Loss of Function of the GdpP Protein Leads to Joint \( \beta \)-Lactam/Glycopeptide Tolerance in \textit{Staphylococcus aureus}

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

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 \( \beta \)-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 \textit{Staphylococcus aureus}.

\( \beta \)-Lactam-tolerant variants of \textit{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\textsubscript{600}) of 0.5 and challenged with 100 \( \mu \)g/ml OXA (200\( \times \) the MIC) for \( \sim 16 \) h. Aliquots (200 \( \mu \)l) 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 \( \approx 90\% \) drop in viability after a 6-h challenge with 12.5 \( \mu \)g 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 \( \beta \)-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 \( \mu \)g 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 OXA15, exhibited tolerance without a concomitant increase in OXA MIC.
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′-TATAAGCTTGAATGTCATTTCTGAAT

and 5′-TGTGGTACCTACTTTCAT

were used to 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 protein. Complementation was performed by PCR amplifying gdpP from SH1000 using oligonucleotide primers 5′-AAAGAGCTCCTAA

AAAGTGAATAGAGGTGG and 5′-TGTGGTACCTACTTTCAT

GCATCTTCACTC and introducing this amplicon into RN4220 containing pEPSA5 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 RN4220gdpP 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 0.2 (±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 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>OXA</th>
<th>VAN</th>
</tr>
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<tbody>
<tr>
<td>RN4220</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>RN4220gdpP</td>
<td>34.9</td>
<td>33.2</td>
</tr>
<tr>
<td>RN4220gdpP (pEPSA5)</td>
<td>37.0</td>
<td>30.4</td>
</tr>
<tr>
<td>RN4220gdpP (pEPSA5gdpPΔ418)</td>
<td>0.1</td>
<td>1.6</td>
</tr>
</tbody>
</table>

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.

REFERENCES