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Loss of Function of the GdpP Protein Leads to Joint β-Lactam/Glycopeptide Tolerance in *Staphylococcus aureus*

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The genetic basis of tolerance to inhibitors of peptidoglycan biosynthesis in *Staphylococcus aureus* was investigated by generating tolerant mutants *in vitro* and characterizing them by comparative genome sequencing. Two independently selected tolerant mutants harbored nonsynonymous mutations in *gdpP*, a gene encoding a putative membrane-located signaling protein. Insertional inactivation of *gdpP* also conferred tolerance. Our findings further implicate altered signal transduction as a route to antibiotic tolerance in *S. aureus*.

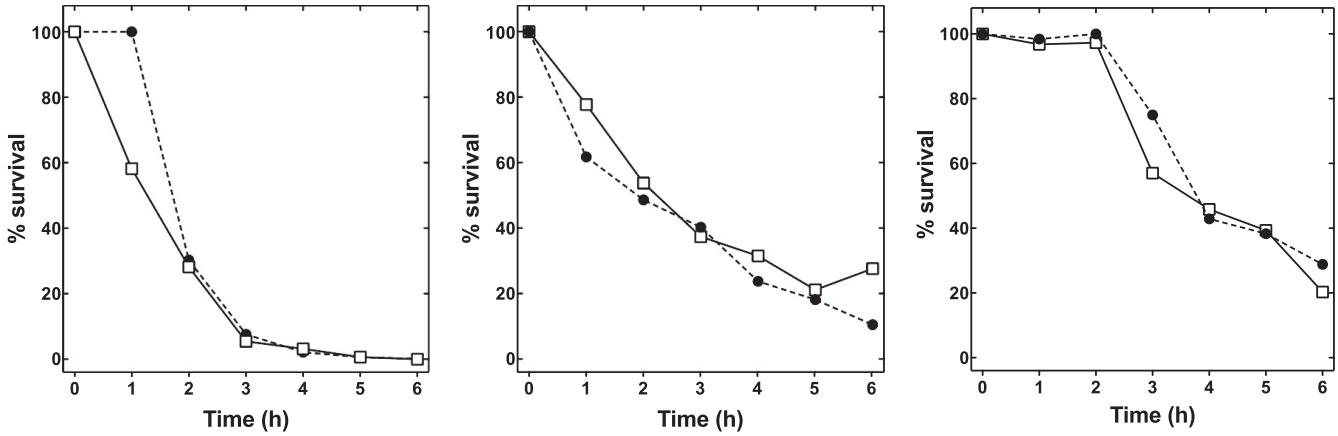

FIG 1 Representative time-kill responses for *S. aureus* SH1000 (left) and two tolerant derivatives (OXA15 [middle] and OXA10 [right]) following challenge with 12.5 μg oxacillin/ml (white squares) or 20 μg vancomycin/ml (black circles).

Tolerance describes the ability of a small minority of strains of a bacterial species to exhibit a bacteriostatic response to an antibacterial challenge that is bactericidal for the majority (2, 12). Among clinical isolates, tolerance is primarily observed for strains of Gram-positive genera in response to challenge with inhibitors of peptidoglycan biosynthesis (7). The presence of tolerant (TOL) strains in deep-seated infections has been reported to negatively impact treatment with β-lactams and/or glycopeptides and lead to therapeutic failure (7, 10, 12). The molecular basis for tolerance remains poorly understood. Here we describe investigations on the genetic basis of tolerance in *Staphylococcus aureus*.

β-Lactam-tolerant variants of *S. aureus* SH1000 (9, 13) were selected by repeated exposure to high concentrations of oxacillin (OXA). Cultures were grown in tryptone soya broth (TSB) to an optical density at 600 nm (OD_{600}) of 0.5 and challenged with 100 μg/ml OXA (200× the MIC) for ~16 h. Aliquots (200 μl) of culture were harvested by centrifugation, washed twice in fresh TSB to remove OXA, and used to inoculate fresh 10-ml volumes of TSB. Tolerance, defined here as a ≤90% drop in viability after a 6-h challenge with 12.5 μg OXA/ml (10, 11), was observed in mutants recovered from two independent cultures of SH1000 after 10 (culture 1) and 15 (culture 2) cycles of OXA exposure (Fig. 1). These mutants, respectively designated OXA10 and OXA15, exhibited tolerance without a concomitant increase in OXA MIC.

Since β-lactam tolerance can be associated with cross-tolerance to glycopeptides (7), we examined whether OXA10 and OXA15 displayed reduced killing compared with SH1000 in the presence of 20 μg vancomycin (VAN)/ml. In both cases, VAN cross-tolerance was observed (Fig. 1). No changes in susceptibility or killing compared with SH1000 were observed for the other bactericidal agents tested (ciprofloxacin, daptomycin) (data not shown).

Genetic changes in both TOL strains were identified by comparative genome sequencing (CGS) as previously described (4, 13) and were verified by PCR and DNA sequencing. OXA10 and

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TABLE 1 Mutations identified by CGS in β-lactam-tolerant strains OXA10 and OXA15, relative to SH1000

Genome position	Nucleotide change	Amino acid change	Locus tag	Comments
OXA10				
19096	C-A	$T_{260}K$	SAOUHSC_00015	*gdpP*
452322	A-T		SAOUHSC_R0006	23S rRNA
496601	A-T		SAOUHSC_R0007	23S rRNA
1905209	A-G		SAOUHSC_R0003	16S rRNA
2762204	T-C	$N_{1515}S$	SAOUHSC_02990	Hypothetical protein
OXA15				
19114	G-A	$G_{266}D$	SAOUHSC_00015	*gdpP*
2272936	A-T		SAOUHSC_02448/SAOUHSC_02449	Intergenic

OXA15 carried five and two mutations per genome, respectively (Table 1). Both harbored nonsynonymous mutations in SAOUHSC_00015 (also known as *gdpP*), a gene encoding a putative membrane-located signaling protein (8) (Table 1; Fig. 2). We focused our subsequent investigations on this gene.

The role of *gdpP* in tolerance was further examined by insertional inactivation using suicide plasmid pMUTIN4 (17). Oligonucleotide primers 5′-TATAAGCTTGAATGTCATTTCTGAATCTGT and 5′-TAACGGATCCTTTTTTCACGTAATTGACTC (engineered restriction sites are underlined) were used to PCR amplify a DNA fragment for targeting insertion of the plasmid into *gdpP*, and inactivation was performed as described previously (3). Disruption of *gdpP* in *S. aureus* RN4220 conferred tolerance (Table 2), implying that the *gdpP* mutations identified in OXA10 and OXA15 cause loss of function of the GdpP protein. Complementation was performed by PCR amplifying *gdpP* from SH1000 using oligonucleotide primers 5′-AAAGAGCTCCTAAAAAGTGAATAGAGGTGG and 5′-TGTGGTACCTACTTTCATGCATCTTCACTC and introducing this amplicon into RN4220*gdpP* in *trans* on plasmid pEPSA5 (5). In the presence of pEPSA5:$gdpP_{SH1000}$, loss of tolerance and restoration of wild-type susceptibility to OXA- and VAN-mediated killing were observed (Table 2).

To examine whether *gdpP* impacts tolerance in *S. aureus* strains other than those of the 8325 lineage (SH1000 and RN4220), we transduced (6) the pMUTIN4-inactivated *gdpP* gene from RN4220*gdpP* into *S. aureus* Newman (1) using Φ11. Disruption of *gdpP* in strain Newman conferred β-lactam tolerance, increasing survival following OXA challenge from 0.2 (±0.2)% to 14.5 (±3.8)%. Survival following VAN exposure increased from 0.8 (±0.5)% in the wild-type Newman strain to 9.1 (±0.8)% in the *gdpP*-inactivated strain. Although disruption of *gdpP* therefore failed to make *S. aureus* Newman fully VAN tolerant according to the definition given above, it nonetheless conferred a considerable (>10-fold) increase in survival in the presence of VAN.

The precise biological role of GdpP is unknown. However, the homologous YybT protein from *Bacillus subtilis*, which exhibits ca. 50% identity with GdpP, has been shown to hydrolyze cyclic dinucleotide second messengers involved in intracellular signaling (15). Deletion of *yybT* results in increased tolerance of *B. subtilis* to acid-mediated killing (15), as does disruption of the *yybT*/*gdpP* counterpart in *Lactococcus lactis* (14). Acid challenge experiments at pH 2.5 established that inactivation of *gdpP* also confers increased acid tolerance upon *S. aureus*, with cultures of RN4220*gdpP* containing ~100× more survivors than RN42220 after 6 h (data not shown). Taken together, the data clearly implicate this family of proteins in stress signaling and response (15). To assess whether GdpP and YybT are functionally equivalent, *yybT* was expressed from plasmid pEPSA5 in RN4220*gdpP*. The *yybT* gene, though conferring some reduction in tolerance, was unable to restore wild-type susceptibility to OXA-mediated killing (data not shown), suggesting that these proteins may not perform identical or fully interchangeable roles in bacilli and staphylococci.

Bioinformatic analysis of the GdpP protein reveals at least two functional domains (Fig. 2). The N-terminal domain contains a diguanylate cyclase (GGDEF) motif, a feature of proteins capable of synthesizing the second nucleotide messenger, c-di-GMP. However, the GGDEF motif in GdpP shows considerable divergence from the canonical sequence and lacks residues critical for catalytic activity, implying that GdpP is unable to catalyze formation of cyclic diguanylate (8). Thus, the nature of the biological activity provided by the N-terminal domain of GdpP is currently unknown. The C-terminal domain of GdpP has a Desert hedgehog (DHH) motif characteristic of phosphodiesterases, and, at least in YybT, this domain mediates hydrolysis of cyclic dinucleotides such as c-di-AMP (15).

The amino acid substitutions identified in GdpP for OXA10 and OXA15 both lie within the GGDEF domain (Fig. 2), implying that tolerance is associated with altered functioning of this region of the protein. To provide corroboration for this idea, and to establish whether the DHH domain has a role in antibiotic-mediated killing and tolerance, we performed mutagenesis of pEPSA5:$gdpP_{SH1000}$ to create GdpP mutants with defects in the

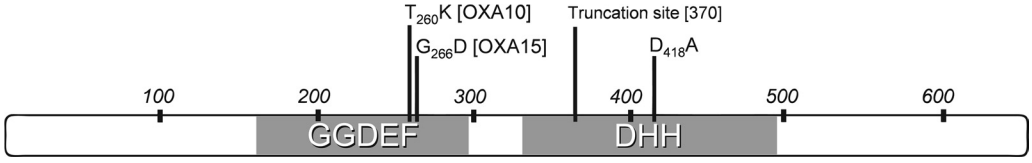

FIG 2 Schematic of the GdpP protein, showing the location of the GGDEF and DHH domains. Amino acid substitutions identified in TOL mutants OXA10 and OXA15 are indicated, as are the sites of engineered modification (truncation, substitution of $D_{418}A$) of GdpP performed in this study.

TABLE 2 Effect of disruption of the *gdpP* gene on survival of *S. aureus* RN4220 in the presence of peptidoglycan biosynthesis inhibitors[a]

Strain	% survival (±SD)	
	OXA	VAN
RN4220	1.6 (1.3)	1.4 (1.4)
RN4220*gdpP*	34.9 (9.8)	33.2 (9.7)
RN4220*gdpP* (pEPSA5)	37.0 (7.9)	30.4 (8.5)
RN4220*gdpP* (pEPSA5:$gdpP_{SH1000}$)	0.1 (0.2)	1.6 (0.5)

[a] Strains were exposed to 12.5 μg OXA/ml or 20 μg VAN/ml for 6 h. For strains carrying plasmid pEPSA5, expression was induced by inclusion of 0.5% (wt/vol) xylose in the growth medium. Results are the means (±standard deviations) of between 3 and 10 determinations.

DHH domain (Fig. 2), and we examined their ability to complement RN4220*gdpP*. The loss of the conserved aspartate at position 418 (GdpP numbering) substantially reduces the phosphodiesterase activity of the DHH domain of YybT *in vitro*, while the loss of D_{418} and D_{497} completely abolishes catalytic activity (15). Neither substitution of D_{418}A in GdpP nor truncation of GdpP to 370 amino acids (thereby leading to loss of both catalytic residues from the DHH domain) prevented the ability of the protein to restore susceptibility to OXA-mediated killing in RN4220*gdpP* (data not shown). Consequently, it appears that only the N-terminal domain of GdpP participates in antibiotic-mediated killing/tolerance. While the biological function of this domain remains to be established, the available evidence suggests a role in the metabolism of nucleotide second messengers.

In conclusion, loss of activity of the membrane-associated signaling protein GdpP protects *S. aureus* from killing by β-lactams, glycopeptides, and acid. This finding further implicates altered transduction of environmental signals as a route to antibiotic tolerance (7) and reinforces the idea that β-lactam- and glycopeptide-mediated killing is not simply a passive consequence of a blockade in peptidoglycan biosynthesis but an active process which involves a signaling cascade (16).

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