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Emergence of Polymyxin B Resistance Influences Pathogenicity in *Pseudomonas aeruginosa* Mutators

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The interplay between polymyxin B pharmacodynamics and pathogenicity was examined in *Pseudomonas aeruginosa* PAO1 and isogenic DNA repair-deficient mutators (*mutM* and *mutS* strains). Against *mutS* mutators, polymyxin B initial killing was concentration dependent, with >99.9% bacterial reduction at 2 h followed by regrowth and resistance. The pre- versus postexposed strains were inoculated real time into *Galleria mellonella* waxworms, resulting in increased median survival times from 20 h to 23 h ($P < 0.001$). Emergence of resistance in *mutS P. aeruginosa* resulted in attenuation of virulence.

Pseudomonas aeruginosa is a versatile opportunistic human pathogen with an exceptional ability to adapt to antimicrobial therapy and has remarkable pathogenicity (1, 2). Virulence in *P. aeruginosa* is primarily under the control of quorum-sensing two-component systems, such as *las* and *rhl*, which allow for regulation of a wide array of virulence genes in a cell-density-dependent manner (3, 4). The immediate impact of antibiotic exposure on *P. aeruginosa* virulence factor production likely varies, depending on the pharmacological mechanism (5–7). Although cellular control of *P. aeruginosa* virulence is well characterized, the impact that antibiotic exposure and emergence of resistance have on bacterial virulence is incompletely understood.

Persistent *P. aeruginosa* infections are commonly populated by strains that lack full DNA repair capabilities (8). These strains may be classified as mutators given their increased propensity to develop stochastic mutations and rapidly respond to dynamic environments (9, 10). Mutators also tend to have considerably higher rates of antibiotic resistance (11, 12). Deficiency in *mutS*, a gene involved in the mismatch repair (MMR) system, is common in chronic infections caused by *P. aeruginosa*. Deficiency in *mutM* involves the 7,8-dihydro-8-oxo-deoxyguanine (GO) base excision repair (BER) system, which is a critical pathway involving repair of DNA damage induced by reactive oxygen species (ROS) (8, 13). The rapidly evolving nature of the *P. aeruginosa mutM* and *mutS* mutators is therefore an ideal tool for examining the interplay between emergence of antibiotic resistance and virulence. Uncovering the collateral impact of antibiotic resistance on virulence may be particularly important in the context of difficult-to-treat, high-bacterial-density infections where last-line treatment options such as polymyxin antibiotics are critical. In these situations, patients may receive antibiotic therapy for an extended duration, which provides an ideal environment for *P. aeruginosa* adaption to occur. Therefore, our objective was to define the pharmacodynamics and time course of killing of the *P. aeruginosa mutM* and *mutS* mutators by polymyxin B and to assess their virulence in real time, before and after exposure to polymyxin B.

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The studied *P. aeruginosa* mutators were an oxidized guanine BER system-deficient *mutM* strain (containing *mutM*::IS*lacZ*/*hah* [loss of function for *mutM*]) and a mismatch-repair-deficient *mutS* strain (containing *mutS*::IS*phoA*/*hah* [loss of function for *mutS*]). An isogenic PAO1 parental strain was utilized as a comparator in all experiments. The construction of the mutants and their characteristics were previously described (8, 13). For virulence experiments, the bacteria are designated PAO1-0 h, PAO1-48 h, mutM-0 h, mutM-48 h, mutS-0 h, and mutS-48 h to denote the number of hours exposed to polymyxin B in time-kill experiments. Polymyxin B was purchased from Sigma-Aldrich (St. Louis, MO). Luria-Bertani (LB) broth (Difco, Detroit, MI) supplemented with 25 mg/liter calcium and 12.5 mg/liter magnesium was utilized for all experiments. MICs were determined in quadruplicate using a broth microdilution method according to CLSI (14). Time-kill experiments were performed as previously described (15). Polymyxin B concentrations of 2, 4, and 8 mg/liter were evaluated against a starting bacterial inoculum of $\sim 10^8$ CFU/ml, and samples were obtained after 0 (predose), 1, 2, 4, 6, 8, 24, 28, 32, and 48 h. Experiments were performed in duplicate, and data are plotted as the average ± standard deviation (SD). For the virulence experiments, bacterial suspensions were removed from each time-kill experiment at 0 (predose) and 48 h (for the polymyxin B 8-mg/liter arm). These suspensions were immediately centrifuged (2,000 × *g* for 5 min), washed twice with normal saline, and designated PAO1-0 h, PAO1-48 h, mutM-0 h, mutM-48 h, mutS-0 h, and mutS-48 h. These bacterial suspensions

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were directly inoculated into the *Galleria mellonella* waxworm assay to assess for their virulence capacity in real time.

The established *Galleria mellonella* assay (16) was adapted as described below. Ten microliters each of PAO1-0 h, mutM-0 h, and mutS-0 h, not exposed to antibiotics, was centrifuged (2,000 × *g* for 5 min at 23°C), washed two times in normal saline, and then injected into the waxworm (Vanderhorst, Inc., St. Mary's, OH) at an inoculum of 10^2 CFU/larva. The inoculum was confirmed by viable counting on LB agar. The Kruskal-Wallis one-way analysis of variance by ranks test was used to compare inocula across all experiments. Similarly, 10^2 CFU/larva were injected for each strain in real time, following exposure to polymyxin B, taken directly from the time-kill environment at 48 h after exposure to 8 mg/liter (PAO1-48 h, mutM-48 h, and mutS-48 h). MICs of polymyxin B were determined at 0 h (predose) and 48 h (14). Twenty waxworms were used for every experimental group and incubated at 37°C after inoculation; the number of dead waxworms was scored at 1-h intervals up to 24 h postinoculation. Waxworms were censored if they were still living at the end of the experiment; no more than one waxworm was censored in any treatment group. Two control groups were used as the reference in conjunction with each experiment (no injection and normal saline injection). Virulence experiments were completed in two replicates.

Prior to antibiotic exposure, the polymyxin B MICs for *P. aeruginosa* were 1.0 μg/ml for PAO1-0 h and mutM-0 h and 2.0 μg/ml for mutS-0 h. These MICs are currently considered susceptible (17). After 48 h of exposure to 8 mg/liter polymyxin B in time-kill experiments, *P. aeruginosa* became resistant, with post-exposure MICs of 8 μg/ml for PAO1-48 h and 16 μg/ml for mutM-48 h and mutS-48 h. For all *P. aeruginosa* strains, bacterial killing within the first 8 h was concentration dependent (Fig. 1), eventually followed by regrowth to growth control levels by 24 h. Following exposure to 2 and 4 mg/liter polymyxin B for 8 h, viable counts declined by <1.0 $\log_{10}$ compared to the growth control for all three strains. For 8 mg/liter polymyxin B, viable counts after 8 h declined by 3.71 $\log_{10}$ for PAO1, 3.43 $\log_{10}$ for the *mutM* strain, and 3.60 $\log_{10}$ for the *mutS* strain compared to the growth control. Interestingly, despite slightly more killing at 1 to 4 h for 8 mg/liter polymyxin B against the *mutM* and *mutS* strains compared to the PAO1 wild-type (Fig. 1), the mutator strains started to regrow earlier, approaching the level of the growth control by 24 h for polymyxin B at a concentration of 8 mg/liter.

The waxworm survival results (Fig. 2) revealed the relative bacterial virulence between *P. aeruginosa* strains. Time to death for waxworms in any sample began as early as 14 h, but 81.7% (196 out of 240) died between 18 and 24 h. Before antibiotic exposure, there was no difference in virulence between the mutM-0 h and mutS-0 h mutators and their isogenic parent strain, PAO1-0 h (Fig. 2A). The median survival time was 20 h each for PAO1-0 h, mutM-0 h, and mutS-0 h. After 48 h of antibiotic exposure, survival of waxworms was significantly longer following injection with mutS-48 h compared to either PAO1-48 h or mutM-48 h ($P < 0.001$) (Fig. 2B). The median survival time was 23 h for PAO1-48 h compared to 20 h for mutM-48 h and mutS-48 h. Pairwise comparisons of virulence yielded no significant difference between PAO1-0 h and PAO1-48 h or mutM-0 h and mutM-48 h (Fig. 2C and D). However, there was a statistically significant increase in waxworm survival for mutS-48 h versus mutS-0 h ($P < 0.001$) (Fig. 2E).

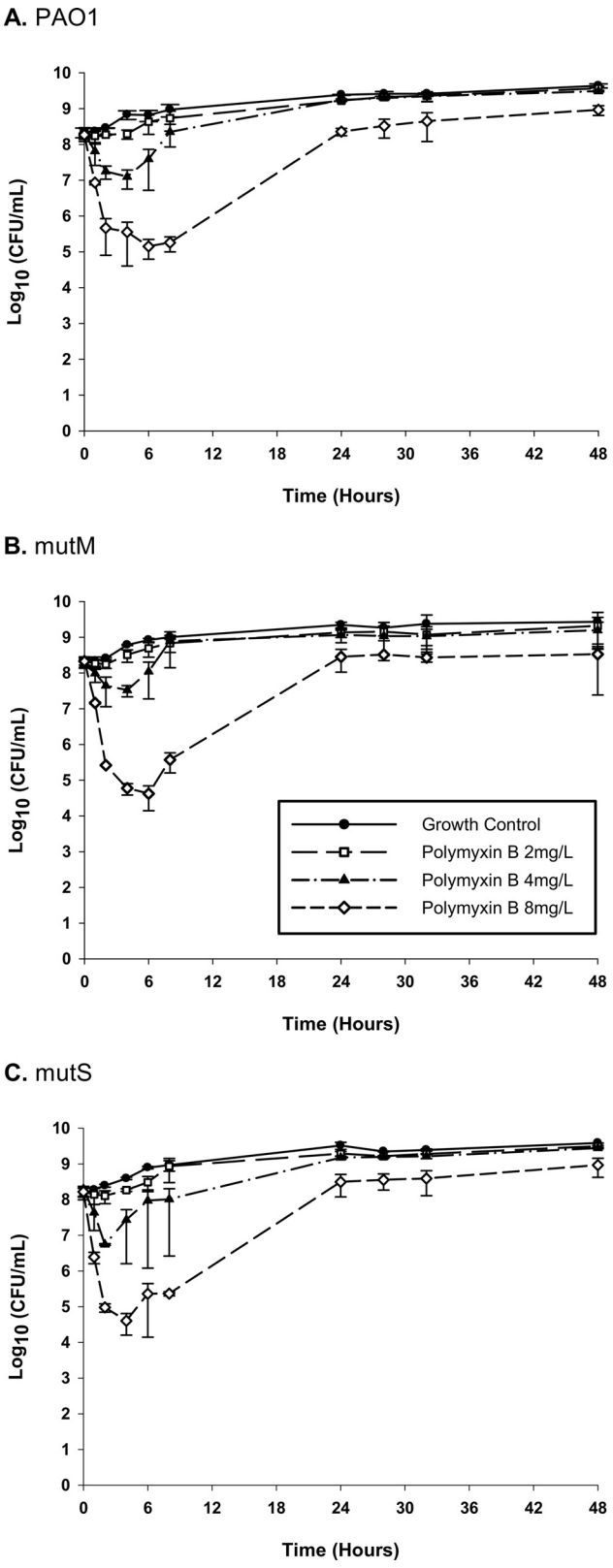

FIG 1 Polymyxin B pharmacodynamics evaluating bacterial killing activity (mean ± standard deviation) over 48 h versus the *P. aeruginosa* wild-type strain PAO1 and the isogenic *mutM* and *mutS* strains deficient in DNA repair. (A) Polymyxin B versus PAO1; (B) polymyxin B versus *mutM* strain; (C) polymyxin B versus *mutS* strain.

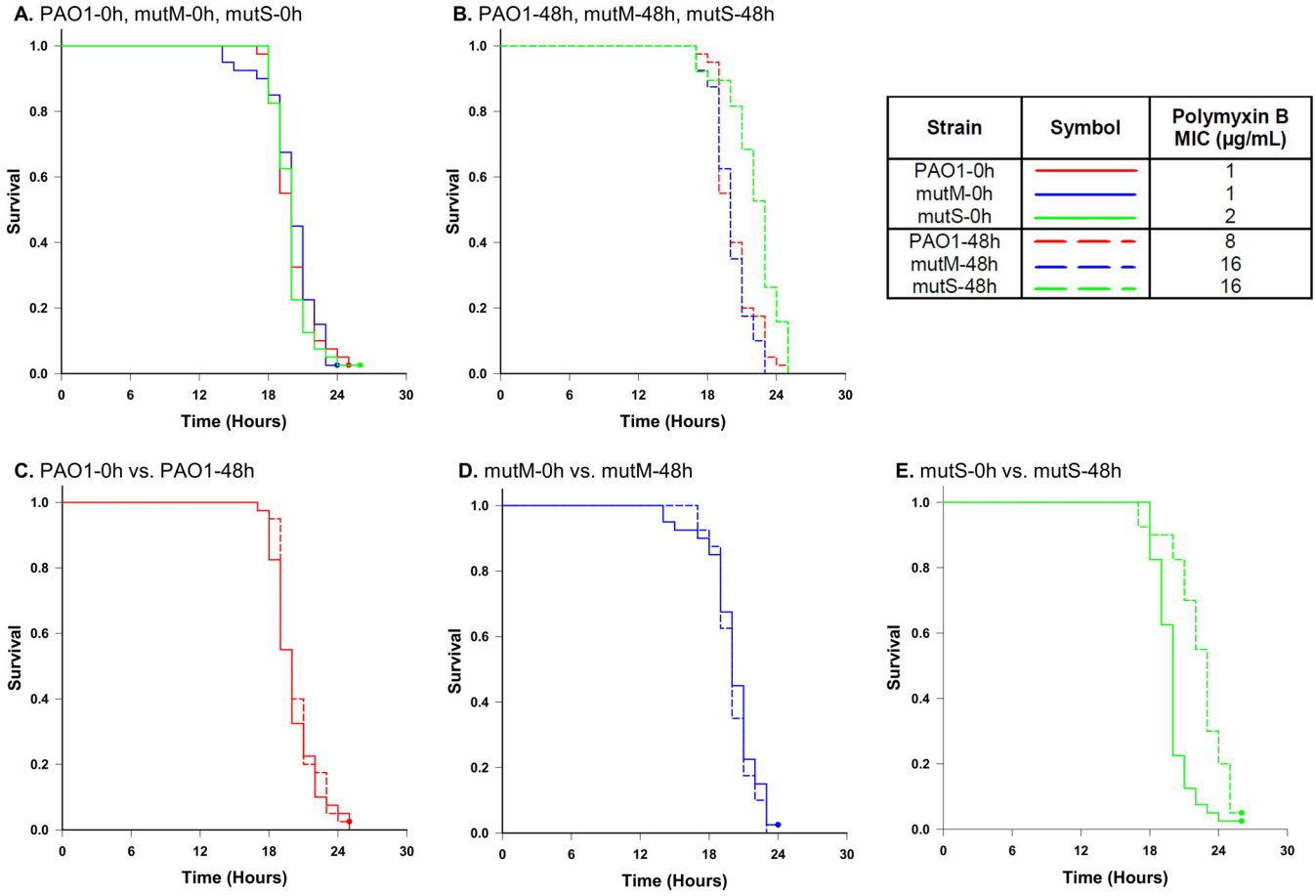

FIG 2 Kaplan-Meier curves for the *G. mellonella* virulence assay assessing survival of *P. aeruginosa* strains before and after antibiotic exposure taken from the time-kill experiments performed in real time. For each strain, 10^2 CFU/larva were injected before (0 h; polymyxin B susceptible) and directly following (48 h; polymyxin B resistant) treatment with polymyxin B (8 mg/liter). Wild-type *P. aeruginosa*, PAO1-0 h, and the isogenic mutators mutM-0 h and mutS-0 h represent strains not previously exposed to antibiotics. PAO1-48 h, mutM-48 h, and mutS-48 h represent bacteria harvested after 48 h of exposure to polymyxin B (8 mg/liter).

In the present study, we examined the relationship between polymyxin B pharmacodynamics and virulence and compared the pre- and postexposed *P. aeruginosa* PAO1 with isogenic mutants containing different DNA repair deficiencies. We determined that there was a prolonged survival in the *G. mellonella* waxworm assay only following inoculation with the 48-h-exposed *mutS P. aeruginosa* strain. Polymyxin B exposure over 48 h had no collateral impact on virulence for the PAO1 parent strain or the *mutM* strain. These findings are of importance as *mutS* deficiency in the mismatch repair (MMR) system is the most prevalent naturally occurring mutator (18). Additionally, the *mutS* strain is considered the strongest mutator and displays the highest mutation frequency compared to the *mutM* and PAO1 strains (19).

Our study is the first in *P. aeruginosa* to show a potential cost associated with development of polymyxin resistance. A similar relationship has been recently uncovered in *Staphylococcus aureus*, where attenuation of virulence was a result of the development of vancomycin-intermediate *S. aureus* (16, 20). The collateral impact of polymyxin B exposure on virulence may have important clinical implications in patients who receive long-term administration of antibiotics, such as in cases of nosocomial pneumonia or cystic fibrosis. In these persistent or chronic infections, there is a high prevalence of defective *mutS P. aeruginosa* strains (greater than 60%) (18, 21, 22) in which long-term administration of antimicrobials may alter the virulence capacity of *P. aeruginosa*. Therefore, understanding the pathogenic consequences of polymyxin administration may be important clinically and may ultimately provide novel approaches to combat *P. aeruginosa*.

Importantly, our data provide information regarding the factors influencing *P. aeruginosa* adaptation that lead to altered bacterial virulence. In the present study, we determined that development of resistance as measured by MIC alone was insufficient to explain the alterations. While all strains displayed similar MICs (8, 16, and 16 μg/ml) after antibiotic exposure, only the *mutS* strain exhibited attenuated virulence. These results support that the combination of a DNA mutation predisposition with an intensive resistance selection pressure (due to high polymyxin B concentrations) results in downstream virulence alterations. Further, we showed that this attenuation of virulence following polymyxin B exposure is not universal to all mutator phenotypes but occurs specifically with DNA mismatch repair deficiency. As the *mutS* strain has a considerably higher mutation rate than either the *mutM* or PAO1 strain, our data cannot rule out the possibility that the higher mutation rate causes attenuation of virulence (19).

Thus, the relationship between the emergence of antibiotic resistance and virulence may be pathogen or even antibiotic specific. Further studies using other antibiotics and mutator phenotypes and involving genomic/transcriptomic approaches are required to strengthen these findings.

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