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PHARMACOLOGY

Influence of *rhlR* and *lasR* on Polymyxin Pharmacodynamics in *Pseudomonas aeruginosa* and Implications for Quorum Sensing Inhibition with Azithromycin

Zackery P. Bulman,[a,b] Neang S. Ly,[a,b] Justin R. Lenhard,[c] Patricia N. Holden,[a,b] Jürgen B. Bulitta,[d] Brian T. Tsuji[a,b]

Laboratory for Antimicrobial Pharmacodynamics, University at Buffalo School of Pharmacy and Pharmaceutical Sciences, Buffalo, New York, USA[a]; New York State Center of Excellence in Life Sciences and Bioinformatics, Buffalo, New York, USA[b]; California Northstate University College of Pharmacy, Elk Grove, California, USA[c]; Center for Pharmacometrics and Systems Pharmacology, Department of Pharmaceutics, College of Pharmacy, University of Florida, Orlando, Florida, USA[d]

ABSTRACT The impact of quorum sensing on polymyxin and azithromycin pharmacodynamics was assessed in *Pseudomonas aeruginosa* PAO1 and an isogenic *rhlR*/*lasR* double knockout. For polymyxin B, greater killing against the *rhlR*/*lasR* knockout than against PAO1 was observed at 10^8 CFU/ml (polymyxin B half-maximal effective concentration [EC_{50}], 5.61 versus 12.5 mg/liter, respectively; $P < 0.005$). Polymyxin B combined with azithromycin (256 mg/liter) was synergistic against each strain, significantly reducing the respective polymyxin B EC_{50} compared to those with monotherapy ($P < 0.005$), and is a promising strategy by which to combat *P. aeruginosa*.

KEYWORDS azithromycin, *P. aeruginosa*, polymyxin B, colistin, quorum sensing, pharmacodynamics, mechanism-based modeling, *Pseudomonas aeruginosa*, polymyxins

Pseudomonas aeruginosa is a leading nosocomial pathogen with infections that are associated with unacceptably high rates of treatment failure, up to 40% (1, 2). The polymyxin antibiotics (colistin [polymyxin E] and polymyxin B) have become important last-line agents against multidrug-resistant *P. aeruginosa* infections and are being increasingly utilized as salvage therapy (3). However, current dosage regimens for colistin and polymyxin B result in plasma concentrations that are suboptimal in a number of critically ill patients (4–7). Despite the initial susceptibility of many strains, mortality rates for polymyxin monotherapy remain high; however, increasing the dose may promote further resistance amplification against a high bacterial density (8–12).

Azithromycin, a macrolide antibiotic, is often used to treat community-associated respiratory tract infections but has no intrinsic activity against *P. aeruginosa*. Earlier *in vitro* time-kill and checkerboard studies suggested that azithromycin may enhance killing in combination with the polymyxins (13, 14). However, the precise time course of bacterial response and the mechanistic basis for this combination remain unknown. Furthermore, the pharmacokinetic-pharmacodynamic relationship of the polymyxin-azithromycin combination has yet to be studied at a range of clinically relevant concentrations, including those in serum (~0.5 mg/liter) and in neutrophils (>500 mg/liter), where azithromycin is concentrated (15, 16).

Recent studies have also concluded that there is a clinical benefit to using azithromycin in patients with cystic fibrosis or diffuse panbronchiolitis who are chronically infected with *P. aeruginosa* (17, 18). It has been hypothesized that one mechanism for the salutary effect of azithromycin against *P. aeruginosa* is through inhibition of quorum sensing, which is a mechanism of bacterial communication that coordinates a multi-

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Address correspondence to Brian T. Tsuji, btsuji@buffalo.edu.

Z.P.B. and N.S.L. contributed equally to this work.

tude of cellular behaviors, such as formation of virulence factors and biofilms (19, 20). Azithromycin interferes with quorum sensing by inhibiting the synthesis of signaling molecules employed through the *las* and *rhl* systems (20), preventing intercellular coordination among *P. aeruginosa* cells. Since quorum sensing functions at a high bacterial density of *P. aeruginosa*, which has been shown to reduce the activity of polymyxins (21), understanding the interrelationships among quorum sensing, polymyxin-azithromycin pharmacodynamics, and inoculum size is of scientific relevance. Our objectives were to (i) profile the pharmacodynamic activity of colistin and polymyxin B against *P. aeruginosa* PAO1 and an *rhlR/lasR* double-knockout strain to determine the impact of quorum sensing on the rate and extent of bacterial killing by polymyxins, and (ii) compare the pharmacodynamics of polymyxin B and azithromycin combinations against quorum sensing-proficient (PAO1) and -deficient (*rhlR/lasR* knockout) strains.

The bacterial strains utilized in this study were wild-type *P. aeruginosa* PAO1 and an isogenic *rhlR/lasR* knockout (Δ*rhlR*::*GmR* and Δ*lasR*::*TcR* cassettes) (22). Colistin, polymyxin B, and azithromycin MICs were determined using broth microdilution in duplicate according to CLSI (23). The colistin, polymyxin B, and azithromycin MICs for PAO1 were 1, 1, and 512 mg/liter, respectively, and the MICs for the *rhlR/lasR* knockout were 2, 2, and 512 mg/liter, respectively. Colistin (sulfate) and polymyxin B (sulfate) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and azithromycin was purchased from AK Scientific, Inc. (Union City, CA, USA). Time-kill experiments were carried out as previously described (24). LB broth supplemented with magnesium chloride (12.5 Mg^{2+}/liter final concentration) and calcium chloride (25 Ca^{2+}/liter final concentration) acted as growing media in all experiments. Serial samples throughout the 48-h experiment were withdrawn to quantify viable cell density after vortexing and visual inspection, which verified that the system was homogenous and planktonic. Colistin or polymyxin B killing was evaluated at different bacterial densities ($CFU_{0\ h}$) of $\sim 10^6$, 10^8, or 10^9 CFU/ml. Polymyxin B and azithromycin combination experiments were performed at a $CFU_{0\ h}$ of $\sim 10^8$ CFU/ml. Polymyxin concentrations ranging from 0 to 128 mg/liter (4, 25) and azithromycin concentrations of 0, 0.5, 2, 128, and 256 mg/liter (15, 16) were used.

Synergy was defined as a $\geq$2 $\log_{10}$-CFU/ml reduction compared to the more active agent as monotherapy at 24 h. To characterize pharmacodynamic activity over time (0 to 48 h), the area under the CFU (AUCFU) curve was calculated using the linear-up, log-down trapezoidal rule. The log ratio change was calculated to compare killing at individual time points, whereas the log ratio area was used to assess activity throughout the time-kill experiments, as described previously (26). To characterize the interplay between inoculum effect and quorum sensing, previously developed mechanism-based models for colistin were used (21). Three preexisting subpopulations with different susceptibilities to colistin or polymyxin B were considered with the modification of the growth model. Data were modeled using a population approach in S-ADAPT software (version 1.57) with the SADAPT-TRAN pre- and postprocessing tool to fit all data simultaneously (see mathematical model development in the supplemental material).

Colistin and polymyxin B alone displayed concentration-dependent killing for all three initial inocula with regrowth toward the growth control (Fig. 1 and 2). Both polymyxins caused rapid initial killing of up to 6.00 (PAO1) and 6.16 (*rhlR/lasR* knockout) $\log_{10}$ CFU/ml at the 10^6 $CFU_{0\ h}$ inoculum. However, at higher starting inocula (10^8 and 10^9 $CFU_{0\ h}$), there was attenuation of bacterial killing, which resulted in a stepwise half-maximal effective concentration (EC_{50}) increase for each strain (Fig. 2). Colistin and polymyxin B achieved better killing against the *rhlR/lasR* knockout than against PAO1, especially at higher concentrations. The discordance in killing was most prominent at the 10^8 $CFU_{0\ h}$ starting inoculum, where the E_{max} (maximal effect) increased from 2.18 to 4.03 ($P < 0.001$) for colistin and from 2.67 to 3.36 ($P = 0.09$) for polymyxin B against the PAO1 and *rhlR/lasR* knockout strains, respectively (Fig. 2A2 and B2). Colistin and polymyxin B also displayed a higher EC_{50} for PAO1 than for the *rhlR/lasR* knockout strain (8.72 mg/liter [24.6% standard error (SE)] versus 7.04 mg/liter [2.64% SE] for colistin, $P = 0.47$; 12.45 mg/liter [16.0% SE] versus 5.61 mg/liter [5.71% SE] for polymyxin B, $P < 0.005$) at this inoculum. At the 10^6 and 10^9 $CFU_{0\ h}$ inocula, the strains

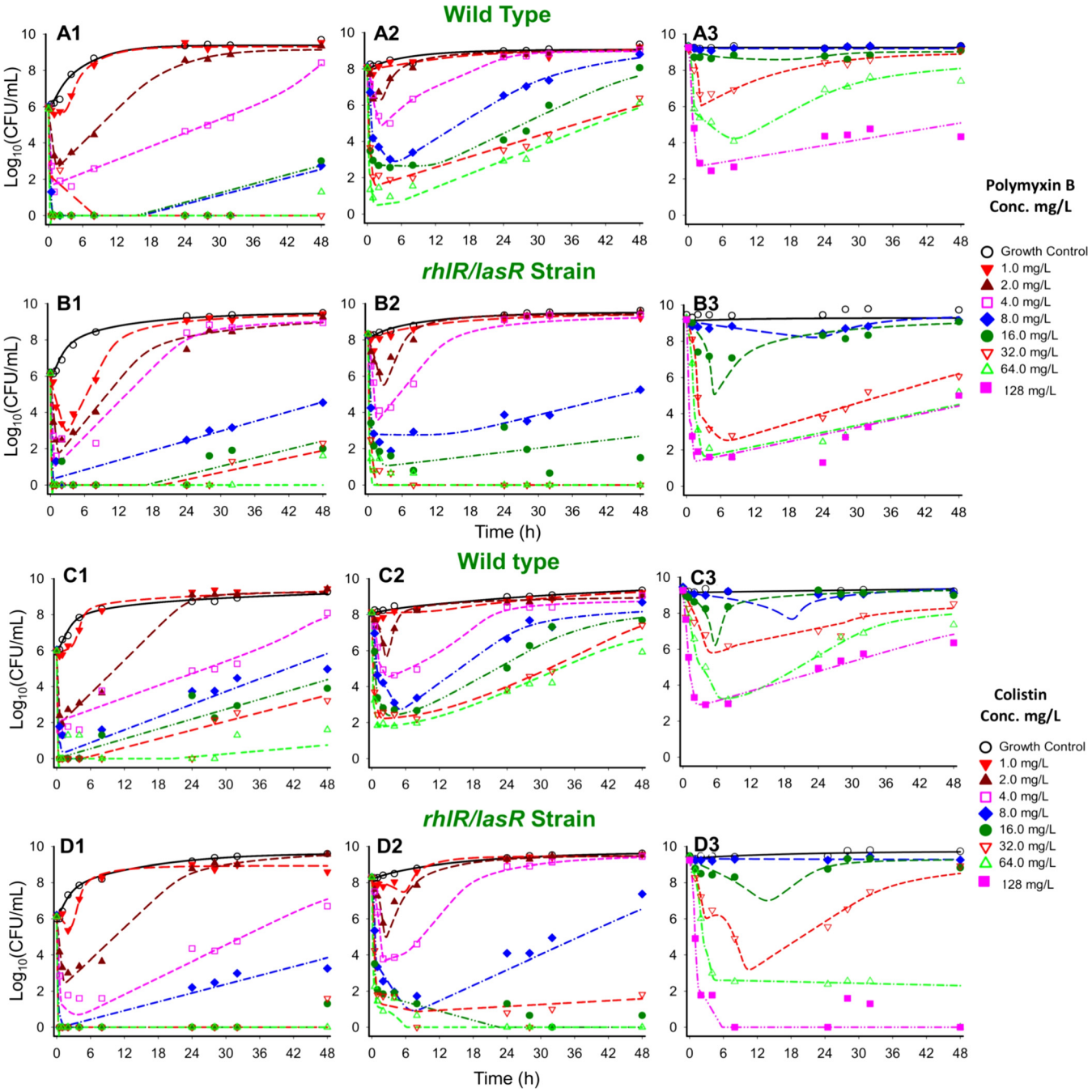

FIG 1 Time course of *P. aeruginosa* strains PAO1 (rows A and C) and the *rhlR/lasR* knockout strain (rows B and D) against polymyxin B (A1 to B3) or colistin (C1 to D3) at three different initial inocula: 10^6 $CFU_{0\ h}$ (column 1), 10^8 $CFU_{0\ h}$ (column 2), and 10^9 $CFU_{0\ h}$ (column 3). Polymyxin B and colistin concentrations range from 0 to 128 mg/liter. Individual points represent observed viable colony counts (CFU/ml) from time-kill experiments, whereas lines represent expected bacterial killing as predicted by the previously validated mechanism-based model.

displayed similar concentration-response curves (Fig. 2A3 and B3); however, the bacterial killing profiles against the *rhlR/lasR* knockout appeared greater (Fig. 1A to D3). Among the polymyxins, colistin performed marginally better than polymyxin B against the *rhlR/lasR* knockout, most markedly at concentrations of ≥4 mg/liter for 10^6 and 10^8 $CFU_{0\ h}$ and ≥32 mg/liter for 10^9 $CFU_{0\ h}$. Against PAO1, the polymyxins achieved comparable killing at each inoculum, with the only perceptible difference occurring in favor of polymyxin B at 10^6 $CFU_{0\ h}$ (≥8 mg/liter). The final mechanism-based pharmacodynamic model, which consists of three preexisting subpopulations and uses the

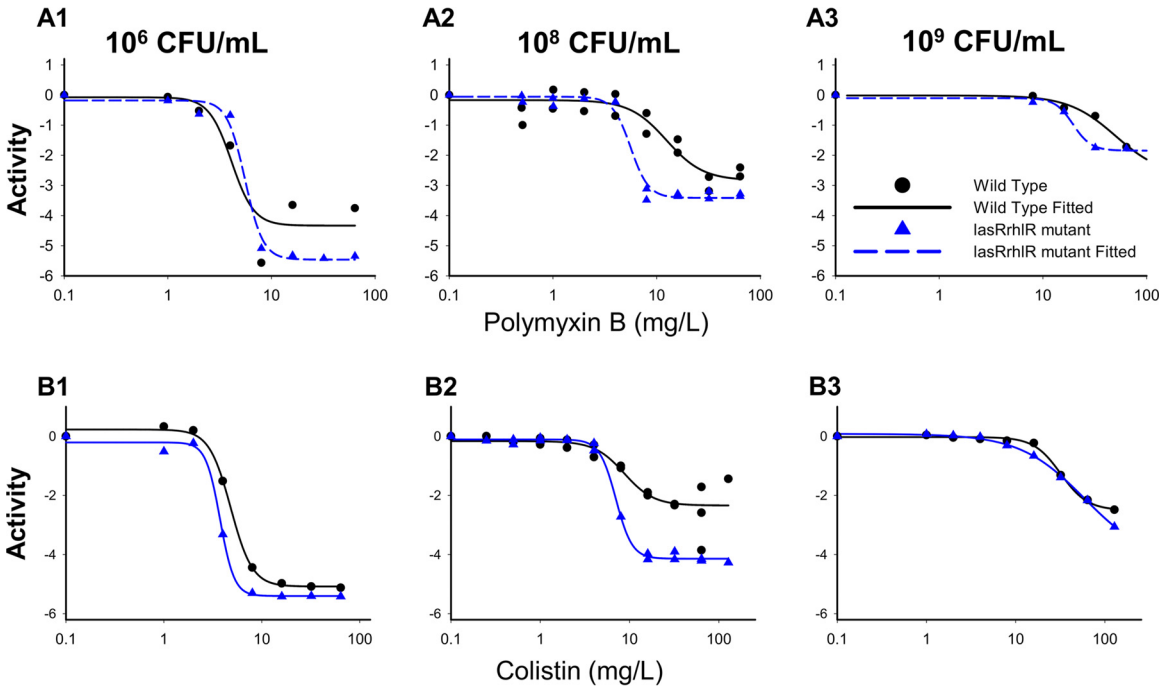

FIG 2 Comparative pharmacodynamic responses between PAO1 wild-type (black) and *rhlR/lasR* knockout (blue) strains at each inoculum to either polymyxin B (row A) or colistin (row B). Activity is defined as the $\log_{10}$ ratio of the AUCFU of treatment to the AUCFU of growth control (log ratio area) based on the observed response throughout the 48-h time-kill experiments. Parameter estimates for the Hill-type models are found in Table S2 in the supplemental material.

target site binding of polymyxin to LPS (see Fig. S1 in the supplemental material), excellently characterized (see Table S1 in the supplemental material, correlation coefficient individual fit >0.994 and population fit >0.905) the time course of bacterial killing, inoculum effect, and regrowth (Fig. 1, dashed lines).

Our time-kill data and pharmacodynamic modeling of colistin and polymyxin B monotherapies show that the rapid bactericidal activity achieved was not sustained, suggesting potential utility for combinations. Polymyxin B was investigated in addition to azithromycin at 10^8 $CFU_{0\ h}$ due to the favorable pharmacokinetics of polymyxin B (not administered as a prodrug) (27) and the similar performance of both polymyxins at clinically achievable concentrations at this inoculum (Fig. 2A2 and B2). In contrast to the regrowth seen against polymyxins alone, azithromycin (256 mg/liter) and polymyxin B achieved synergy at 24 h against PAO1 regardless of the polymyxin B concentration (Fig. 3A1 to 4). Remarkably, after the rapid initial bacterial killing within 8 h by polymyxin B and azithromycin (128 or 256 mg/liter) combinations, apparent bacteriostasis was achieved and persisted through 48 h. Against the *rhlR/lasR* knockout, the combination with azithromycin 256 mg/liter was synergistic at polymyxin B concentrations of 4 and 6 mg/liter. Comparison of bacterial killing seen in PAO1 and the *rhlR/lasR* knockout using the log ratio area, showed minimal differentiation (<0.82) at azithromycin 0.5, 2, and 128 mg/liter (Fig. 3C1 to 3). The progressive decreases in log ratio area between these azithromycin concentrations were well fit to linear functions (standard error of estimate, <0.2). The highest azithromycin concentration (256 mg/liter) displayed less total killing against the *rhlR/lasR* knockout than against PAO1, and both were excellently fit to a Hill-type model (R^2, >0.99). At an azithromycin concentration of 256 mg/liter, the largest difference in killing was seen at 2 mg/liter of polymyxin B, in which combination treatment achieved 2.86 and 2.51 $\log_{10}$ CFU/ml less killing against the *rhlR/lasR* knockout strain at 24 and 48 h, respectively. For the polymyxin combinations with 256 mg/liter azithromycin, the polymyxin B EC_{50}s against the *rhlR/lasR* knockout and PAO1 increased from 0.66 to 2.80 mg/liter ($P < 0.001$).

In the current work, we explored the interrelationship between quorum sensing and polymyxin pharmacodynamics at various bacterial densities. Interestingly, we deter-

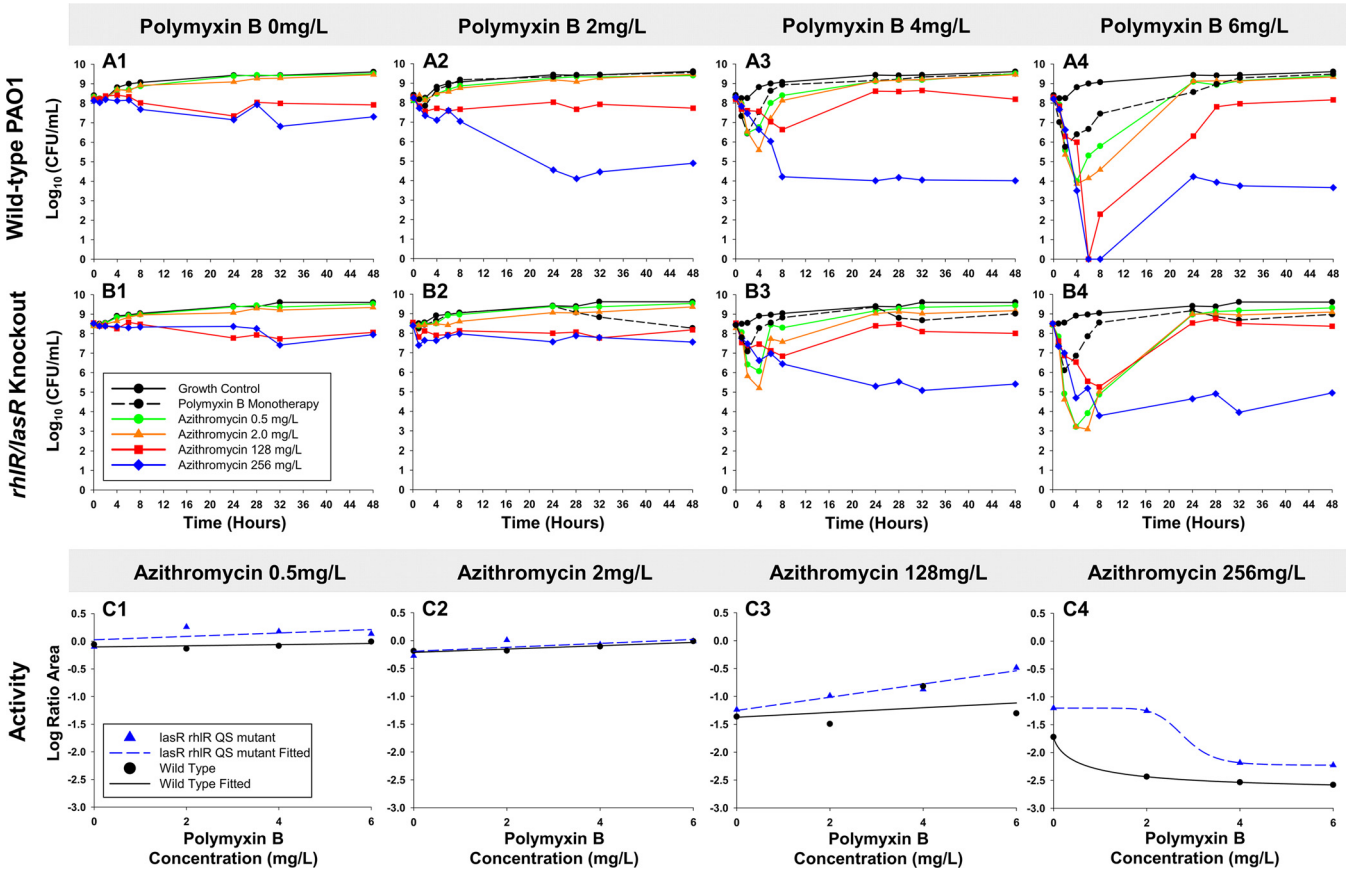

FIG 3 Time-kill experiments evaluating bacterial killing activity of polymyxin B and azithromycin combinations versus the *P. aeruginosa* wild-type PAO1 (row A) and the *rhlR/lasR* knockout (row B) strains. Comparative pharmacodynamic responses between PAO1 (black) and the *rhlR/lasR* knockout (blue) at increasing azithromycin concentrations in combination with polymyxin B and data fit with linear (C1 to 3) or Hill-type (C4) functions. Data for polymyxin B monotherapy are not shown for C1 to C4. Activity is defined as the $\log_{10}$ ratio of the AUCFU of treatment to the AUCFU of growth control (log ratio area) based on observed response throughout the 48-h time-kill experiments.

mined that *rhlR/lasR* deficiency enhanced the pharmacodynamic activity of colistin and polymyxin B monotherapies, especially with the 10^8 $CFU_{0\ h}$ inoculum. The hypothesized quorum sensing-driven alterations to killing were well characterized by our mathematical model, which was based on the known mechanisms of action of the polymyxin antibiotics. Furthermore, the model used signaling compartments that altered killing and growth to account for the observed inoculum effect. Consistent with these results, quorum sensing has been shown to influence susceptibility to other antimicrobials. Kayama et al. (28) demonstrated that quorum sensing-deficient *P. aeruginosa* strains (*lasR*/*lasI* but not *rhlR*/*rhlI*) exposed to ofloxacin had >40 times lower survival rates than the quorum sensing-proficient strain. Modulation of gene expression is thought to be important in adaptation to polymyxin exposure and has been proposed to be regulated by the two-component regulatory systems phoP-phoQ (29), pmrA-pmrB (30), and ParR-ParS (31). The role of such systems may be inoculum dependent, as we previously found that colistin exposed to *pmrA* and *phoP* mutant strains achieved greater killing than the wild-type strain at low initial inocula (32). Unlike our discovery in the current study, Ly et al found that there was no disparity of bacterial killing between the mutant and wild-type strains at the 10^8 $CFU_{0\ h}$ inoculum. Collectively, these observations suggest that the adaptive response to polymyxin exposure may be regulated by rhl- and las-mediated quorum sensing directly or by downstream oversight of other two-component regulatory systems associated with polymyxin resistance.

As an adjuvant to polymyxin B, high concentrations (128 or 256 mg/liter) of the known quorum sensing inhibitor azithromycin caused bacterial killing of *P. aeruginosa* that persisted below the growth control level. Thus, early bactericidal activity by

polymyxin B monotherapy against a high-density infection may be sustained longer with polymyxin-azithromycin combinations. Perhaps the static concentrations of *P. aeruginosa* below growth control levels indicate a persister phenotype. Persister cells become tolerant to antibiotic therapy but are unable to replicate (33). By lowering the bacterial burden and forcing *P. aeruginosa* into a static phenotype, the polymyxin B-azithromycin combination may improve the immune system's likelihood of eradicating the infection.

In contrast to our results with polymyxin B monotherapy, we determined that combinations with high azithromycin concentrations were more active against the wild-type strain versus the *rhlR/lasR* knockout. This finding supports previous data that showed that increasing azithromycin concentrations inhibited growth of a *P. aeruginosa* wild-type strain more than quorum sensing-deficient mutants (*lasR*) (19). Similar bacterial killing may have been anticipated between the wild-type and *rhlR/lasR* knockout strains in the presence of complete quorum sensing inhibition by azithromycin. Therefore, an enhanced susceptibility of the wild-ype strain to the polymyxin-azithromycin combination may be explained in part by the production of rhamnolipids, an exoproduct that is controlled by quorum sensing (*rhl* and *las* systems) and thus only expressed in the wild-type strain before inhibition by azithromycin. Rhamnolipids may increase the uptake of azithromycin into *P. aeruginosa*, making the wild-type strain more susceptible to the polymyxin-azithromycin combination, especially at higher concentrations (34). Furthermore, evidence supports the benefit of azithromycin in prevention of *P. aeruginosa* ventilator-associated pneumonia in patients colonized with *P. aeruginosa* producing high levels of rhamnolipids (35). A potential limitation of this study is that we did not directly quantify quorum sensing activity or rhamnolipid expression in our *in vitro* system. Additional studies are warranted to fully elucidate the mechanism(s) of azithromycin attack on *P. aeruginosa* in the presence of polymyxin B.

In conclusion, our study demonstrated that bacterial modulation by quorum sensing may decrease *P. aeruginosa* susceptibility to polymyxin antibiotics. Considering the utility of azithromycin in cystic fibrosis exacerbations and its ability to inhibit quorum sensing, expanding its niche to other *P. aeruginosa* infections is worth consideration. Azithromycin may therefore provide a multifactorial attack on *P. aeruginosa* and prove useful as an adjuvant to polymyxins in a range of clinical scenarios. Further *in vivo* investigations with polymyxin-azithromycin combinations are needed to better understand the dual benefit of quorum sensing inhibition and potentiation of polymyxin activity.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00096-16.

TEXT S1, PDF file, 0.5 MB.

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