Antimicrobial Tolerance of *Pseudomonas aeruginosa* Biofilms Is Activated during an Early Developmental Stage and Requires the Two-Component Hybrid SagS

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A hallmark characteristic of biofilms is their extraordinary tolerance to antimicrobial agents. While multiple factors are thought to contribute to the high level of antimicrobial tolerance of biofilms, little is known about the timing of induction of biofilm tolerance. Here, we asked when over the course of their development do biofilms gain their tolerance to antimicrobial agents? We demonstrate that in *Pseudomonas aeruginosa*, biofilm tolerance is linked to biofilm development, with transition to the irreversible attachment stage regulated by the two-component hybrid SagS, marking the timing when biofilms switch to the high-level tolerance phenotype. Inactivation of *sagS* rendered biofilms but not planktonic cells more susceptible to tobramycin, norfloxacin, and hydrogen peroxide. Moreover, inactivation of *sagS* also eliminated the recalcitrance of biofilms to killing by bactericidal antimicrobial agents, a phenotype comparable to that observed upon inactivation of *brlR*, which encodes a MerR-like transcriptional regulator required for biofilm tolerance. Multicopy expression of *brlR* in a Δ *sagS* mutant restored biofilm resistance and recalcitrance to killing by bactericidal antibiotics to wild-type levels. In contrast, expression of *sagS* did not restore the susceptibility phenotype of Δ *brlR* mutant biofilms to wild-type levels, indicating that BrlR functions downstream of SagS. Inactivation of *sagS* correlated with reduced BrlR levels in biofilms, with the produced BrlR being impaired in binding to the previously described BrlR-activated promoters of the two multidrug efflux pump operons *mexAB-oprM* and *mexEF-oprN*. Our findings demonstrate that biofilm tolerance is linked to early biofilm development and SagS, with SagS contributing indirectly to BrlR activation.

Biofilms are complex communities of surface-associated microorganisms embedded within a self-produced extracellular matrix (1, 2). Biofilm communities exhibit increased tolerance to antimicrobial agents, often leading to persistent and chronic infections that are difficult to eradicate (3, 4). Bacteria living in biofilms can be up to 1,000 times more tolerant to antibacterial compounds than their planktonic counterparts (5). This enhanced tolerance is thought to be multifactorial, requiring a combination of different mechanisms, and is distinct from mechanisms more commonly associated with planktonic cells, such as acquisition of resistance through random mutation or uptake of plasmid-borne resistance markers (6–11). Mechanisms contributing to high antimicrobial tolerance in biofilms are thought to include reduced metabolic and growth rates (12–14), the presence of dormant persister cells that are not killed by exposure to antibiotics (15), restricted diffusion of certain types of antimicrobial agents (11, 16, 17), starvation-induced growth arrest (18), and the maturity or developmental stage of the biofilm (13, 19). The notion of multifactorial mechanisms contributing to biofilm tolerance is supported by several observations. For instance, bacteria dispersed from biofilms into liquid medium have been shown to only partially regain susceptibility compared to planktonic cells, indicating that reduced metabolic/divisional rates alone do not fully account for the biofilm resistance phenotype (14, 20). Restricted penetration has only been demonstrated for a limited number of antibiotics, including ampicillin and aminoglycosides, but not for ciprofloxacin (8, 10, 17). Furthermore, the extent of retardation of antibiotic diffusion due to sorption or inactivation does not appear to be sufficient to account for reduced biofilm susceptibility (8, 17, 21). This is further supported by the finding that antimicrobials are able to inhibit biofilm growth (no reduction in the

MIC compared to that in planktonic cells), indicating that they are able to diffuse (in part with delay) through the biofilm and act normally against their targets (8).

More recent evidence suggests a contribution of the biofilm physiology to tolerance, as biofilm bacteria are physiologically distinct from planktonic bacteria, expressing specific protective factors, such as multidrug (MDR) efflux pumps and stress response regulons (6, 7, 9, 10, 13, 22–25). Moreover, bacteria within microbial communities were found to employ a specific regulatory mechanism to resist the action of antimicrobial agents. Liao et al. (26) demonstrated that in the human pathogen *Pseudomonas aeruginosa*, biofilm tolerance is conferred, in part, by BrlR, a member of the MerR family of multidrug efflux pump activators. BrlR, which is produced in a biofilm-specific manner, was found to contribute to the high-level drug tolerance of *P. aeruginosa* biofilms by affecting the MIC and recalcitrance to killing by bactericidal antimicrobial agents, including tobramycin, tetracycline, chloramphenicol, trimethoprim, and norfloxacin (26). This raises the question of the timing of induction of biofilm tolerance mechanisms and whether the developmental stage and physiology of the biofilm bacteria contribute to the high levels of resistance observed in biofilms. Anwar et al. (13) reported that 2-day-old *P. aeruginosa* biofilm cells formed on pieces of silicone tubing placed

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
<i>Escherichia coli</i>		
DH5α	F [−] ϕ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r _k [−] m _k ⁺) phoA supE44 thi-1 gyrA96 relA1 tonA	Invitrogen Corp.
BL21	F [−] ompT hsdS _B (r _B [−] m _B [−]) gal dcm rne131 (DE3)	Invitrogen Corp.
<i>Pseudomonas aeruginosa</i>		
PAO1	Wild-type strain PAO1	B. H. Holloway
ΔsagS mutant	PAO1 ΔsagS (PA2824)	29
ΔbfiS mutant	PAO1 PA4197::ISphoA Tet ^r	35
ΔbfmR mutant	PAO1 PA4101::ISlacZ Tet ^r	35
ΔbrlR mutant	PAO1 ΔbrlR (PA4878)	26
Plasmids		
pCR2.1-TOPO	TA cloning vector; Km ^r Ap ^r	Invitrogen Corp.
pET101D	Vector for directional cloning and V5/His ₆ fusion protein expression; Amp ^r	Invitrogen Corp.
pRK2013	Helper plasmid for triparental mating; mob tra Km ^r	55
pMJT1	araC-P _{BAD} cassette of pJN105 cloned into pUCP18; Amp ^r (Carb ^r)	31
pJN105	Arabinose-inducible gene expression vector; pBRR-1 multiple cloning site; araC-P _{BAD} Gm ^r	30
pMini-CTX-lacZ	attB site-specific integration vector; Tet ^r	56
pJN-sagS	sagS cloned into pJN105	29
pJN-brlR	brlR cloned into pJN105	26
pMJT-brlR-V6/His ₆	C-terminally V5/His ₆ -tagged brlR cloned into pMJT	26
pmexAB-oprM	mexAB-oprM operon cloned into pMJT1 at NheI-SacI; Amp ^r Carb ^r	This work
pmexEF-oprN	mexEF-oprN operon cloned into pJN105 at NheI-SacI; Gm ^r	This work
CTX-P _{brlR} -brlR-V5/His ₆	C-terminally V5/His ₆ -tagged brlR under the control of native promoter cloned into pMini-CTX	This work

in a chemostat-controlled biofilm reactor can be effectively eradicated with a combination of piperacillin and tobramycin over the course of 7 days of antibiotic therapy, which was evident by the lack of regrowth upon termination of treatment. In contrast, biofilms grown for 7 days on silicone tubing were found to be less susceptible, as the same antibiotic treatment regimen did not achieve eradication of 7-day-old biofilms (13). Instead, the viability was found to be reduced by only 20% (13). While the authors did not define the biofilm developmental stages of the young and old biofilms, the findings nevertheless indicated that biofilm drug tolerance may coincide with the developmental stage or maturity of the biofilm (19). The notion of regulation of biofilm tolerance being age specific is further reinforced by the finding that biofilm formation occurs in a regulated and stage-specific manner (22, 23, 27, 28). At least four two-component regulatory systems, namely, SagS, BfiRS, BfmRS, and MifRS, are required to coordinate the progression of *P. aeruginosa* biofilm development in a stage-specific manner. Together, these systems form a coordinated signaling network that regulates three committed steps of the *P. aeruginosa* biofilm life cycle, in particular the transition to three later biofilm developmental stages following initial attachment, namely, the transition to initiation of biofilm formation (BfiRS), biofilm maturation (BfmRS), and microcolony formation (MifRS). SagS coordinates the transition to the irreversible attachment stage via direct interaction with and modulation of the phosphorylation state of BfiS (29).

Here, we asked when over the course of development do *P. aeruginosa* biofilms gain their extraordinary tolerance to antimicrobial agents? We demonstrate that biofilm tolerance is linked to biofilm development, with transition to the irreversible attachment stage regulated by the two-component hybrid SagS, marking

the timing when biofilms switch to the high-level tolerance phenotype. Subsequent growth accompanied by increased biofilm biomass accumulation did not result in further increased tolerance. Moreover, we demonstrate that SagS is linked to the activation of the MerR-like transcriptional regulator BrlR, which was previously demonstrated to be required for biofilm tolerance.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strain PAO1 was used as the parental strain. All planktonic cultures were grown in Lennox broth (LB) (BD Biosciences) in flasks at 220 rpm. Biofilms were grown as described below at 22°C in 20-fold-diluted LB. Antibiotics for plasmid maintenance were used at the following concentrations: 50 to 75 μg/ml gentamicin, 60 μg/ml tetracycline, and 200 to 250 μg/ml carbenicillin for *P. aeruginosa* and 20 μg/ml gentamicin, 20 μg/ml tetracycline, and 50 μg/ml ampicillin for *Escherichia coli*.

Strain construction. Complementation and overexpression of sagS or brlR were accomplished by placing the respective genes under the control of an arabinose-inducible promoter in the pJN105 (30) or pMJT-1 (31) vectors and introducing the vectors into *P. aeruginosa* via conjugation or electroporation (32, 33). The primers used for strain construction are listed in Table 2.

Planktonic antibiotic susceptibility testing. To determine the role of SagS in antimicrobial susceptibility, *P. aeruginosa* strains grown planktonically in LB medium at 37°C to the exponential phase were treated with tobramycin (50 μg/ml), norfloxacin (150 μg/ml), or 0.3% hydrogen peroxide for 1 h and subsequently homogenized, serially diluted, and spread plated onto LB agar. Hydrogen peroxide was neutralized by the addition of 100 mM sodium thiosulfate prior to homogenization. Viability was determined via CFU counts. Susceptibility is expressed as log reduction in CFU counts. Under the conditions tested, a total of 5 × 10⁷ ± 2 × 10⁶ CFU/ml were detected on average for exponential-phase planktonic cells.

TABLE 2 Primers used in this study

Oligonucleotide	Sequence
RT-PCR	
mreB-for	CTGTGATCGACCTGGG
mreB-rev	CAGCCATCGGCTCTTCG
brlR_RT-for	GCAACGACACCAGCACAC
brlR_RT-rev	GAAGCGTTCCAGAGCTG
mexA-for	CAGCAGCTCTACCAGATCGAC
mexA-rev	GTATTGGCTACCGTCTCCAG
mexE-for	GTCATCGAACAACCGCTG
mexE-rev	GTCGAAGTAGGCGTAGACC
ndvB-for	GTGATAGTCGGCAACGAGG
ndvB-rev	CGGTGAAGTTGAACTTGGCC
Cloning	
PbrlR-His6V5-pET-for	CACCTCCCTTTGCGATGGGTTTAC
brlR-pET-rev	ATAGATGGGGATATACAGGTCGAC
PbrlR-brlR-His6V5-CTX-XbaI-for	GCGCGCGCTCTAGACTCCCTTTCG
	GATGGGTTTAC
PbrlR-brlR-His6V5-CTX-XmaI-rev	GCGCGCGCCCCGGTCAATGGTG
	ATGGTGATG
mexAB-oprM-NheI-for	GCGCGCGCTAGCGTAAGTATTTTG
	CCTGCCTTCTTC
mexAB-oprM-SacI-rev	GCGCGCGAGCTCGATCAAGCCTG
	GGGATCTTCC
mexEF-oprN-for	GCGCGCGCTAGCGAGTCAAGCAT
	GGAACAGTCATC
mexEF-oprN-rev	GCGCGCGAGCTCCTGGAGTGGC
	CGATTTCATC
Streptavidin-coupled bead assays	
PmexAF ^a	GTAGTTTCATTGGTTTGGCC
PmexAR	CATAGCGTTGTCTCATG
PmexEF ^a	GGATCAGCATGTTTCATCG
PmexER	CTGTTCCATGCTTGACTC

^a Primers were biotinylated; unbiotinylated primers were used for competition.

Biofilm formation. For biofilm antibiotic susceptibility testing, biofilms were grown in a continuous flow tube reactor system (1-m-long size 13 silicone tubing; Masterflex, Cole Parmer, Inc.) with an inner surface area of 25 cm² at a flow rate of 0.1 ml/min and in flow cell reactors (BioSurface Technologies), which also allowed for the analysis of biofilm architecture, as previously described (23, 34, 35). Biofilms were grown at 22°C in 20-fold-diluted LB medium. The same growth conditions were used to cultivate biofilms to obtain protein and RNA. For plasmid maintenance, antibiotics were used in tube reactors at the following concentrations: 2 µg/ml gentamicin and 10 µg/ml carbenicillin. Confocal laser scanning microscopy (CLSM) images of flow-cell-grown biofilms were acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), and quantitative analysis of the images was performed using COMSTAT (36).

Biofilm antibiotic susceptibility assays. To determine when over the course of biofilm formation biofilms gain their tolerance, biofilms were grown for 1, 2, 3, 4, and 6 days under flowing conditions (0.1 ml/min) and subsequently treated for 1 h under flowing conditions with the following antimicrobial agents: tobramycin (150 µg/ml), norfloxacin (450 µg/ml), and hydrogen peroxide (0.3%). For all other susceptibility assays, biofilms were grown for 3 days. Following exposure of biofilms to the respective antimicrobial agents, biofilms were harvested by squeezing the tubing, followed by the extrusion of the cell paste as previously described (22). To ensure complete disaggregation of cell aggregates, the resulting suspension was first homogenized using a Tissue Tearor and then serially diluted and spread plated onto LB agar. Hydrogen peroxide was neutralized by the addition of 100 mM sodium thiosulfate. Viability was determined via

CFU counts. Susceptibility is expressed as log reduction. Under the conditions tested, a total of $5 \times 10^8 \pm 3 \times 10^7$ CFU were detected on average per biofilm tube reactor following 1 day of biofilm growth, which increased to $1.5 \times 10^9 \pm 5 \times 10^8$ CFU, $3 \times 10^9 \pm 1.5 \times 10^8$ CFU, $4 \times 10^9 \pm 4 \times 10^8$ CFU, and $1.5 \times 10^{10} \pm 1.5 \times 10^9$ CFU following 2, 3, 4, and 6 days of biofilm growth, respectively.

The biofilm minimum bactericidal concentration (MBC) has been defined as the concentration at which no further increase in log reduction is observed (37–39). To determine whether SagS affects the biofilm MBC and resistance to killing, PAO1 and Δ sagS strain biofilms were grown for 3 days, after which time the medium was switched to the same medium containing increasing concentrations of tobramycin or norfloxacin ranging from 0.5 to 400 µg/ml. Following 24 h of exposure to the antibiotic under continuous flow at 0.1 ml/min, biofilms were harvested, and the surviving bacteria were enumerated by viable counts.

Immunoblot analysis. For immunoblot analysis, total cell extracts from biofilms were obtained by sonication as previously described (23), followed by centrifugation for 2 min at 21,200 × g to pellet unbroken cells. The protein concentrations were determined using a modified Lowry assay and bovine serum albumin as a standard (40). Total cell extracts were resolved on an 11% polyacrylamide gel and subsequently transferred onto polyvinylidene difluoride (PVDF) membrane using a TurboTransblot apparatus (Bio-Rad). Western blots were probed with anti-V5 antibodies (Life Technologies) and developed using the Immobilon-Star WesternC chemiluminescence kit (Bio-Rad). Following transfer, SDS-PAGE gels were Coomassie stained to ensure equal loading. Analysis of phosphorylated BrlR-V5/His₆ levels in protein extracts of wild-type and Δ sagS biofilms was accomplished using phosphoprotein purification via metal oxide affinity chromatography (MOAC) as previously described (35), followed by the detection of BrlR-V5/His₆ by immunoblotting with anti-V5 antibodies.

qRT-PCR. Isolation of mRNA and cDNA synthesis was carried out as previously described (28, 35, 41, 42). Quantitative reverse transcription (qRT)-PCR was performed using the Bio-Rad CFX Connect real-time PCR detection system and SsoAdvanced SYBR green supermix (Bio-Rad) with the oligonucleotides listed in Table 2. *mreB* was used as a control. Relative transcript quantitation was accomplished using the CFX Manager software (Bio-Rad), first by normalization of transcript abundance (based on the threshold cycle [*C_T*] value) to *mreB*, followed by determination of transcript abundance ratios. Melting curve analyses were employed to verify specific single product amplification.

Streptavidin-coupled magnetic bead DNA binding assay. BrlR binding to the promoter regions of the *mexAB-oprM* and *mexEF-oprN* operons was determined using the streptavidin-coupled magnetic bead DNA binding assay as previously described (43). Briefly, a total of 1 pmol of target DNA (biotinylated *P_{mexA}* or *P_{mexE}*) (Table 2) was incubated for 30 min at room temperature with 15 µg of total cell extract containing the equivalent of 5 pmol of V5/His₆-tagged BrlR (as indicated by immunoblot analysis using purified V5/His₆-tagged BrlR) in a mixture containing 25 mM Tris-Cl (pH 8), 5 mM MgCl₂, 0.5 mM dithiothreitol, 1 mM EDTA, and 50 ng/µl poly(dI-dC) as the nonspecific competitor DNA. For specific competition, nonbiotinylated target DNA (up to 20-fold excess of target DNA) was used. Streptavidin-coupled magnetic beads (100 µg) (Thermo Scientific) were used to capture biotinylated DNA. Following four washes, the proteins copurified with the biotinylated DNA were separated by 11% SDS-PAGE and assessed by immunoblot analysis for the presence of BrlR using anti-V5 antibodies (Invitrogen). An aliquot prior to addition of streptavidin-coupled magnetic beads was used to determine total BrlR present in each DNA binding assay (loading control).

Statistical analysis. Student's *t* test was performed for pairwise comparisons of groups, and multivariate analyses were performed using a 1-way analysis of variance (ANOVA) followed by an *a posteriori* test using Sigma Stat software.

FIG 1 Tolerance of *P. aeruginosa* biofilms is not related to biofilm age, biofilm biomass accumulation, or biofilm thickness. Shown are the results of susceptibility determination of *P. aeruginosa* PAO1 cells grown planktonically to the exponential phase and as biofilms under flowing conditions for 1, 2, 3, 4, or 6 days. Under the conditions tested, a total of 5×10^7 CFU/ml were detected for exponential-phase planktonic cells, while on average, $5 \times 10^8 \pm 3 \times 10^7$ CFU, $1.5 \times 10^9 \pm 5 \times 10^8$ CFU, $3 \times 10^9 \pm 1.5 \times 10^8$ CFU, $4 \times 10^9 \pm 4 \times 10^8$ CFU, and $1.5 \times 10^{10} \pm 1.5 \times 10^9$ CFU were detected for biofilms grown for 1, 2, 3, 4, and 6 days, respectively. (A) Biofilms were treated for 1 h with tobramycin (150 $\mu\text{g}/\text{ml}$), while planktonic cells were treated for 1 h with 50 $\mu\text{g}/\text{ml}$ tobramycin. (B) Biofilms were treated for 1 h with norfloxacin (450 $\mu\text{g}/\text{ml}$) or hydrogen peroxide (0.3%), while planktonic cells (0 days) were treated for 1 h with 150 $\mu\text{g}/\text{ml}$ norfloxacin or for 30 min with 0.3% hydrogen peroxide. Susceptibility is expressed as log reduction. *, significantly different from log reduction obtained under planktonic growth conditions ($P < 0.01$). (C and D) Biofilm biomass accumulation (C) and biofilm thickness (D) of *P. aeruginosa* biofilms grown for 1, 3, and 6 days in flow cells, as determined using confocal images and subsequent COMSTAT analysis. Experiments were carried out at least in triplicate. Error bars denote standard deviations.

RESULTS

Biofilm tolerance to antimicrobial agents is not related to biofilm age, biofilm biomass accumulation, or biofilm thickness.

To begin addressing the question of when over the course of development biofilms gain their profound tolerance to antimicrobial agents, biofilms were grown for 1, 2, 4, and 6 days under flowing conditions and subsequently treated with the aminoglycoside tobramycin. The timing was chosen to reflect biofilms grown to different developmental stages (irreversible attachment, transition from initial attachment to maturation stage, and early and late maturation stages of biofilm development, respectively) (23, 35). Compared to planktonic cells grown to exponential phase, biofilms regardless of age were significantly more tolerant to tobramycin (Fig. 1A). Similar results were obtained when biofilms were treated with norfloxacin or hydrogen peroxide (Fig. 1B). Moreover, biofilm tolerance was independent of biofilm biomass accumulation and biofilm thickness, as no difference in susceptibility was noted among the biofilms representing different developmental stages (Fig. 1C and D).

Biofilms inactivated in *sagS* are more susceptible than wild-type and $\Delta bfiS$ mutant biofilms. The findings suggested that biofilm tolerance is linked to the transition to surface-associated

growth or is induced soon after attachment. To further elucidate the timing of biofilm tolerance, we made use of a set of mutants previously described to arrest biofilm formation at various stages over the course of biofilm development. Inactivation of the two-component system (TCS) hybrid *sagS* and the TCS *bfiSR* has been demonstrated to impair initiation of biofilm formation by arresting biofilm development at the transition to the irreversible attachment stage, while inactivation of *bfiMR* was found to impair biofilm maturation (29, 35, 43, 44). Overall, *SagS*, *BfiRS*, and *BfiMR* form a coordinated signaling network that regulates two committed steps of the *P. aeruginosa* biofilm life cycle (35).

Compared to wild-type biofilms, no difference in susceptibility was noted for *bfiMR* and *bfiS* mutant biofilms following treatment with norfloxacin, tobramycin, and hydrogen peroxide (Fig. 2A). However, while exhibiting similar biomass accumulation and thickness as $\Delta bfiS$ and $\Delta bfiMR$ biofilms (Fig. 2B and C), $\Delta sagS$ mutant biofilms did not develop high-level biofilm-specific drug tolerance compared to the wild type. This was evidenced by the finding that treatment of 6-day-old biofilms with tobramycin (150 $\mu\text{g}/\text{ml}$) for 1 h resulted in a 3.5-fold log reduction for $\Delta sagS$ biofilm in contrast to a 1-log reduction in wild-type biofilms (Fig. 2A). Increased susceptibility of $\Delta sagS$ biofilms was not limited to

FIG 2 Mutant biofilm susceptibility to antimicrobial agents is not dependent on biomass accumulation. (A) Determination of susceptibility of *P. aeruginosa* PAO1 and mutant biofilms inactivated in *sagS*, *bfiS*, and *bfmR* to tobramycin (150 $\mu g/ml$), norfloxacin (450 $\mu g/ml$), and hydrogen peroxide (0.3%). Susceptibility is expressed as log reduction. *, significantly different from PAO1 ($P < 0.01$). (B and C) Biofilm biomass accumulation (B) and average thickness (C) of *P. aeruginosa* wild-type and mutant biofilms following 6 days of growth in flow cells, as determined using confocal images and subsequent COMSTAT analysis. *, significantly different from PAO1 ($P < 0.01$). (D) Susceptibility determination of *P. aeruginosa* PAO1 and mutants inactivated in *sagS*, *bfiS*, and *bfmR* grown planktonically to the exponential phase to tobramycin (50 $\mu g/ml$), norfloxacin (150 $\mu g/ml$), and hydrogen peroxide (0.3%) following 1 h of treatment. Susceptibility is expressed as log reduction. Experiments were carried out at least in triplicate. Error bars denote standard deviations.

tobramycin. Instead, inactivation of *sagS* also coincided with a significant increase in log killing upon treatment with norfloxacin and hydrogen peroxide (Fig. 2A). With the exception of $\Delta bfmR$ mutants being less susceptible to tobramycin compared to the wild type, inactivation of *sagS*, *bfiS*, or *bfmR* had no effect on the susceptibility of planktonic cells (Fig. 2D).

SagS plays a role in drug tolerance of *P. aeruginosa* biofilms.

To further elucidate the role of SagS in biofilm drug tolerance, the susceptibility of 3-day-old rather than 6-day-old biofilms was determined. Treatment with tobramycin or norfloxacin resulted in 3.2- and 3.3-log reductions, respectively, in the viability of the $\Delta sagS$ mutant biofilms compared to a 1-log reduction in the viability of wild-type biofilms (Fig. 3A and B). The reduction in viability was comparable to that observed for 6-day-old biofilms (Fig. 2A), indicating that the contribution of SagS to biofilm tolerance was independent of biofilm age. Expression of *sagS* in 3-day-old $\Delta sagS$ mutant biofilms restored drug tolerance to tobramycin and norfloxacin to wild-type levels (Fig. 3A and B). No difference in susceptibility was noted for the vector control (Fig. 3). Interestingly, while inactivation of *sagS* rendered 3-day-old biofilms more susceptible to tobramycin and norfloxacin, overexpression of *sagS* in *P. aeruginosa* wild-type biofilms did not render biofilm cells more resistant (Fig. 3A and B). Moreover, the contribution of

SagS to drug tolerance was biofilm specific, as neither inactivation nor overexpression of *sagS* had an effect on the susceptibility of exponential-phase planktonic cells (Fig. 3C and D).

Considering that biofilms formed by *sagS*, *bfiS*, and *bfmR* mutants did not significantly differ in biofilm biomass accumulation or biofilm thickness (Fig. 2B and C) and that both BfiS and SagS are required for *P. aeruginosa* biofilms to transition to the irreversible attachment stage, with SagS directly interacting with and activating BfiS (29), our findings strongly support the notion of biofilm tolerance being linked to biofilm development rather than biofilm thickness or biomass accumulation. Moreover, our findings indicate that biofilm tolerance is likely induced upon transition to the irreversible attachment stage.

SagS contributes to the recalcitrance of *P. aeruginosa* biofilms to killing by bactericidal antibiotics. While the hallmark characteristic of biofilm populations is their increased antimicrobial tolerance, recent reports suggest that biofilm drug tolerance describes an increased recalcitrance of biofilm cells to killing (45, 46). For instance, despite demonstrating increased susceptibility to antimicrobial agents, biofilms such as those formed by mutants inactivated in both MexAB-OprM and MexEF-OprN multidrug efflux pumps are still capable of surviving prolonged treatment at elevated bactericidal antibiotic concentrations (47). To determine

FIG 3 Inactivation of *sagS* renders *P. aeruginosa* biofilms but not planktonic cells more susceptible to antimicrobial agents. (A and B) *P. aeruginosa* wild-type and mutant biofilms inactivated in or overexpressing *sagS* were grown in tube reactors for 3 days and subsequently treated with (A) tobramycin (150 μ g/ml) or (B) norfloxacin (450 μ g/ml) for 1 h under flowing conditions. *P. aeruginosa* harboring an empty pJN105 vector was used as a control. *, significantly different from PAO1 ($P < 0.01$). (C and D) *P. aeruginosa* wild-type cells and cells of mutants inactivated in or overexpressing *sagS* were grown planktonically to the exponential phase and subsequently treated with (C) tobramycin (50 μ g/ml) or (D) norfloxacin (150 μ g/ml) for 1 h. Susceptibility is expressed as log reduction. Experiments were carried out at least in triplicate. Error bars denote standard deviations.

whether SagS affects resistance to killing of biofilms by bactericidal antibiotics, we made use of tube reactor-grown biofilms and the two antibiotics tobramycin and norfloxacin. *P. aeruginosa* wild-type and Δ sagS mutant cells were grown for 3 days, after which time the medium was switched to the same medium containing increasing concentrations of tobramycin ranging from 0.5 to 200 μ g/ml. Following 24 h of exposure to the antibiotic under continuous flow, biofilms were harvested, and the surviving bacteria were enumerated. The biofilm MBC has been defined as the concentration at which no further increase in log reduction is observed (37–39). For *P. aeruginosa* wild-type biofilms, no further increase in log reduction was observed at concentrations higher than 80 μ g/ml tobramycin (Fig. 4A and B). Higher concentrations resulted neither in increased log reduction nor in complete killing of *P. aeruginosa* wild-type biofilms (Fig. 4A and B). In contrast, complete killing of Δ sagS mutant biofilms was accomplished following treatment with tobramycin concentrations exceeding 100 μ g/ml (Fig. 4A and B). Similar results were obtained when biofilms were treated with increasing concentrations of norfloxacin (Fig. 4C and D). Total killing of Δ sagS biofilm cells was achieved at concentrations exceeding 200 μ g/ml norfloxacin, while wild-type PAO1 biofilms maintained a steady level of survivors through the highest concentrations tested (Fig. 4C and D). The findings indicated that *P. aeruginosa* wild-type biofilms were, overall, more resistant to killing by tobramycin and norfloxacin than Δ sagS mu-

tant biofilms, for which no viable cells were recovered at concentrations exceeding the biofilm MBCs.

Drug tolerance of Δ sagS mutant biofilms can be restored by BrlR. The role of SagS in biofilm drug tolerance was comparable to that of the transcriptional regulator BrlR, a member of the MerR family of multidrug efflux pump activators, which was previously demonstrated to contribute to the recalcitrance of biofilms to killing by bactericidal antimicrobial agents (26). However, while complete killing of Δ sagS biofilms was accomplished at concentrations exceeding 100 μ g/ml tobramycin and 200 μ g/ml norfloxacin (Fig. 4), much lower antibiotic concentrations were previously reported to result in complete killing of Δ brlR biofilms (26). Direct comparison of the viability of *P. aeruginosa* PAO1, Δ sagS, and Δ brlR biofilms following 24 h of treatment with tobramycin and norfloxacin at sub-biofilm-MBCs (as determined for Δ sagS biofilms in Fig. 4A to D) confirmed that Δ sagS biofilms were more resilient to bactericidal antibiotics than Δ brlR biofilms (Fig. 4E). While total killing of Δ brlR biofilms was observed at 75 μ g/ml tobramycin and 100 μ g/ml norfloxacin, the same concentrations did not eradicate wild-type or Δ sagS biofilms (Fig. 4E).

Considering that inactivation of both *sagS* and *brlR* resulted in elimination of resistance to killing by bactericidal antibiotics, we hypothesized that SagS and BrlR are part of the same pathway involved in regulating biofilm tolerance, with SagS potentially playing a role in activating BrlR. Therefore, we asked whether

FIG 4 SagS contributes to resistance to killing in *P. aeruginosa* biofilms. *P. aeruginosa* PAO1 and Δ sagS biofilms were grown for 3 days and subsequently treated for 24 h under continuous flow conditions before recovery and enumeration of the surviving cells. (A) Biofilm susceptibility to tobramycin as determined by viable counts (CFU). Biofilm CFU were obtained from biofilm tube reactors having an inner surface area of 25 cm². The number of viable Δ sagS mutant cells was below the detection limit at the highest concentrations of tobramycin tested. (B) Biofilm susceptibility to tobramycin as determined by log reduction. Total killing of Δ sagS biofilm cells was achieved at 120 μ g/ml of tobramycin. In contrast, *P. aeruginosa* PAO1 biofilms maintained a steady level of persisting survivors at concentrations higher than 40 μ g/ml of tobramycin. (C and D) Biofilm susceptibility to norfloxacin was determined similarly. Total killing of Δ sagS biofilm cells was achieved at 250 μ g/ml of norfloxacin. (E) Susceptibility of *P. aeruginosa* PAO1, Δ sagS, and Δ brlR biofilms to 75 μ g/ml tobramycin and 100 μ g/ml norfloxacin (sub-MBC for Δ sagS biofilms) as determined by viable counts (CFU). (F) Susceptibility of *P. aeruginosa* PAO1 and Δ brlR, Δ sagS, and Δ sagS/pJN-brlR biofilms to 300 μ g/ml tobramycin and norfloxacin as determined by viable counts (CFU). *, significantly different from PAO1 ($P < 0.01$). Experiments were carried out at least in triplicate. Error bars denote standard deviations.

expression of *brlR* in a Δ sagS mutant restores resistance of biofilms to antimicrobial agents. Expression of *brlR* in Δ sagS biofilms resulted in significantly reduced susceptibility to tobramycin compared to that in Δ sagS biofilms, with *brlR* expression rendering Δ sagS biofilms comparable in resistance to wild-type and complemented Δ sagS biofilms (Fig. 5A). Similar results were obtained when biofilms were treated with norfloxacin (Fig. 5B). In contrast, however, expression of *sagS* did not restore the susceptibility phenotype of Δ brlR mutant biofilms to wild-type levels. Instead, the resistance phenotype of this mutant was only restored

by complementation with *brlR* (Fig. 5A and B). Expression of *brlR* in Δ sagS mutant biofilms furthermore, restored resistance of biofilm cells to killing by tobramycin and norfloxacin (Fig. 4F). While total killing of Δ sagS biofilm cells was achieved at concentrations exceeding 100 μ g/ml tobramycin and 200 μ g/ml norfloxacin, viable cells of Δ sagS/pJN-brlR biofilms were detected following 24 h of treatment with 300 μ g/ml tobramycin and 300 μ g/ml norfloxacin. The viability of Δ sagS/pJN-brlR biofilms was comparable to that of wild-type biofilms (Fig. 4F). The findings suggested BrlR acts downstream of SagS. The contribution of BrlR to the drug

FIG 5 Multicopy expression of *brlR* restores the susceptibility phenotype of Δ *sagS* biofilms to wild-type levels. *P. aeruginosa* wild-type and Δ *sagS* and Δ *brlR* mutant biofilms expressing *brlR* or *sagS* in *trans* were grown in tube reactors for 3 days and subsequently treated with (A) tobramycin (150 μ g/ml) or (B) norfloxacin (450 μ g/ml) for 1 h under flowing conditions. *P. aeruginosa* harboring the empty vector pJN105 was used as a control. Cells of the *P. aeruginosa* wild type or Δ *sagS* and Δ *brlR* mutants expressing *brlR* or *sagS* were grown planktonically to the exponential phase and subsequently treated with (C) tobramycin (50 μ g/ml) or (D) norfloxacin (150 μ g/ml) for 1 h. Susceptibility was determined via viability counts and is expressed as log reduction. Experiments were carried out at least in triplicate. Error bars denote standard deviations. *, significantly different from PAO1 ($P < 0.01$).

tolerance of Δ *sagS* biofilms, however, was biofilm specific, as expression of *brlR* in Δ *sagS* under planktonic conditions had no effect on susceptibility (Fig. 5C and D). This finding indicated that under planktonic growth conditions, inactivation of *sagS* abrogates the resistance phenotype of a *brlR*-overexpressing strain, suggesting the contribution of BrlR to tolerance is *SagS* dependent.

Expression of *brlR* does not affect the biofilm architecture of Δ *sagS* mutant biofilms. To confirm that restoration of biofilm resistance of Δ *sagS* mutant biofilms to wild-type levels upon *brlR* expression is not due to differences in growth rates, growth in liquid of the wild type and Δ *sagS* mutant in the presence and absence of *brlR* was determined by using growth curves. However, no difference in growth rate was noted, suggesting that the difference in susceptibility was not a result of a general increase in growth rate. Moreover, following inactivation or overexpression of *sagS*, no difference in colony morphology (not shown) or expression of *ndvB* (0.9 ± 0.3 -fold change in transcript levels), which is required for glucan synthesis (25), was noted.

To exclude the possibility that expression of *brlR* altered the biofilm architecture of Δ *sagS* biofilms, Δ *sagS* biofilms expressing *brlR* were grown in flow cell reactors for 6 days, after which time the biofilm architecture was viewed using confocal microscopy. As previously described (29), Δ *sagS* cells formed thin biofilms that lacked large cellular aggregates and microcolonies, and as determined by COMSTAT analysis were composed of ≥ 10 -fold less biomass than the wild type (Fig. 6; Table 3). Expression of *brlR* had

no effect on the biofilm architecture of Δ *sagS* mutants. In contrast, expression of *sagS* restored the biofilm architecture to wild-type levels (Fig. 6; Table 3). Over the course of biofilm formation, biofilm architecture was monitored daily by bright-field microscopy, with similar trends observed throughout the 6-day growth period. These findings indicated that while expression of *brlR* restores the drug tolerance of Δ *sagS* biofilms, BrlR does not contribute to the biofilm architecture, underscoring the notion of biomass- and architecture-independent biofilm resistance mechanisms.

***SagS* contributes to BrlR abundance and transcript levels of multidrug efflux pumps.** Given that the observed restoration of the biofilm resistance phenotype of the Δ *sagS* mutant to wild-type levels upon multicopy expression of *brlR* was independent of differences in growth rates, biofilm biomass accumulation, and biofilm architecture, and considering the link between *SagS* and BrlR in biofilm drug tolerance, with BrlR likely acting downstream of *SagS*, we next asked whether *SagS* plays a role in the regulation of *brlR* expression. qRT-PCR analysis indicated that the *brlR* transcript levels were reduced 25.3 ± 0.4 -fold in Δ *sagS* biofilms compared to wild-type biofilms. All fold changes in transcript levels compared to wild-type biofilms were significantly different ($P < 0.01$). However, given that the qPCR threshold cycle (C_T) for the *brlR* transcripts was above 30 (but lower than that for the no-RT control), the data suggested that *brlR* is still transcribed in Δ *sagS* biofilms. Detection of *brlR* expression in Δ *sagS* biofilms was surprising, considering that Δ *sagS* mutant biofilms were comparable in their susceptibility to tobramycin and norfloxacin to *brlR* mu-

FIG 6 Multicopy expression of *brlR* does not restore the biofilm architecture of Δ *sagS* mutants to wild-type levels. Confocal images were acquired from flow-cell-grown biofilms following 6 days under flowing conditions. Biofilms were stained with the LIVE/DEAD BacLight viability stain (Invitrogen Corp.). Confocal images were acquired using a Leica TCS SP5 confocal microscope. White bars, 100 μ m.

tant biofilms (Fig. 3 and 5) (26), with resistance being restored to wild-type levels upon *brlR* expression (Fig. 5). To confirm that BrlR is indeed being produced in Δ *sagS* biofilms, we generated a chromosomally located V5/His₆-tagged BrlR variant under the control of its own promoter in both *P. aeruginosa* wild-type and Δ *sagS* strains. Detection of BrlR levels in biofilms was done by immunoblot analysis. While BrlR was detected in both wild-type

and Δ *sagS* biofilms, inactivation of *sagS* correlated with significantly decreased BrlR levels (Fig. 7A).

We next asked whether BrlR is active by determining the transcript levels of genes previously described to be expressed in a BrlR-dependent manner in biofilms. These included *mexA* and *mexE*, encoding components of the MexAB-OprM and MexEF-OprN multidrug efflux pumps, respectively (47). Expression of

TABLE 3 COMSTAT quantitative analysis of the architecture of biofilms formed by the wild-type PAO1 strain and the Δ *sagS* and Δ *sagS* mutants expressing *sagS* and *brlR* in *trans*^a

Strain	Total biomass (μ m ³ /μm ²)	Substratum coverage (%)	Thickness (μm)		Roughness coefficient ^b
			Avg	Maximum	
PAO1	11.92 ± 9.28	28.85 ± 13.29	11.97 ± 11.48	37.17 ± 26.78	1.25 ± 0.28
Δ <i>sagS</i> mutant	0.82 ± 0.63*	7.07 ± 5.14*	0.83 ± 0.62*	9.00 ± 2.83*	1.74 ± 0.20*
Δ <i>sagS</i> / <i>sagS</i> complemented	11.89 ± 8.48	25.34 ± 11.36	11.90 ± 8.99	47.67 ± 29.96	1.38 ± 0.23
Δ <i>sagS</i> /pJN- <i>brlR</i>	1.5 ± 0.8*	5.7 ± 2.2*	1.9 ± 0.7*	13.6 ± 5.2*	1.72 ± 0.23*

^a Biofilms were grown in flow cells for 6 days prior to confocal image acquisition and subsequent COMSTAT analysis. COMSTAT analysis was carried out with biofilms grown in triplicate using at least 6 images per replicate. *, significantly different from PAO1 ($P \leq 0.05$), as determined by ANOVA and SigmaStat.

^b The roughness coefficient is dimensionless.

FIG 7 SagS contributes to BrlR levels and BrlR-DNA binding to the BrlR target promoters *P_{mexA}* and *P_{mexE}*. (A) Inactivation of *sagS* correlates with reduced BrlR levels in biofilms. Total cell extracts (TCE) obtained from *P. aeruginosa* wild-type and Δ *sagS* biofilms expressing a chromosomally located V5/His₆-tagged BrlR under the control of its own promoter (*P_{brlR}-brlR-V5/His₆*) were probed for the presence of BrlR by immunoblot analysis (anti-V5) using anti-V5 antibodies. A total of 15 μ g total cell extract was loaded. The corresponding SDS-PAGE gel image obtained posttransfer demonstrates equal loading. (B) Detection of V5/His₆-tagged BrlR encoded in *trans* (*pMJT-brlR-V5/His₆*) in total cell extracts (TCE) or metal oxide affinity chromatography (MOAC)-enriched phosphoproteomes of wild-type and Δ *sagS* biofilms. (C and D) Streptavidin-coupled magnetic bead binding assays using cell extracts obtained from Δ *sagS* and PAO1 biofilms expressing V5/His₆-tagged BrlR in *trans*. Images on the right demonstrate BrlR is present in similar amounts in aliquots removed prior to addition of streptavidin-coupled magnetic beads in Δ *sagS* and PAO1 biofilm samples (loading control). Binding assays were carried out using a total of 5 pmol BrlR-V5/His₆ protein and (C) 1 pmol biotinylated *P_{mexA}* or (D) 1 pmol of biotinylated *P_{mexE}* in the wild-type and Δ *sagS* strains. Nontinylated *P_{mexA}* and *P_{mexE}* (*P_{mexA}*-NB and *P_{mexE}*-NB) were used as specific competitor DNA in 20-fold excess. BrlR binding to *P_{mexA}* and *P_{mexE}* was detected by immunoblot analysis using anti-V5 antibodies. + indicates the presence of specified cell extract. (E) Expression of multidrug efflux pump genes *mexAB-oprM* or *mexEF-oprN* in Δ *sagS* restores Δ *sagS* biofilm susceptibility to tobramycin and norfloxacin. The *P. aeruginosa* wild-type strain harboring the empty plasmid pMJT1 and Δ *sagS* and Δ *sagS* mutant biofilms overexpressing *mexAB-oprM* or *mexEF-oprN* in *trans* (Δ *sagS*/pMJT-*mexABoprM*, Δ *sagS*/pJN-*mexEFoprN*, and Δ *sagS*/pMJT-*mexABoprM*/pJN-*mexEFoprN*) were grown in tube reactors for 3 days and subsequently treated with tobramycin (150 μ g/ml) or norfloxacin (450 μ g/ml) for 1 h under flowing conditions. *, significantly different from PAO1 ($P < 0.01$). Experiments were carried out in triplicate. Representative images are shown. Error bars denote standard deviations.

these genes was found to be reduced 2.7 ± 0.2 -fold and 3.7 ± 0.3 -fold, respectively, in $\Delta sagS$ biofilms compared to wild-type biofilms, as described above. The expression levels of *mexA* and *mexE* in $\Delta sagS$ biofilms were comparable to the previously reported *mexA* and *mexE* transcript levels in $\Delta brlR$ biofilms relative to wild-type biofilms (-3.3 ± 0.2 -fold and -3.8 ± 0.3 -fold, respectively) (47). This finding suggested that BrlR potentially fails to act as an activator of multidrug efflux pump gene expression in the absence of SagS. The finding is in agreement with $\Delta sagS$ mutants being as susceptible as $\Delta brlR$ mutant biofilms, despite the presence of detectable BrlR levels, with plasmid-borne expression of *brlR* restoring the resistance phenotype to wild-type levels.

BrlR binding to its target promoters requires SagS. Considering that SagS harbors a histidine kinase and a response regulator receiver domain and has been previously implicated in phosphotransfer events with regulatory proteins modulating attachment and biofilm formation, including HptB and BfiS (29, 48), we asked whether SagS may regulate BrlR activity by affecting its phosphorylation state. However, no difference in phosphorylation of BrlR was noted in wild-type and $\Delta sagS$ biofilms (Fig. 7B). To further determine whether BrlR in the $\Delta sagS$ mutant is active, we asked whether BrlR is impaired in DNA binding to its target promoters of the two multidrug efflux pumps *mexAB-oprM* and *mexEF-oprN* in the absence of SagS. To do so, we made use of streptavidin-coupled magnetic bead DNA binding assays using cell extracts obtained from wild-type and $\Delta sagS$ biofilms overexpressing V5/His₆-tagged *brlR* (pMJT-*brlR*). BrlR binding to the *mexA* promoter (1 pmol) was observed using cell extracts obtained from wild-type biofilms, with addition of 20-fold excess of unlabeled competitor significantly reducing the interaction of BrlR with the *mexA* promoter DNA. Despite having levels of BrlR comparable to those in wild-type biofilms (Fig. 7C, image to the right), significantly reduced binding of BrlR to *PmexA* was observed in the absence of SagS in $\Delta sagS$ /pMJT-*brlR* biofilms (Fig. 7C). Moreover, while binding to the *PmexE* promoter DNA was observed using cell extracts obtained from wild-type biofilms, with addition of 20-fold excess of unlabeled competitor significantly reducing the interaction of BrlR with the *mexE* promoter DNA, no binding was observed in the absence of SagS (Fig. 7D).

To determine whether the reduced expression of the multidrug efflux pump genes contributes to the susceptibility phenotype of *sagS* mutant biofilms, plasmid-borne copies of *mexAB-oprM* and *mexEF-oprN* operons under the control of the *P_{BAD}* promoter were introduced into the $\Delta sagS$ strain, and the resulting strains ($\Delta sagS$ /pMJT-*mexAB-oprM* and $\Delta sagS$ /pJN-*mexEF-oprM*) were subsequently evaluated for their susceptibility to tobramycin and norfloxacin. While treatment of $\Delta sagS$ biofilms with tobramycin for 1 h resulted in a 3-log reduction, wild-type biofilms were only reduced by 1 log following the same treatment. Plasmid-borne expression of *mexAB-oprM* or *mexEF-oprN* in the $\Delta sagS$ mutant restored the tobramycin resistance phenotype to wild-type levels (Fig. 7E). Likewise, while norfloxacin treatment resulted in a 3.3-log reduction in viability of $\Delta sagS$ biofilms compared to a 1-log reduction observed for wild-type biofilms following the same treatment, plasmid-borne expression of *mexAB-oprM* or *mexEF-oprN* similarly restored the norfloxacin resistance phenotype of $\Delta sagS$ biofilms to wild-type levels (Fig. 7E). Multicopy expression of both efflux pumps in $\Delta sagS$ biofilms equally restored resistance to tobramycin to wild-type levels (Fig. 7E). The findings suggested

a contribution of multidrug efflux pump gene expression to the susceptibility phenotype of $\Delta sagS$ mutant biofilms.

DISCUSSION

A hallmark of biofilms is their profound tolerance against antimicrobial agents. The tenacious biofilm phenotype is believed to arise from a multiplicity of factors, including reduced metabolic and divisional rates (13, 14, 20, 49), starvation-induced growth arrest (18), the presence of persister cells that neither grow nor die in the presence of bactericidal antibiotics (15, 46, 50–52), and restricted penetration of antimicrobials into a biofilm (8, 10, 11, 16, 17, 21, 53, 54). The goal of the present study was to determine when over the course of development *P. aeruginosa* biofilms gain their extraordinary tolerance to antimicrobial agents. A previous study by Anwar et al. (13) hinted at biofilm development being a contributing factor to drug tolerance. However, while those authors determined the kinetics of biofilm formation in their reactor system by viability counts, the developmental stages of the biofilms used in the study were not defined. Our findings demonstrated that association with the surface, rather than biofilm age or the extent to which biofilm biomass accumulation occurs over time, contributes to biofilm drug tolerance. Moreover, using a suite of mutants arrested at distinct biofilm developmental stages, we were able to demonstrate that the timing of when biofilm cells become tolerant is linked to biofilm development. Inactivation of either SagS or BfiSR has been previously shown to impair biofilm formation at the irreversible attachment stage. However, while impaired at similar stages, inactivation of *bfiS* did not affect biofilm tolerance, strongly suggesting that the transition to the irreversible attachment stage, regulated by the two-component hybrid SagS, marks the timing when biofilms switch to the high-level tolerance phenotype. The role of SagS, and not BfiSR, in biofilm tolerance is of particular interest, considering that SagS interacts directly with BfiS, with the direct and specific interaction between these two membrane-bound sensors resulting in the modulation of the phosphorylation state of BfiS in a growth mode-dependent manner. Thus, SagS appears to function as a master regulator, with SagS activation enabling both biofilm formation and biofilm tolerance.

SagS-dependent biofilm tolerance was found to be linked to BrlR, which was previously demonstrated to be required for biofilm tolerance (26, 47). Inactivation of *sagS* resulted in a biofilm phenotype similar to that of $\Delta brlR$ biofilms, in that both mutant biofilms were significantly more susceptible to antimicrobial agents, including tobramycin and norfloxacin. Moreover, inactivation of either *sagS* or *brlR* eliminated the recalcitrance of biofilms to killing by bactericidal antimicrobial agents (Fig. 4) (26). However, the concentrations of antibiotics at which total killing of $\Delta brlR$ and $\Delta sagS$ mutant biofilms was achieved differed. While complete killing of $\Delta sagS$ biofilms was accomplished at concentrations exceeding 100 $\mu\text{g/ml}$ tobramycin and 200 $\mu\text{g/ml}$ norfloxacin (Fig. 4), complete killing of $\Delta brlR$ biofilms only required concentrations exceeding 30 $\mu\text{g/ml}$ tobramycin and 20 $\mu\text{g/ml}$ norfloxacin (26). The difference in antibiotic concentrations required for complete killing is consistent with BrlR acting downstream of SagS, with SagS contributing to BrlR abundance and BrlR binding to the promoter regions of the multidrug efflux pump gene operons *mexAB-oprM* and *mexEF-oprN*. Thus, SagS contributes 2-fold to BrlR-dependent biofilm drug tolerance, by contributing to *brlR* transcript levels and by enhancing BrlR-DNA

binding to its target promoters. Moreover, we demonstrate that the contribution of BrlR to tolerance is SagS dependent, as inactivation of *sagS* abrogated the high-resistance phenotype of a *brlR*-overexpressing strain. We previously demonstrated that overexpression of *brlR* rendered both biofilms and planktonic cells more resistant to antimicrobial agents (26). However, while our findings suggest a role of SagS in contributing to *brlR* expression, with *brlR* expression failing to render Δ *sagS* planktonic cells more resistant to antibiotics, additional factors may play a role in biofilms. This is based on the findings that inactivation of *sagS* significantly reduced, but did not abolish, *brlR* expression and that *sagS* inactivation did not reduce susceptibility to killing to Δ *brlR* biofilm levels.

While the direct mechanism by which SagS contributes to *brlR* expression and BrlR-DNA binding remains to be elucidated, our findings strongly suggest biofilm tolerance is linked to biofilm development, with tolerance to antimicrobial agents being induced upon transitioning to the surface-associated lifestyle. While our observations do not exclude the possibility of other mechanisms, including the presence of persister cells, reduced metabolic and divisional rates, and drug indifference of slow-growing, nutrient-limited cells, to contribute to biofilm tolerance, the present results demonstrate that biofilm tolerance and recalcitrance to killing by bactericidal antimicrobial agents require the two-component hybrid SagS. SagS not only directly contributes to the motile-sessile switch to enable *P. aeruginosa* biofilm formation but also contributes indirectly to both *brlR* expression and BrlR-DNA binding, further supporting the existence of a classical, biofilm-specific mechanism of drug tolerance in *P. aeruginosa*.

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