Daptomycin Tolerance in the *Staphylococcus aureus* *pitA6* Mutant Is Due to Upregulation of the *dlt* Operon

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Understanding the mechanisms of how bacteria become tolerant toward antibiotics during clinical therapy is a very important object. In a previous study, we showed that increased daptomycin (DAP) tolerance of *Staphylococcus aureus* was due to a point mutation in *pitA* (inorganic phosphate transporter) that led to intracellular accumulation of both inorganic phosphate (P_i) and polyphosphate (polyP). DAP tolerance in the *pitA6* mutant differs from classical resistance mechanisms since there is no increase in the MIC. In this follow-up study, we demonstrate that DAP tolerance in the *pitA6* mutant is not triggered by the accumulation of polyP. Transcriptome analysis revealed that 234 genes were at least 2.0-fold differentially expressed in the mutant. Particularly, genes involved in protein biosynthesis, carbohydrate and lipid metabolism, and replication and maintenance of DNA were downregulated. However, the most important change was the upregulation of the *dlt* operon, which is induced by the accumulation of intracellular P_i . The GraXRS system, known as an activator of the *dlt* operon (D-alanylation of teichoic acids) and of the *mprF* gene (multiple peptide resistance factor), is not involved in DAP tolerance of the *pitA6* mutant. In conclusion, DAP tolerance of the *pitA6* mutant is due to an upregulation of the *dlt* operon, triggered directly or indirectly by the accumulation of P_i .

Daptomycin (DAP) is a cyclic lipopeptide antibiotic that was approved by the Food and Drug Administration (FDA) for treatment of complicated skin infections and bacteremia, as well as endocarditis caused by Gram-positive bacteria (1). DAP is highly efficient not only against growing cells but also against stationary-phase cultures of *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA) strains, and is used as a last-resort antibiotic (2). Interestingly, a MIC creep for DAP has not been observed since its approval in 2003 (3); however, the number of cases with DAP therapy failure due to DAP-resistant (DAP-R) *S. aureus* strains has increased (1, 4).

Daptomycin is known to bind to the bacterial membranes of Gram-positive bacteria and kills the cell by disrupting the proton motive force (1). It is known that most of the adaptations in DAP-R strains concern the bacterial cell membrane and cell wall (5); however, the precise mode of action of DAP is still enigmatic.

In addition to antibiotic resistance, antibiotic tolerance can also be responsible for the failure of antibiotic therapy (4, 6). In contrast to resistant cells, tolerant strains are unable to grow in the presence of DAP; however, they survive the bactericidal action of DAP at least for a restricted period of time (7).

Antibiotic tolerance can be categorized into phenotypic and genetic tolerance. Phenotypic tolerance is independent from genetic alterations and becomes apparent, for instance, in bacterial persister cells, representing a tolerant subpopulation within isogenic bacterial populations (8, 9). Persisters are dormant or non-growing, and their tolerance extends to different kind of antibiotics (6). Drug indifference is another example of phenotypic tolerance which is distributed, however, over the entire population and is caused by the inhibition of proliferation due to nutrient starvation (9, 10). Nonproliferating stationary-phase cells that are less sensitive toward treatment with most antibiotics can be named as a well-known example of drug indifference. In comparison, genetic tolerance is caused by genetically manifested and

vertically transmitted mutations (8). All types of tolerance share the fact that an increase in the MIC does not occur. Although genetic antibiotic tolerance has been known for several decades, little is known about its generation (11).

In a recent study, we selected a mutant of *S. aureus* strain HG003 with a 10,000-fold-increased DAP tolerance (7). The tolerance of the corresponding mutant HG003*pitA6* differs from that of the wild-type strain as it fulfills all criteria of genetic tolerance with a rather slow but linear decrease of viability during DAP treatment (8, 12). In contrast, the wild-type strain shows a biphasic killing behavior after the addition of DAP, which is typical of phenotypic tolerance by persister cells (6, 12). The DAP tolerance of HG003*pitA6* was shown to be restricted to the stationary growth phase and associated with an increased uptake of inorganic phosphate (P_i), which was stored as polyphosphate (polyP) (7). We showed that the genetic basis of this increased DAP tolerance is represented by an adaptive single point mutation (*pitA6*) in the P_i transporter-encoding gene, *pitA*, which is part of the *pitRA* operon.

In this follow-up study, we found that heterologous expression of exopolyphosphatase (PPX) in the tolerant strain HG003*pitA6*

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diminishes polyP to a wild-type level; however, the intracellular P_i level and growth phase-dependent DAP tolerance were not affected. Transcriptome analysis followed by quantitative real-time PCR (qRT-PCR) revealed that the *dlt* operon is upregulated in the *pitA6* mutant, which is most likely responsible for the DAP tolerance. The responsibility of the *dlt* operon in the *pitA6* DAP tolerance phenotype was confirmed as the deletion of *dltA* completely eliminated DAP tolerance caused by the *pitA6* allele.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains used in this study are listed in Table S2 in the supplemental material. *Escherichia coli* DC10B (13) was used for molecular cloning of plasmids pMAD (14) and pRAB11 (15). *E. coli* cells bearing pMAD, pRAB11, or their derivatives were grown at 37°C in basic medium (BM; 1% [wt/vol] soy peptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl, 0.1% [wt/vol] K_2HPO_4 , 0.1% [wt/vol] glucose), supplemented with 100 µg/ml ampicillin (Carl Roth). *S. aureus* cells with episomal pMAD plasmids were incubated at 30°C in BM or tryptic soy broth (TSB; 1.7% [wt/vol] peptone from casein, pancreatic digest, 0.3% [wt/vol] soy peptone, 0.5% [wt/vol] NaCl, 0.25% [wt/vol] K_2HPO_4 , 0.25% [wt/vol] glucose, pH 7.3 ± 0.2) supplemented with 2.5 µg/ml erythromycin, and those carrying pRAB11 were incubated at 37°C in medium supplemented with 10 µg/ml chloramphenicol (Carl Roth).

Molecular cloning and DNA isolation. Genomic DNA from *S. aureus* was isolated as described previously (16). Qiagen kits were used for isolation of bacterial plasmids. Successful cloning of plasmid constructs was verified by colony PCR and sequencing (GATC Biotech). RbCl was used for preparation of chemically competent *E. coli* cells (17), and electrocompetent cells of *S. aureus* were prepared as described previously by Mechler et al. (7).

Allelic exchange constructs and homologous recombination. We used Gibson assembly (18) for cloning of the constructs for chromosomal deletion of genes *dltA*, *mprF*, *graR*, and *graS* in *S. aureus* HG003 wild type and HG003pitA6. DNA from *S. aureus* HG003 was used as a template. The primer pairs LM_graR_up_f /LM_graR_up_r and LM_graR_down_f /LM_graR_down_r (see Table S3 in the supplemental material for oligonucleotides) were used to amplify the upstream and downstream regions of *graR* in *S. aureus* HG003. The deletion region for *graR* includes 9 bp upstream from the start codon of *graR*, including parts of the putative ribosomal binding site. *graXS* fulfills all requirements for functionality in the corresponding strains. The primer pairs LM_graSup1/LM_graSup2 and LM_graSdown1/LM_graSdown1 were used for the deletion construct of *graS*. Regions consisting of 12 bp encoding the N-terminal part and 21 bp encoding the C terminus remained, resulting in functional *graR* and *graX*. For *dltA* deletion, the primer pairs LM_dlt_up1/LM_dlt_up2 and LM_dlt_down1/LM_dlt_down2 were used for amplification of regions bracketing *dltA*, leaving *dltX*, *dltB*, *dltC*, and *dltD* intact. The deletion part contains 10 bp upstream of *dltA*, disrupting the putative Shine-Dalgarno (SD) sequence, but retains 21 bp encoding the N terminus and encompassing the putative SD sequence of *dltB*. For deletion of *mprF*, the primer pairs LM_mprFup1/LM_mprFup2 and LM_mprFdown1 /LM_mprFdown2 were used. In this construct, 6 bp encoding the N terminus and 16 bp encoding the C terminus remain, resulting in a frameshift. For cloning of all deletion constructs, the vector pMAD was digested with the enzymes BglII and SalI, and PCR products were incubated in a 2.5-fold excess of the plasmid backbone for 60 min at 50°C, in a final volume of 20 µl of reaction mixture (0.08 U of T5 exonuclease, 0.5 U of Phusion DNA polymerase, 80 U of *Taq* DNA ligase [all from New England BioLabs]) and 4 µl of 5× isothermal reaction buffer (25% [wt/vol] polyethylene glycol [PEG] 8000, 500 mM Tris-HCl [pH 7.5], 50 mM $MgCl_2$, 50 mM dithiothreitol [DTT], 1 mM concentrations of each of the four deoxynucleoside triphosphates [dNTPs], and 5 mM NAD). The procedure of homologous recombination was performed as described previously by Mechler et al. (7).

Inducible expression using plasmid pRAB11. The exopolyphosphatase (*ppx*) gene, A284_02120, including its native ribosome-binding sequence, was PCR amplified from genomic DNA of *Staphylococcus warneri* (19), using the primer pair *ppx*(warneri)fw and *ppx*(warneri)rev. Plasmid pRAB11 was digested with BglII. Again, Gibson assembly gave rise to pRAB11-*ppx*, harboring *ppx* downstream of the anhydrotetracycline (ATc)-inducible promoter $P_{xyl-tet}$. pRAB11-*ppx* was finally used to transform HG003, as well as HG003pitA6. For heterologous expression and downstream experiments, strains carrying pRAB11-*ppx* were inoculated to an optical density at 578 nm (OD_{578}) of 0.07 and were grown for 2 h in TSB at 37°C with agitation before the addition of ATc (0.4 µM). Incubation was continued for a total of 16 h, and then polyP levels, intracellular P_i levels, and DAP tolerance were determined.

Determination of MIC and antibiotic tolerance. MIC values were determined as described previously (20). The MIC of DAP is 1 µg/ml for both HG003 and HG003pitA6. For antibiotic tolerance assays, cells were grown for 16 h at 37°C in TSB with agitation. Afterwards, 2.24 ml of the overnight culture was transferred to 14-ml round-bottom tubes. DAP and $CaCl_2$ were added to final volumes of 100 µg/ml and 50 µg/ml, respectively. The number of CFU/milliliter was determined at time points indicated in the figures.

Intracellular inorganic phosphate (P_i) and polyphosphate (polyP) determination. Intracellular P_i was quantified as described previously (7) using a commercially available colorimetric approach (ab65622; Abcam). In brief, cells were grown at 37°C in TSB with agitation, harvested at time points indicated in the figures, washed with double-distilled H_2O (ddH_2O), and disrupted using a FastPrep (MP Biomedicals) instrument. Cell debris was pelleted, and supernatant was used to quantify P_i according to the manual of the colorimetric approach (ab65622; Abcam). Intracellular polyP was determined using 4'-6-diamidino-2-phenylindole (DAPI; Sigma) as described previously (7, 21, 22). Again, cells were grown in TSB at 37°C with agitation before being harvested and washed twice in Tris-HCl buffer (100 mM Tris; pH 7.5). Samples were adjusted to an OD_{578} of 0.5, and DAPI was added to a final concentration of 20 µM. The polyP fluorescence signal was determined using a microplate reader (TECAN Infinite 200M) with excitation at 415 nm and an emission wavelength of 550 nm.

RNA isolation for microarray and qRT-PCR. RNA for microarray and qRT-PCR was isolated as described previously (7). In brief, cells were adjusted to an OD_{578} of 0.07 in TSB and cultured at 37°C. At the time points indicated in the figures, cells were harvested and adjusted to an OD_{578} of 70 before lysis with 1 ml Trizol (Life Technologies). DNA was removed by 2-fold DNase I digestion as described previously (23). A pool of 3 µg of total RNA for each condition was reverse transcribed using SuperScript II (Invitrogen, Basel, Switzerland). A volume of 5 µl of a 1:100-fold dilution was used for quantitative PCRs (qPCRs). Gene-specific primers and 6-carboxyfluorescein (FAM)-coupled probes for *hu* and *mprF* (see Table S4 in the supplemental material) were designed using Primer Express, version 3.0 (Applied Biosystems), and mixed in qPCR reagents (ABgene). Other genes were quantified by using a Brilliant SYBR Green master mix (Agilent) with primers described in Table S4 in the supplemental material. Reactions were performed in a Bio-Rad CFX96 and normalized using intensity levels recorded for the *hu* gene as described previously (24).

Microarray manufacturing and microarray design. The microarray was manufactured by *in situ* synthesis of 60-base-long oligonucleotide probes (Agilent), selected as previously described (25). The microarray consists of a glass slide with 15,000 printed oligonucleotides, covering >95% of all open reading frames (ORFs) annotated in strains NCTC 8325 (26), UAMS-1 (27), and SA564 (28) as well as Newman (29), including their respective plasmids.

Preparation of labeled nucleic acids for expression microarrays. Total RNA was purified from two independent cultures from strain HG003 and its *pitA6* mutant. After additional DNase treatment, the absence of DNA traces was confirmed by quantitative PCR (SDS 7700; Applied Bio-

FIG 1 DAP tolerance of HG003pitA6 is independent of polyP. Graphs represent the following: quantification of polyP levels in DAPI-stained cells with excitation at 415 nm and emission at 550 nm (A), intracellular P_i concentration (B), and killing kinetics of stationary-phase cultures treated with 100 μ g/ml DAP (C). Strains harboring the empty plasmid are indicated by -ppx, whereas those expressing *ppx* are indicated by +ppx. All values are averages from at least three independent experiments. Error bars indicate the standard deviations, and significance compared to results with HG003 with the empty plasmid pRAB11 was determined using Student's *t* test. Asterisks indicate *P* values of <0.05. wt, wild type; RFU, relative fluorescence units.

systems) with assays specific for 16S rRNA (30). Batches of 5 μ g of total *S. aureus* RNA were labeled by Cy3-dCTP, using SuperScript II (Invitrogen) according to the manufacturer's instructions. Labeled products were then purified onto QiaQuick columns (Qiagen). Purified genomic DNA was extracted (DNeasy; Qiagen) from the different sequenced strains used for the design of the microarray, labeled with Cy5-dCTP using the Klenow fragment of DNA polymerase I (BioPrime), and used for the normalization process (31). Cy5-labeled DNA (500 ng) and a Cy3-labeled cDNA mixture were diluted in 50 μ l of Agilent hybridization buffer and hybridized at a temperature of 60°C for 17 h in a hybridization oven (Robbins Scientific). Slides were washed, dried under nitrogen flow, and scanned (Agilent) using 100% photon multiplier tube power for both wavelengths.

Microarray analysis. Fluorescence intensities were extracted using Feature Extraction software (version 9; Agilent). Local background-subtracted signals were corrected for unequal dye incorporation or unequal loading of the labeled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS (locally weighted linear regression) method. Data consisting of three independent biological experiments were expressed as log₁₀ ratios and analyzed using GeneSpring, version 8.0 (Silicon Genetics). A filter was applied to select oligonucleotides mapping ORFs in the Newman genome, yielding approximately 95% coverage. Statistical significance of differentially expressed genes was calculated by analysis of variance (32) using GeneSpring, including the Benjamini-Hochberg false discovery rate correction of 5% (*P* value cutoff, 0.05) and an arbitrary cutoff of 2-fold for expression ratios.

FITC-poly-L-lysine binding assay. To determine the relative surface charge, overnight cultures were washed twice with HEPES buffer (20 mM, pH 7.25) and resuspended to an OD₅₇₈ of 0.1. fluorescein isothiocyanate (FITC)-poly-L-lysine (FPLL) was added to a final concentration of 5 μ g/ml, and the percentage of bound FPLL was determined as described previously (33, 34).

Microarray data accession numbers. The complete microarray data set has been deposited in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE77069 and GPL10537 (for platform design).

RESULTS

DAP tolerance of HG003pitA6 is independent from polyP. Previously, we selected a mutant of *S. aureus* HG003 which was extremely tolerant against DAP. The corresponding strain HG003pitA6 further showed an increased level of intracellular inorganic phosphate (P_i) and inorganic polyphosphate (polyP) (Fig. 1) (7). We heterologously expressed the exopolyphosphatase gene (*ppx*) from *Staphylococcus warneri* using the inducible plasmid pRAB11 to artificially hydrolyze the polyP level in both the

HG003 wild type and mutant HG003pitA6 (Fig. 1A). Overexpression of *ppx* (Fig. 1, +ppx) in HG003pitA6 decreased polyP to a basal wild-type level (Fig. 1A). The P_i level and DAP tolerance, however, remained high in HG003pitA6 irrespective of *ppx* expression (Fig. 1B and C). Heterologous expression of *ppx* in HG003 wild type did not influence the polyP or intracellular P_i level or DAP tolerance (Fig. 1A to C).

Transcriptome analysis disclosed predominant downregulation of genes from several functional categories. We performed transcriptome analysis using the microarray technique to better understand molecular processes affecting the increase of DAP tolerance in HG003pitA6.

We showed previously that DAP tolerance and increased P_i level were restricted to the stationary growth phase (7). We therefore compared relative gene expression of HG003pitA6 with that of the wild type after 6 h (early stationary phase) and 8 h (advanced stationary phase) of growth. The complete results are summarized in Table S1 in the supplemental material. A total of 234 genes were found to be at least 2.0-fold differentially expressed in HG003pitA6 compared to expression in the HG003 wild-type strain after 6 h and/or 8 h of growth.

The majority of 170 genes were downregulated, in contrast to 65 genes that were upregulated (Fig. 2A). In general, differences in gene expression between the two strains were mostly moderate, in a range of 2- to 5-fold (see Table S1 in the supplemental material); however, downregulation was common in most functional categories (Fig. 2B). Predominantly genes involved in translation (100%), transcription (90%), and amino acid (65%) and nucleotide (67%) metabolism, as well as posttranslational modification (82%), were repressed in HG003pitA6 (Fig. 2B; see also Table S1). The percentage reflects the portion of downregulated genes in the corresponding functional category. Furthermore, a high number of repressed genes are involved in carbohydrate metabolism (77%), energy production (100%), or lipid transport (100%).

Interestingly, many genes associated with the synthesis of the cell envelope, which was shown before to be affected in DAP-nonsusceptible strains (35–38), are dysregulated, with no clear tendency to up- or downregulation (Fig. 2B). Among them are the *murC*, *murD*, and *murG* genes that are 2- to 4-fold upregulated in HG003pitA6 (see Table S1 in the supplemental material). These genes are involved in the synthesis of peptidoglycan (39). However, *murA*, which represents the first step in peptidoglycan syn-

FIG 2 Transcriptome analysis of genes differentially expressed in HG003pitA6 compared to expression in the HG003 wild-type strain. (A) Venn diagrams showing numbers of genes that are at least 2.0-fold up- or downregulated after 6 h and/or 8 h of growth in TSB. (B) COG (clusters of orthologous groups of proteins) functional categories with the number of genes being at least 2.0-fold up- or downregulated.

thesis (39), was found to be 2-fold downregulated in our approach (see Table S1).

The *dlt* operon is upregulated in the HG003pitA6 mutant. The *mprF* gene and the *dlt* operon are well-known hot spots with increased activity in DAP-nonsusceptible strains (35, 36, 40). Table 1 shows the relative expression profiles of *mprF* and the *dlt* operon during log and stationary phases. Neither *mprF* nor the *dlt* operon was upregulated during exponential growth according to quantitative real-time PCR (qRT-PCR) data. In contrast, the *dlt* operon was moderately downregulated. Both qRT-PCR and microarray analyses showed an upregulation of the *dlt* operon in stationary phase, in contrast to *mprF*, which was mildly downregulated.

TABLE 1 Expression profiles of *mprF* and *dltABCD* in HG003pitA6 compared to those in HG003

Method of analysis ^a	Relative expression (HG003pitA6 vs HG003)				
	<i>dltA</i>	<i>dltB</i>	<i>dltC</i>	<i>dltD</i>	<i>mprF</i>
qRT-PCR (4 h)		−1.40	−1.27	−1.41	−1.05
qRT-PCR (8 h)	1.20	3.10	2.70	2.70	−1.84
Microarray (8 h)		2.40			−1.52

^a Times in parentheses refer to growth periods.

Deletion of *dltA* removes DAP tolerance in HG003pitA6. As shown in Fig. 3A, markerless deletion of *mprF* from the HG003 wild-type strain and the HG003pitA6 mutant had only minor effects on DAP susceptibility of both strains in the time-kill curve. The additional deletion of *dltA*, however, increased the susceptibility of both wild-type HG003 and mutant HG003pitA6. This is reflected by the complete sterilization of the HG003pitA6 culture after 8 to 24 h of DAP treatment (Fig. 3B) and by an even faster eradication of the wild-type culture within 5 h of exposure to the drug. The DAP MIC was decreased in all deletion mutants (0.125 μg/ml) compared to the MICs of the HG003 wild-type strain and HG003pitA6 with intact *mprF* and *dltA* alleles (1 μg/ml).

Surface charge is unaffected in HG003pitA6. An increase in the surface charge was shown earlier to be a possible phenotype for the upregulation of the *dlt* operon (33); however, no differences in the relative surface charges of the HG003 wild-type and HG003pitA6 strains could be detected when the FITC-poly-L-lysine (FPLL) approach was used (Fig. 4).

Upregulation of the *dlt* operon is independent from GraXRS. The GraXRS-VraFG five-component system is known to increase the expression of both *mprF* and the *dlt* operon (41). We found, however, that neither the markerless deletion of *graR* nor that of

FIG 3 Time-dependent killing of $\Delta mprF$ and $\Delta mprF \Delta dltA$ strains by DAP. CFU counts were determined for stationary-growth-phase cultures of the HG003 $\Delta mprF$ and HG003 $\Delta mprF \Delta dltA$ strains (A) and for the HG003pitA6 $\Delta mprF$ and HG003pitA6 $\Delta mprF \Delta dltA$ strains (B) upon challenge with 100 $\mu\text{g/ml}$ DAP. Values represent the arithmetic means from three independent experiments. Error bars represent standard deviations.

graS in either wild-type HG003 or HG003pitA6 was able to alter DAP tolerance (Fig. 5).

DISCUSSION

A fundamental understanding of the mechanisms by which bacteria are able to escape bactericidal activity of antibiotics is essential for the development of new and possibly better antimicrobial therapies. Antibiotic tolerance is one aspect explaining the recalcitrance of relapsing and chronic infection by genetically susceptible bacteria (6). In an earlier study, we developed a strain showing decreased levels of DAP susceptibility in stationary phase (7). This phenotype differed from classical resistance since there was no increase in the MIC. The only adaptation required to decrease DAP susceptibility was a nonsynonymous mutation within the inorganic phosphate transporter gene *pitA*. Additionally, we showed that this kind of DAP tolerance in the mutant *S. aureus* HG003pitA6 was restricted to the stationary growth phase and correlated with an increased level of intracellular P_i and polyP. Previous studies by Gerdes and Maisonneuve demonstrated that the accumulation of polyP increased antibiotic tolerance by persister cells in *E. coli* (42). As polyP levels were also increased in the mutant HG003pitA6 (Fig. 1A), we wanted to elucidate a potential

role of increased amounts of polyP in the DAP tolerance phenotype. PolyP is synthesized by polyphosphate kinases (PPKs) and hydrolyzed by exopolyphosphatases (PPXs) (43). *S. aureus* HG003 contains a putative exopolyphosphatase gene (SAOUHSC_01812) and a putative inorganic polyphosphate/ATP-NAD kinase gene (SAOUHSC_00943), but they are poorly characterized and different from the well-known *ppk-ppx* operon of *E. coli* (44). For heterologous expression, we decided to use the *S. warneri ppx* gene (A284_02120), which is a homologue of *ppx* from *E. coli* but closely related to the *S. aureus* gene (45). According to expectations (43), inducible overexpression of *ppx* reduced the amount of polyP in HG003pitA6/pRAB11-*ppx* to a basal wild-type level (Fig. 1A). Concentrations of intracellular P_i and the increase in DAP tolerance, however, remained unaffected (Fig. 1B and C), a fact clearly indicating that DAP tolerance mediated by the *pitA6* allele is independent from polyP.

Transcriptome analysis using a microarray technique was applied to unravel the cellular basis of the HG003pitA6 phenotype (Fig. 2A and B; see also Table S1 in the supplemental material). As the increase in DAP tolerance is restricted to the stationary phase (7), we checked the expression of HG003pitA6 relative to that of the HG003 wild-type strain after 6 h (early stationary phase) and 8 h (advanced stationary phase) of growth.

Among a total of 234 genes that were at least 2.0-fold differentially expressed after 6 h and/or 8 h (Fig. 2A; see also Table S1 in the supplemental material), 65 were significantly upregulated, and 170 were repressed, in most cases with moderate changes (2- to

FIG 4 Relative surface charge of HG003 and HG003pitA6. The percentage of FPLL bound to HG003 and HG003pitA6 grown for 16 h in TSB and incubation of cells with FPLL for 10 min at room temperature are depicted. All values are averages from at least three independent experiments. Error bars indicate the standard deviations, and significance was determined using Student's *t* test ($P < 0.05$). n.s., not significant.

FIG 5 Time-dependent killing of *S. aureus* strains with markerless deletion of *graR* or *graS* by DAP. Stationary-growth-phase cultures of the HG003 $\Delta graS$, HG003 $\Delta graR$, HG003pitA6 $\Delta graS$, and HG003pitA6 $\Delta graR$ strains were challenged with 100-fold the MIC of DAP, and CFU counts were recorded. Values are the averages from three independent experiments. Error bars represent standard deviations.

5-fold). A broad range of differentially expressed genes from central metabolic function was observed, with a predominant downregulation in most functional categories (Fig. 2B). A prevalent downshift of expression profiles for genes being involved in translation, transcription, amino acid metabolism, nucleotide metabolism, or posttranslational modification (Fig. 2B; see also Table S1) indicates that protein biosynthesis is low in HG003pitA6. Likewise, downregulation of genes implicated in carbohydrate or lipid metabolism and especially those responsible for energy generation suggests that a metabolic downshift prevails in the mutant HG003pitA6 at stationary growth phase. This is endorsed by the fact that HG003pitA6 is impaired in growth in comparison to HG003 wild type as soon as it reaches stationary phase (7). An increase in the cell wall thickness is assumed to be one reason for decreased susceptibility to DAP (36–38, 46), and indeed we found several genes involved in cell wall, membrane, and envelope maintenance or synthesis to be significantly upregulated. Among them were *murC*, *murD*, and *murG* (Fig. 2B; see also Table S1), which are responsible for the synthesis of peptidoglycan, a major component of the bacterial cell wall (39). However, MurA executes the first step in peptidoglycan synthesis (39) and is 2-fold downregulated in our data set, which contradicts an increased cell wall thickness phenotype. Accordingly, differences in cell wall thickness of the HG003 wild-type and HG003pitA6 strains were not detected by transmission electron microscopy (see Fig. S1 in the supplemental material). Furthermore, the content of wall teichoic acids (WTA) does not differ significantly in these strains (see Fig. S2), indicating that DAP tolerance is independent from alteration of cell wall thickness.

Beside an increase in thickness, alteration in composition of the bacterial cell wall as well as the membrane is a common adaptation in DAP-R mutants (1, 5). Genes involved in these processes and well-known hot spots for modification in DAP-nonsusceptible strains are *mprF* and the *dltXABCD* operon (1, 35, 36).

The multi-peptide resistance factor gene *mprF* transfers lysyl from an L-lysyl-tRNA to membrane-bound phosphatidylglycerol, forming lysyl-phosphatidylglycerol (LPG) (47). The *dlt* operon is known to link positively charged D-alanine to both lipid and wall teichoic acids (40). Enhanced expression or activity of either or both is known to cause DAP nonsusceptibility (35, 36, 40, 48).

We found a time-dependent upregulation of the *dlt* operon but not the *mprF* gene in HG003pitA6 compared to the level in the wild type that could be linked to an increased level of intracellular P_i and DAP tolerance (Table 1 and Fig. 1B and C) (7). In accordance with previous studies (49), we found that the markerless deletion of *mprF* in both wild-type HG003 and mutant HG003pitA6 increased the general susceptibility for these strains, with a MIC of DAP of 0.125 $\mu\text{g/ml}$ in contrast to a MIC of 1 $\mu\text{g/ml}$ for HG003 and HG003pitA6 with an intact *mprF* gene. However, no dramatic increase in DAP susceptibility was detectable in time-kill studies in any of the strains (Fig. 3A). Interestingly, the subsequent deletion of *dltA* did not further decrease the MIC of DAP but completely reversed tolerance for both the HG003 $\Delta mprF \Delta dltA$ and HG003pitA6 $\Delta mprF \Delta dltA$ strains, with a complete sterilization within 24 h of DAP treatment (Fig. 3A and B).

According to our results, the *dlt* operon seems to be necessary for both the basal tolerance of the wild-type strain and the increased DAP tolerance of HG003pitA6. The growth phase-dependent upregulation of the *dlt* operon in the mutant HG003pitA6 is therefore the most plausible explanation for the increase in DAP

FIG 6 Putative model for DAP tolerance due to *pitA6*. A mutation in *pitA6* generating PitA6 increases intracellular P_i levels and polyP levels. Elevated concentrations of intracellular P_i directly or indirectly promote the upregulation of the *dlt* operon, which is responsible for DAP tolerance. PitR is a PhoU-like protein that interacts with itself to most likely form dimers. The precise function of PitR is not known; however, it seems to act as a helper protein for phosphate transport and is mandatory for increased intracellular amounts of P_i and DAP tolerance in HG003pitA6.

tolerance in the HG003pitA6 strain. Nevertheless, it should be mentioned that many more genes were dysregulated in HG003pitA6 according to our microarray results, and their possible involvement in DAP tolerance was not further investigated and thus cannot be excluded entirely.

It further remains an open question as to how the upregulation of *dlt* is mediated. The five-component system GraXRS-VraFG is known to increase the expression of both *mprF* and the *dlt* operon (41, 50). We found, however, that the deletion of *graR* or *graS*, which will disrupt the function of the system (50), had no effect on DAP tolerance in the HG003 wild-type strain or HG003pitA6 (Fig. 5), an observation indicating that the regulation is mediated by a different mechanism.

The prevailing hypothesis of how *dlt* decreases DAP susceptibility is that there is an increase in the relative surface charge due to an increased D-alanylation of teichoic acids, resulting in repulsion of cationic peptides like DAP (48). Previous studies suggested that increased *dlt* expression would possibly, but not necessarily, increase the surface charge (36). Our experiments using HG003pitA6 support these findings as higher expression in HG003pitA6 of *dlt* did not produce an increase in the net charge (Fig. 4), clearly indicating that *dlt* contributes to a DAP-nonsusceptible phenotype by a means other than an increase in the cell surface charge.

According to our results, we propose a graphical summary of the DAP tolerance associated with *pitA6* in Fig. 6. We conclude that the growth phase-dependent increase of intracellular P_i and DAP tolerance are linked to the upregulation of the *dlt* operon. As this was shown before to be causative for DAP nonsusceptibility (35, 36, 48), it

is reasonable to argue that *dlt* overexpression is causative for DAP tolerance of HG003pitA6, which once more emphasizes the potential role of this operon in DAP nonsusceptibility.

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