatekeeper of ion transporting channels (most prominently that of potassium transporters, KdpFABC and KtrAB/AD potassium importers, and the CpaA potassium/sodium cation-proton antiporter) (16, 30), thereby controlling ion homeostasis in bacterial cells. It is thus imperative that elevated CDA concentrations due to GdpP loss of function would lead to reduced potassium ion concentrations in bacterial cells, which in turn would increase bacterial turgor pressure forcing bacteria to alter their cell structure and cell wall stiffness. Indeed, similar to our observation, GdpP loss-of-function mutants have previously been shown to display 30% smaller staphylococcal cell size (12). Since changes in cell shape in bacteria have recently been shown to influence antibiotic efficacy (31), it will be tempting to determine if changes in physical parameters, such as cell shape or stiffness brought in by CDA-mediated ionic imbalance, could contribute to β-lactam tolerance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains were grown in Trypticase soy broth (TSB) as previously described (21). Allelic replacements and complementation were carried out with the plasmids *pKOR1* and *pTX$_\Delta$*, respectively (10). Tetracycline-resistant SF8300 (SF8300[tet]) was created by chromosomal integration of the plasmid *pLL29* as previously described (22). Bacterial strains, plasmids, and primers used in this study are listed in Tables S1a and b, S2, S3, and S4 in the supplemental material.

Antibiotic resistance measurements. MICs were determined by broth microdilution method as described previously (32). Briefly, 1×10^5 CFU were incubated for 48 h at 37°C in 0.2 mL cation-adjusted Mueller-Hinton broth (CAMHB) (BD Biosciences) containing increasing concentrations of antibiotic. MIC was recorded as the lowest concentration without growth at 48 h.

Population analyses were done by the agar method as previously described (21). Tetracycline (12.5 μg/mL) was added to the agar plates to select for the plasmid *pTX$_\Delta$* whenever stated. Briefly, a 10-μl volume of serially diluted culture was spotted onto agar plates containing various concentrations of antibiotic. The plates were incubated at 37°C for 72 h. Plates were read and expressed as CFU mL^{-1}. Ceftobiprole and ceftaroline were provided by Johnson and Johnson Pharmaceutical Research and Development and (former) Forest Laboratories, respectively. All other antibiotics were purchased from Sigma-Aldrich.

CDA measurement in bacterial cell lysates. CDA measurement was carried out as previously described (33) with a few modifications. Briefly, bacterial cells were collected after 6 h postculture, washed, and lysed in phosphate-buffered saline (PBS) containing 1 mM EDTA using a FastPrep-24 homogenizer (MP Biomedicals). Bacterial cytosolic fraction was collected upon centrifugation. Aliquots of 40-μl cytosolic samples were mixed with 10 μL internal standard (20 ng/mL tenofovir, a phosphate group containing compound that is negatively charged in solution, similar to CDA), and 10 μL of the resulting mixture was injected into a liquid chromatography-tandem mass spectrometer (LC-MS/MS) system. The LC-MS/MS system consisted of AB Sciex API 5000 tandem mass spectrometer, Shimadzu Prominence 20AD^{XR} ultra-fast liquid chromatography (UFLC) pumps, and SIL-20AC^{XR} autosampler. A Hypercarb analytical column (100 by 2.1 mm, 3 mm; Thermo Fisher Scientific, USA) and mobile phases

100 mM ammonium acetate (Buffer A) and acetonitrile (Buffer B) were used for separation. Electrospray ionization in negative ion mode as the ion source and multiple reactions monitoring with ion pairs m/z 657/133 for CDA and m/z 286/133 for the internal standard were used for quantification. Calibration standards were prepared with synthetic CDA (Biolog) dissolved in the PBS containing 1 mM EDTA. The calibration range was 10 to 500 ng/mL with lower limit of quantification at 10 ng/mL.

Identification of GdpP function among clinical strains. We cloned the *gdpP* gene from the clinical strains that contained missense mutations with a constitutively expressing plasmid, pTX_Δ, and transformed the construct into a heterologous host, SF8300. The intracellular CDA abundance for each of these strains was measured through LC-MS/MS.

Bioinformatics and statistical analysis. All statistical analyses were performed using GraphPad Prism. Comparisons between groups and determination of P values were carried out using two-tailed t test unless otherwise stated. Error bars represent standard deviation of the mean for experiments performed in triplicates. Each experiment was performed in biological duplicates to ensure reproducibility. DNA sequence analysis was performed using DNAstar software. All other methods used in this study are presented in detail in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

REFERENCES

1. Chatterjee SS, Otto M. 2013. Improved understanding of factors driving methicillin-resistant *Staphylococcus aureus* epidemic waves. Clin Epidemiol 5:205–217. https://doi.org/10.2147/CLEP.S37071.

2. Lowy FD. 1998. *Staphylococcus aureus* infections. N Engl J Med 339:520–532. https://doi.org/10.1056/NEJM199808203390806.

3. Bui LMG, Conlon BP, Kidd SP. 2017. Antibiotic tolerance and the alternative lifestyles of *Staphylococcus aureus*. Essays Biochem 61:71–79. https://doi.org/10.1042/EBC20160061.

4. Levin-Reisman I, Brauner A, Ronin I, Balaban NQ. 2019. Epistasis between antibiotic tolerance, persistence, and resistance mutations. Proc Natl Acad Sci U S A 116:14734–14739. https://doi.org/10.1073/pnas.1906169116.

5. Chambers HF, Deleo FR. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 7:629–641. https://doi.org/10.1038/nrmicro2200.

6. Berger-Bachi B. 1999. Genetic basis of methicillin resistance in *Staphylococcus aureus*. Cell Mol Life Sci 56:764–770. https://doi.org/10.1007/s000180050023.

7. Argudín MA, Roisin S, Nienhaus L, Dodemont M, de Mendonca R, Nonhoff C, Deplano A, Denis O. 2018. Genetic diversity among *Staphylococcus aureus* isolates showing oxacillin and/or cefoxitin resistance not linked to the presence of mec genes. Antimicrob Agents Chemother 62:e00091-18. https://doi.org/10.1128/AAC.00091-18.

8. Dordel J, Kim C, Chung M, Pardos de la Gándara M, Holden MT, Parkhill J, de Lencastre H, Bentley SD, Tomasz A. 2014. Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant *Staphylococcus aureus*. mBio 5:e01000-13. https://doi.org/10.1128/mBio.01000-13.

9. Boonsiri T, Watanabe S, Tan X-E, Thitiananpakorn K, Narimatsu R, Sasaki K, Takenouchi R, Sato'o Y, Aiba Y, Kiga K, Sasahara T, Taki Y, Li F-Y, Zhang Y, Azam AH, Kawaguchi T, Cui L. 2020. Identification and characterization of mutations responsible for the β-lactam resistance in oxacillin-susceptible mecA-positive *Staphylococcus aureus*. Sci Rep 10:16907. https://doi.org/10.1038/s41598-020-73796-5.

10. Chatterjee SS, Chen L, Gilbert A, da Costa TM, Nair V, Datta SK, Kreiswirth BN, Chambers HF. 2017. PBP4 mediates β-lactam resistance by altered function. Antimicrob Agents Chemother 61:e00932-17. https://doi.org/10.1128/AAC.00932-17.

11. Argudín MA, Dodemont M, Taguemount M, Roisin S, de Mendonca R, Deplano A, Nonhoff C, Denis O. 2017. In vitro activity of ceftaroline against clinical *Staphylococcus aureus* isolates collected during a national survey conducted in Belgian hospitals. J Antimicrob Chemother 72:56–59. https://doi.org/10.1093/jac/dkw380.

12. Corrigan RM, Abbott JC, Burhenne H, Kaever V, Grundling A. 2011. c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. PLoS Pathog 7:e1002217. https://doi.org/10.1371/journal.ppat.1002217.

13. Taylor BL, Zhulin IB. 1999. PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol Mol Biol Rev 63:479–506. https://doi.org/10.1128/MMBR.63.2.479-506.1999.

14. Tal R, Wong HC, Calhoon R, Gelfand D, Fear AL, Volman G, Mayer R, Ross P, Amikam D, Weinhouse H, Cohen A, Sapir S, Ohana P, Benziman M. 1998. Three cdg operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. J Bacteriol 180:4416–4425. https://doi.org/10.1128/JB.180.17.4416-4425.1998.

15. Fahmi T, Port GC, Cho KH. 2017. c-di-AMP: an essential molecule in the signaling pathways that regulate the viability and virulence of Gram-positive bacteria. Genes (Basel) 8:197. https://doi.org/10.3390/genes8080197.

16. Corrigan RM, Campeotto I, Jeganathan T, Roelofs KG, Lee VT, Gründling A. 2013. Systematic identification of conserved bacterial c-di-AMP receptor proteins. Proc Natl Acad Sci U S A 110:9084–9089. https://doi.org/10.1073/pnas.1300595110.

17. Corrigan RM, Grundling A. 2013. Cyclic di-AMP: another second messenger enters the fray. Nat Rev Microbiol 11:513–524. https://doi.org/10.1038/nrmicro3069.

18. Huynh TN, Woodward JJ. 2016. Too much of a good thing: regulated depletion of c-di-AMP in the bacterial cytoplasm. Curr Opin Microbiol 30:22–29. https://doi.org/10.1016/j.mib.2015.12.007.

19. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330. https://doi.org/10.1038/nrmicro.2016.34.

20. Greninger AL, Chatterjee SS, Chan LC, Hamilton SM, Chambers HF, Chiu CY. 2016. Whole-genome sequencing of methicillin-resistant *Staphylococcus aureus* resistant to fifth-generation cephalosporins reveals potential

non-mecA mechanisms of resistance. PLoS One 11:e0149541. https://doi
.org/10.1371/journal.pone.0149541.

21. Chan LC, Gilbert A, Basuino L, da Costa TM, Hamilton SM, Dos Santos KR, Chambers HF, Chatterjee SS. 2016. PBP4 mediates high-level resistance to new-generation cephalosporins in *Staphylococcus aureus*. Antimicrob Agents Chemother 60:3934–3941. https://doi.org/10.1128/AAC.00358-16.

22. Luong TT, Lee CY. 2007. Improved single-copy integration vectors for *Staphylococcus aureus*. J Microbiol Methods 70:186–190. https://doi.org/10.1016/j.mimet.2007.04.007.

23. Luo Y, Helmann JD. 2012. Analysis of the role of *Bacillus subtilis* σ^M in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. Mol Microbiol 83:623–639. https://doi.org/10.1111/j.1365-2958.2011.07953.x.

24. Whiteley AT, Garelis NE, Peterson BN, Choi PH, Tong L, Woodward JJ, Portnoy DA. 2017. c-di-AMP modulates *Listeria monocytogenes* central metabolism to regulate growth, antibiotic resistance and osmoregulation. Mol Microbiol 104:212–233. https://doi.org/10.1111/mmi.13622.

25. Pozzi C, Waters EM, Rudkin JK, Schaeffer CR, Lohan AJ, Tong P, Loftus BJ, Pier GB, Fey PD, Massey RC, O'Gara JP. 2012. Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. PLoS Pathog 8:e1002626. https://doi.org/10.1371/journal.ppat.1002626.

26. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ. 2017. Antibiotic tolerance facilitates the evolution of resistance. Science 355: 826–830. https://doi.org/10.1126/science.aaj2191.

27. Griffiths JM, O'Neill AJ. 2012. Loss of function of the GdpP protein leads to joint β-lactam/glycopeptide tolerance in *Staphylococcus aureus*. Antimicrob Agents Chemother 56:579–581. https://doi.org/10.1128/AAC.05148-11.

28. Ba X, Kalmar L, Hadjirin NF, Kerschner H, Apfalter P, Morgan FJ, Paterson GK, Girvan SL, Zhou R, Harrison EM, Holmes MA. 2019. Truncation of GdpP mediates β-lactam resistance in clinical isolates of *Staphylococcus aureus*. J Antimicrob Chemother 74:1182–1191. https://doi.org/10.1093/jac/dkz013.

29. Sommer A, Fuchs S, Layer F, Schaudinn C, Weber RE, Richard H, Erdmann MB, Laue M, Schuster CF, Werner G, Strommenger B. 2021. Mutations in the *gdpP* gene are a clinically relevant mechanism for β-lactam resistance in meticillin-resistant *Staphylococcus aureus* lacking mec determinants. Microbial Genomics 7:000623. https://doi.org/10.1099/mgen.0.000623.

30. Kim H, Youn SJ, Kim SO, Ko J, Lee JO, Choi BS. 2015. Structural studies of potassium transport protein KtrA regulator of conductance of K^+ (RCK) C domain in complex with cyclic diadenosine monophosphate (c-di-AMP). J Biol Chem 290:16393–16402. https://doi.org/10.1074/jbc.M115.641340.

31. Banerjee S, Lo K, Ojkic N, Stephens R, Scherer NF, Dinner AR. 2021. Mechanical feedback promotes bacterial adaptation to antibiotics. Nat Phys 17:403–409. https://doi.org/10.1038/s41567-020-01079-x.

32. Chan LC, Basuino L, Diep B, Hamilton S, Chatterjee SS, Chambers HF. 2015. Ceftobiprole- and ceftaroline-resistant methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 59:2960–2963. https://doi.org/10.1128/AAC.05004-14.

33. Burhenne H, Kaever V. 2013. Quantification of cyclic dinucleotides by reversed-phase LC-MS/MS. Methods Mol Biol 1016:27–37. https://doi.org/10.1007/978-1-62703-441-8_3.