Cadaverine Suppresses Persistence to Carboxypenicillins in *Pseudomonas aeruginosa* PAO1[∇]

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The refractory nature of *Pseudomonas aeruginosa* infections is due in part to the presence of specialized cells, termed persisters, within the population. To identify genes involved in *P. aeruginosa* persister formation, a PAO1 transposon (Tn) library was challenged *en masse* with 1,000 µg/ml of carbenicillin and was enriched for mutants that were able to survive in the presence of this antibiotic. For one mutant that was further characterized, the carbenicillin MIC was equal to that of PAO1, but persister formation exhibited a 20-fold increase after exposure to the antibiotic. Sequence analysis revealed that the Tn had inserted into PA4115, a gene encoding a putative lysine decarboxylase. A PA4115 mutant that produced 48-fold and 20-fold more survivors than PAO1 after 10-h exposures to carbenicillin and ticarcillin, respectively, was generated by allelic exchange. Furthermore, the rate of carboxypenicillin-induced lysis was reduced in the PA4115 mutant. Under certain pH conditions, lysine decarboxylase converts lysine to cadaverine. By measuring cadaverine production, we discovered that the PA4115 mutant had significantly reduced lysine decarboxylase activity. To determine if reduced cadaverine levels are responsible for the increase in carbenicillin and ticarcillin persistence, viability and lysis assays were performed in the presence of exogenous cadaverine. Cadaverine increased the rate of killing and lysis of the PA4115 mutant in the presence of both antibiotics. These findings suggest that cadaverine may be able to enhance the effectiveness of carboxypenicillins against *P. aeruginosa* by reducing persister formation.

Pseudomonas aeruginosa is an opportunistic pathogen that is responsible for a number of infections, including nosocomial pneumonia, chronic respiratory infections in cystic fibrosis patients, hospital-acquired urinary tract infections, and ocular infections (4, 16). The difficulty of treating infections caused by this bacterium may be attributed in part to its ability to form persisters (33). Upon antibiotic exposure, the majority of bacteria in both planktonic and biofilm cultures are killed, but survivors consisting of less than 1% of the original population remain (7, 33). Among the survivors, a subpopulation exists that exhibits various degrees of persistence (22, 43). According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, persisters differ from antibiotic-resistant cells in that they are not mutants and their growth is arrested by the same MIC of a bactericidal antibiotic as the wild type. But in contrast to the wild type, the minimum bactericidal concentration (MBC) of an antibiotic is higher for persister cells (6). Persister development has been attributed to a switch from fast to slow growth (1). Slow growth, however, cannot be the only reason for the refractory nature of persisters, because fluoroquinolones, which are known to be active against nongrowing cells, are unable to eliminate persisters (33, 39). Rather, persisters are believed to exist in a distinct physiological state, since the gene expression profiles of persisters differ from those of stationary-phase cells (31). The mechanism of persister formation remains unknown; however, several unrelated genes that in-

fluence persister formation have been identified in *Escherichia coli*, *P. aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Streptococcus pneumoniae* (24, 26, 32, 39, 40).

Besides specific “persistence-related genes,” environmental factors influence the bacterial response to drug treatment through induction of universal defense mechanisms. For example, the stringent response, a stress response induced under starvation conditions, has been implicated in the ability of *E. coli* (29) and *P. aeruginosa* (40) to persist in the presence of ampicillin and fluoroquinolones, respectively. Furthermore, natural polyamines (e.g., putrescine, spermidine, spermine, and cadaverine) impact bacterial adaptation to physiological stress. Most prokaryotes are able to synthesize polyamines *de novo* by enzymatic modification of precursor amino acids; however, they are also equipped with transport proteins for the utilization of extracellular polyamines (31). In *E. coli*, exogenous putrescine imparts nalidixic acid resistance to cells under oxidative stress through indirect regulation of OmpF translation (37). In a subsequent study, intracellular polyamine levels were found to increase in *E. coli* cells in response to sublethal concentrations of antibiotics (38). Polyamines also regulate the transcription of genes required for cell growth and viability, bacterial pathogenesis, and biofilm formation (30, 34).

The aim of the present study was to identify genes involved in *P. aeruginosa* persister formation. It has been proposed that persistence be defined according to the specific conditions under which the surviving cells were isolated, including the type and length of antibiotic treatment, the culture medium used, and the phase of growth (21). Here persisters are defined as logarithmic-phase bacterial cells that are able to survive a 10-h exposure to carboxypenicillins in Mueller-Hinton broth. We discovered a gene encoding a putative lysine decarboxylase

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype or relevant phenotype ^a	Source or reference
Plasmids		
pCM639	IS <i>phoA</i> /hah Chl ^r Tet ^r	12
pCR2.1	TA cloning vector Amp ^r	Invitrogen
pCR4115	PA4115 in pCR2.1	This study
pCR4115a	pCR4115 with a 300-bp deletion in PA4115	This study
pEX18Ap	Suicide plasmid; Amp ^r	12
pEX4115	PA4115 in pEX18Ap	This study
pUCP22	Broad-host-range vector; Amp ^r Gent ^r	41
pUCP4115	PA4115 in pUCP22	This study
pUCP4115a	pUCP4115 Gent ^r	This study
Strains		
<i>P. aeruginosa</i>		
PAO1	Wild type	11
Mutant 77	PAO1 PA4115::IS <i>phoA</i> /hah	This study
PAO115	PAO1 with a 300-bp deletion in PA4115	This study
PAO4115(pUCP4115a)	PAO4115 harboring pUCP4115a	This study
<i>E. coli</i> SM10λpir		
	<i>thi-1 thr leu tonA lacY supE recA</i> ::RP4-2-Tet::Mu(Kan ^r)	12
Primers		
PA4115-fwd	5'-ATGCCCCCTTGCCCTGGTA-3'	This study
PA4115-rev	5'-CGGGGCGACCTGTTTCGAGA-3'	This study
CEKG 2A	5'-GGCCACGCGTCGACTAGTAC(N) ₁₀ AGAG-3'	12
CEKG 2B	5'-GGCCACGCGTCGACTAGTAC(N) ₁₀ ACGCC-3'	12
CEKG 2C	5'-GGCCACGCGTCGACTAGTAC(N) ₁₀ GATAT-3'	12
CEKG 4	5'-GGCCACGCGTCGACTAGTAC-3'	12
HAH MINUS 114	5'-CCCCGGTTTTCCAGAACAG-3'	12
HAH MINUS 138	5'-CGGGTGCAGTAATATCGCCCT-3'	12
HAH MINUS 166	5'-TCACCCGTTAAACGGCGA-3'	12

^a Amp, ampicillin; Chl, chloramphenicol; Gent, gentamicin; Kan, kanamycin; Tet, tetracycline.

(PA4115) that is able to suppress *P. aeruginosa* persistence to carboxypenicillins. We demonstrate that exogenous cadaverine restores the susceptibility of the PA4115 mutant to carbenicillin and ticarcillin. The results presented here suggest that cadaverine may be involved in persister formation in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *P. aeruginosa* and *E. coli* strains were routinely cultured on Luria-Bertani (LB) agar plates or in LB broth (Difco Laboratories, Detroit, MI) at 37°C. *P. aeruginosa* strains were tested for susceptibility to antibiotics in cation-adjusted Mueller-Hinton broth (CAMHB; Fisher Scientific, Fair Lawn, NJ) supplemented with antibiotics or cadaverine (Sigma-Aldrich Canada, Oakville, ON, Canada) as needed. The penicillinase used in MBC determinations was obtained from Sigma. The antibiotics used in this study, purchased from the Research Products International Corp. (Mt. Prospect, IL), are as follows: ampicillin, carbenicillin, gentamicin, polymyxin E, and ticarcillin. The following antibiotics were purchased from Sigma: ceftazidime, ceftriaxone, chloramphenicol, piperacillin, tazobactam, and tetracycline. Ciprofloxacin was obtained from Bayer (Leverkusen, Germany). Meropenem was obtained as a gift from AstraZeneca (Wilmington, DE).

Isolation of a carbenicillin-persistent mutant. A *P. aeruginosa* PAO1 IS*phoA*/hah transposon (Tn) library was generated through biparental mating of strain PAO1 with *E. coli* SM10λpir (pCM639). Transconjugants were selected on LB agar supplemented with tetracycline (60 µg/ml) and chloramphenicol (10 µg/ml). A total of 5,000 mutants were generated and pooled in LB broth. Skim milk stocks of pooled cultures were made and stored at -80°C. For the isolation of persister mutants, pooled cultures were grown to mid-logarithmic phase in 15 ml of LB broth and were challenged *en masse* with 1,000 µg/ml of carbenicillin for 4 h. The cultures were washed twice with fresh LB broth, challenged a second

time by plating onto LB agar containing 1,000 µg/ml of carbenicillin, and incubated at 37°C for 24 h. The plates were subsequently sprayed with 2,000 U penicillinase and were then incubated at 37°C for an additional 18 h. Colonies that appeared after this treatment were considered putative carbenicillin-persistent mutants. Individual colonies were streaked onto LB agar plates supplemented with 1,000 µg/ml of carbenicillin and were incubated at 37°C overnight. The plates were treated once again with penicillinase and were incubated for 18 h. Mutants that produced confluent growth after treatment with penicillinase were tested for susceptibility to antibiotics as described below.

Identification of the transposon insertion site. To determine the location of the Tn insertion, a two-step semidegenerate PCR procedure was employed as previously described (12), using primers listed in Table 1. The PCR product was sequenced at the University of Calgary Core DNA Services facility, and the sequence was analyzed with the blastn and blastx databases.

Creation of a PA4115 mutant and a PA4115 overexpression plasmid. A 2.7-kb DNA fragment containing the PA4115 gene from PAO1 was PCR amplified using the primers listed in Table 1. The PCR product was cloned into pCR2.1 (Invitrogen, Burlington, ON, Canada) to generate pCR4115. A 300-bp fragment within the PA4115 coding sequence was deleted by digestion with HincII to generate pCR4115a. The 2.4-kb fragment was then excised from pCR4115a and cloned into the suicide plasmid pEX18Ap (10), creating pEX4115. Allelic exchange through biparental mating between *E. coli* SM10(pEX4115) and PAO1 was used to replace the wild-type PA4115 gene with the mutated copy of the gene. Transconjugants were screened on *Pseudomonas* isolation agar (PIA; Fisher Scientific) plates supplemented with 100 µg/ml of carbenicillin. Bacteria that had undergone a double-crossover event were selected on PIA containing 10% sucrose. Putative PA4115 mutants were subsequently plated on carbenicillin-supplemented PIA to confirm the restoration of sensitivity to this antibiotic. The mutation was verified by PCR with the same primers to obtain a 2.4-kb fragment. To generate a vector overexpressing the PA4115 gene, the 2.7-kb fragment from pCR4115 was directionally cloned into the shuttle vector pUCP22 (42) via BamHI and SacI sites. The ampicillin resistance marker was removed

from this construct by digestion with BspHI, creating pUCP4115a. This plasmid was mobilized into PAO4115 for complementation purposes.

Testing of planktonic cultures for susceptibility to antibiotics. MIC and MBC testing was performed via the broth macrodilution method in accordance with CLSI guidelines (6). Briefly, antibiotics were added to 1 ml of CAMHB, and 2-fold serial dilutions were performed. Logarithmic-phase cultures of *P. aeruginosa* were diluted and inoculated into each tube to yield a final concentration of 1×10^6 CFU/ml. Cell cultures were then incubated at 37°C for 24 h. For MIC testing requiring cadaverine, cadaverine was added at specific concentrations to each tube prior to incubation for 24 h. The MIC was defined as the lowest concentration of a given antibiotic that inhibited the growth of the organism within 24 h. A 10- μ l aliquot from tubes containing no growth was diluted and plated onto both LB agar alone and LB agar supplemented with penicillinase for MBC determination. The MBC was defined as the concentration of the antibiotic that killed 99.9% of the organisms.

Biofilm formation and antibiotic susceptibility testing. Biofilm formation was determined using 96-well microtiter plates and was measured spectrophotometrically after staining with crystal violet as described by O'Toole and Kolter (27). The susceptibility of biofilms to antibiotics was assessed by a method devised by Parkins et al. (28) whereby biofilms were grown on pegs attached to the lid of a 96-well microtiter plate (Nunc, Rochester, NY). Briefly, the biofilms were exposed to various concentrations of antibiotics (10 $\times$, 25 $\times$, 50 $\times$, 100 $\times$, 200 $\times$, 400 $\times$, and 800 $\times$ MIC) aliquoted into the microtiter plate. To determine biofilm viability after a 24-h exposure to antibiotics, the lid was transferred to a microtiter plate containing fresh medium and was sonicated for 20 min using a Branson 1510 sonicator (Branson Ultrasonics Corporation, Danbury, CT), and aliquots were plated onto LB agar plates (33). The CFU count per milliliter was determined after 24 h of incubation at 37°C. The minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration inhibiting the growth of the organism.

Concentration-dependent and time-dependent killing assays. For concentration-dependent killing assays, *P. aeruginosa* strains were grown in 10 ml CAMHB in 125-ml flasks to mid-logarithmic phase and were challenged with 0 $\times$, 1 $\times$, 2 $\times$, 4 $\times$, and 8 $\times$ the MIC of carbenicillin for 4 h at 37°C. A 100- μ l aliquot was removed, and 10-fold serial dilutions were performed in 1 $\times$ phosphate buffered saline (PBS). A 50- μ l aliquot of the diluted sample was plated onto LB agar plates using the Autoplate 4000 (Spiral Biotech, Norwood, MA) and was incubated overnight for determination of viability. Time-dependent killing assays were performed in a similar manner; however, mid-logarithmic-phase cultures were subjected to either 8 $\times$ the MIC of carbenicillin or 64 $\times$ the MIC of ticarcillin. A 100- μ l aliquot was removed at 1-h intervals for as long as 10 h, serially diluted, and plated onto LB agar plates for viability determination. For assays requiring cadaverine, cultures were grown to mid-logarithmic phase in the presence of cadaverine prior to the addition of antibiotics.

Antibiotic-induced lysis. *P. aeruginosa* strains were grown to mid-logarithmic phase in 10 ml CAMHB with or without cadaverine and were exposed to carbenicillin or ticarcillin. Culture turbidity (optical density at 600 nm [OD₆₀₀]) was determined using the Ultrospec 500 pro spectrophotometer (Biochrom Ltd., Fisher Scientific) at 1-h intervals for 10 h.

Detection of lysine decarboxylase activity. The lysine decarboxylase assay was performed as described by Lemonnier and Lane (17). Briefly, *P. aeruginosa* strains were grown in 5 ml MHB for 16 h at 37°C. A 5-ml volume of culture was centrifuged at 6,000 rpm, washed in a 1 M NaCl–0.05 M potassium phosphate solution, and resuspended in 200 μ l of 20 mM potassium phosphate (pH 6.8). A drop of CHCl₃ was added to the suspension, which was mixed using a vortex. For the assay, 50 μ l of the suspension was added to a solution of 5 mM lysine, 0.1 mM pyridoxal phosphate, and 16 mM potassium phosphate (pH 6.8) to achieve a final volume of 120 μ l. To determine the background levels of endogenous polyamines, a parallel mixture without lysine was prepared. After the mixtures were incubated at 37°C for 15 min, 120 μ l of 1 M Na₂CO₃ was added, and the mixtures were placed on ice and then treated with 120 μ l of 2,4,6-trinitrobenzenesulfonate (Sigma). The mixtures were incubated at 40°C for 4 min and were subsequently extracted with 1 ml toluene. Lysine decarboxylase activity was measured as the difference in A₃₄₀ between the samples incubated with lysine and those incubated without lysine. Specific activity was calculated as $1,000 \times A_{340}$ unit per min per OD₆₀₀ unit of whole cells.

RESULTS

Isolation of a carbenicillin-persistent mutant. Moyed and Bertrand (24) identified a high-persistence locus (*hipAB*) in *E. coli* whereby *hipA7* mutants exhibited a 1,000-fold increase in

FIG. 1. Killing curves of *P. aeruginosa* PAO1 and mutant 77 in the presence of carbenicillin. (A) Cells were exposed to varying concentrations of carbenicillin (μ g/ml) for 4 h. (B) Cells were exposed to 8 $\times$ the MIC (800 μ g/ml) of carbenicillin for as long as 10 h. $\blacklozenge$, PAO1; $\blacksquare$, mutant 77.

survival upon ampicillin exposure. Since there are no *P. aeruginosa* genes with homology to this *hip* locus, a similar screening procedure for genes involved in β -lactam tolerance was employed. Carbenicillin MICs and MBCs were determined for four mutants isolated from this process. According to the CLSI guidelines, an organism is said to exhibit persistence to an antibiotic if it has an MBC/MIC ratio greater than or equal to 32 (6). None of the mutants met these criteria; however, one mutant, called mutant 77, had a carbenicillin MIC equivalent to that of PAO1 and reproducibly exhibited a carbenicillin MBC 2-fold higher than that of the wild type (data not shown). To confirm that this strain in fact exhibits increased persistence to carbenicillin, mutant 77 was challenged with carbenicillin in a dose-dependent and time-dependent manner. After exposure to carbenicillin concentrations ranging from 1 $\times$ MIC to 8 $\times$ MIC, at the highest concentration (800 μ g/ml), mutant 77 produced 13.5-fold more survivors than the wild type (Fig. 1A). Furthermore, after a 10-h exposure to 8 $\times$ the MIC of carbenicillin, there were 20-fold more survivors in the mutant population than in the PAO1 parent population (Fig. 1B).

Identification of the *P. aeruginosa* persistence gene PA4115. Sequencing of the DNA flanking the Tn insertion in mutant 77 revealed that a gene encoding a putative lysine decarboxylase (PA4115) had been mutated (data not shown). To determine if the observed phenotype is the result of the disruption of this gene, a PA4115 knockout strain (PAO4115) was generated. The carbenicillin MIC for PAO4115 was equal to that of

TABLE 2. Susceptibilities of PAO1 and its derivatives to carboxypenicillins

Strain	Carbenicillin		Ticarcillin	
	MIC ^a	MBC ^a	MIC	MBC
PAO1(pUCP22)	100	200	32	1,024
PAO4115(pUCP22)	100	400	32	1,024
PAO4115(pUCP4115a)	100	200	32	1,024

^a MICs and MBCs are given in micrograms per milliliter.

PAO1, and the carbenicillin MBC was 2-fold greater than that for the wild type (Table 2). MBC determinations were performed using L agar alone and L agar supplemented with penicillinase (2,000 U) to eliminate potential carryover effects from the carbenicillin. The addition of penicillinase did not impact the MBC of carbenicillin (data not shown). After a 10-h exposure to 8× the MIC of carbenicillin, PAO4115 generated 48-fold more survivors than PAO1 (Fig. 2A). Furthermore, the complemented strain, PAO4115(pUCP4115a), was as susceptible to carbenicillin as PAO1 (Fig. 2A). These findings are consistent with the data obtained for mutant 77.

Planktonic cultures of PAO4115 exhibit a higher level of carboxypenicillin persistence than PAO1. The impact of PA4115 on PAO1 multidrug persistence was assessed. MIC and MBC determinations were performed using representatives from different classes of antibiotics as a rudimentary means of identifying persistence. Ceftriaxone, ciprofloxacin, meropenem, piperacillin-tazobactam, polymyxin E, and tobramycin were chosen because of their known activities against *P. aeruginosa*. The MICs of all antibiotics tested for PAO4115 did not deviate from those for the wild type, nor did the MBCs for

TABLE 3. Lysine decarboxylase activities of PAO1 and its derivatives

Strain	Sp act ^a of lysine decarboxylase in whole cells
PAO1(pUCP22)	21.15 (±3.8)
PAO4115(pUCP22)	15.00 (±1.6)
PAO4115(pUCP4115a)	23.87 (±4.0)

^a Calculated as $1,000 \times A_{340}$ unit per min/OD₆₀₀ unit of whole cells. Values in parentheses are standard deviations from three replicates.

the antibiotics tested (data not shown). Since we have shown time-dependent killing assays to be a better means of ascertaining the degree of persistence, these studies were performed. No difference in time-dependent killing was observed between the mutant and the wild type upon exposure to the antibiotics listed above (data not shown). Because PAO4115 exhibited a 2-fold increase in the MBC of carbenicillin (Table 2), we assessed the MBC of a second antibiotic belonging to the same class, namely, ticarcillin. PAO4115 did not exhibit an increase in the MBC of this antibiotic (Table 2); however, it was found to produce 20-fold more survivors than PAO1 after a 10-h exposure to ticarcillin (64× MIC) (Fig. 2B). Susceptibility to ticarcillin was restored in PAO4115(pUCP4115a). For the time-kill assays, cells were exposed to 8× the MIC of carbenicillin and 64× the MIC of ticarcillin, because these concentrations reproducibly resulted in a 0.1% survival rate.

Cell death induced by β-lactams occurs through both the lytic and nonlytic pathways (2). To assess whether PA4115 influences cell lysis, PAO1, PAO4115, and the complemented strain were exposed to carbenicillin and ticarcillin, and turbidity was monitored spectrophotometrically at 1-h intervals. Minimal lysis was observed with carbenicillin; however, of the three strains tested, PAO4115 lysed at a lower rate than the wild type (Fig. 2C). Similarly, ticarcillin-induced lysis of PAO4115 was 1.6-fold and 1.8-fold less than those of the wild-type and complemented strains, respectively (Fig. 2D).

PA4115 does not affect biofilm formation or biofilm tolerance of antibiotics. The abilities of PAO1 and PAO4115 to form biofilms and persist in the presence of antibiotics were assessed. No differences between the two strains were observed (data not shown).

PA4115 is involved in cadaverine biosynthesis. Under certain pH conditions, lysine decarboxylase converts lysine to cadaverine. Because PA4115 encodes a putative lysine decarboxylase, we investigated the involvement of PA4115 in cadaverine production. PAO1, PAO4115, and the complemented strain were assessed for lysine decarboxylase activity. In *E. coli*, the *cadAB* operon and *ldc* are required for the production of lysine decarboxylase induced at an acidic pH (5.6) and a neutral pH, respectively (17, 30). Since cadaverine production is influenced by the pH of the medium, the lysine decarboxylase activities of PAO1 and its derivatives were determined at pH 5.6 and 6.8. At the acidic pH, no difference in lysine decarboxylase activity exists among the three strains (data not shown). However, at the neutral pH, a 1.4-fold decrease in cadaverine production was observed for PAO4115 relative to PAO1, indicating that the product of PA4115 exhibits lysine decarboxylase activity (Table 3).

FIG. 2. Time-dependent killing and lysis assays for bacteria incubated in the presence of carboxypenicillins. Cells were exposed to 8× the MIC of carbenicillin (A and C) or 64× the MIC of ticarcillin (B and D) and were assessed for viability (A and B) and cell lysis (C and D). ♦, PAO1(pUCP22); ■, PAO4115(pUCP22); ▲, PAO4115(pUCP4115a).

TABLE 4. Effects of cadaverine on the susceptibilities of PAO1 and its derivative to carbenicillin and ticarcillin

Strain and antibiotic	MIC ($\mu\text{g/ml}$) of the antibiotic in the presence of cadaverine at the following concn (mM) ^a :						
	0	0.25	0.5	1	5	10	20
PAO1(pUCP22)							
Carbenicillin	100	100	100	50	50	50	12.5
Ticarcillin	32	32	32	16	16	16	8
PAO4115(pUCP22)							
Carbenicillin	100	100	100	50	50	50	12.5
Ticarcillin	32	32	32	16	16	16	8

^a Cadaverine had no effect on bacterial growth at concentrations up to and including 20 mM.

Cadaverine increases the carboxypenicillin-mediated killing of PAO4115. Cadaverine has previously been reported to decrease the MIC of carbenicillin for PAO1 (15). To determine if cadaverine has the same effect on ticarcillin as on carbenicillin, the MICs of the two carboxypenicillins for PAO1 and PAO4115 were assessed in the presence of varying concentrations of cadaverine. As shown in Table 4, increasing the concentration of cadaverine increased the susceptibilities of these strains to both carbenicillin and ticarcillin. The addition of 20 mM cadaverine reduced the MICs of carbenicillin and ticarcillin 8-fold and 4-fold, respectively, for both strains.

Next, we wanted to establish whether the addition of exogenous cadaverine could restore the antibiotic susceptibility of PAO4115 to a wild-type level. Cultures were supplemented with the highest concentration of cadaverine (0.5 mM) that did not impact the MICs of carbenicillin and ticarcillin for PAO1 and PAO4115. Exposure to cadaverine prior to carbenicillin challenge increased the rates of killing and lysis of the PAO4115 mutant over those of the unexposed strain (Fig. 3A and C). The effects of cadaverine on killing and lysis by ticarcillin were even more pronounced (Fig. 3B and D). The wild-type strain, PAO1, was also rendered more susceptible to ticarcillin upon cadaverine exposure; however, this difference was not statistically significant (Fig. 3B and D).

DISCUSSION

During treatment with bactericidal antibiotics, the majority of the bacteria are rapidly killed, while persisters remain to replenish the population once the antibiotic has been removed (33). The presence of persister cells, therefore, can complicate the treatment of bacterial diseases (18, 19, 20). The aim of the current study was to identify genes involved in *P. aeruginosa* persister formation. Transposon mutagenesis of strain PAO1 enabled the discovery of a gene encoding a putative lysine decarboxylase (PA4115) that suppresses the ability of this organism to survive carboxypenicillin exposure. Zhang (43) has proposed that within a persister population, subgroups exist that differ in their responses to antibiotic treatment, and enrichment of these subgroups is dependent on the length of antibiotic exposure (22). “Deep persisters” are isolated after long (for example, 5-day) exposure to antibiotics, while short exposures (<1 day) may lead to the isolation of “shallow/intermediate” persisters (22). The latter group is proposed to

be killed at higher antibiotic concentrations or by longer antibiotic exposure. In this study, mutant 77 was isolated after a 28-h exposure to carbenicillin, and its ability to persist in the presence of this antibiotic was determined after 10 h. Mutant 77 may represent a shallow persister, which could explain the discrepancy observed between the MBC/MIC determinations and time-kill studies. For both Tn mutant 77 and its isogenic derivative PAO4115, the carbenicillin MBC showed only a 2-fold increase over that of the wild type. Yet time-dependent killing assays revealed that PAO4115 exhibits a markedly enhanced ability to persist in the presence of carbenicillin and ticarcillin (Fig. 2A and B). Our results indicate that PA4115 is involved in *P. aeruginosa* persister formation and that time-dependent killing assays are more sensitive than MBC assays for detecting antibiotic persistence. The latter finding is in agreement with those of other groups who have reported that MBC determinations are an ineffective means of assessing persistence (3, 23, 35).

Intracellular levels of polyamines are an important determinant of the susceptibility of an organism to antibiotics. In prokaryotes, polyamines exist at millimolar concentrations and are affected by the presence of polyamines in the medium (34). Kwon and Lu have demonstrated that the addition of polyamines to bacteria altered the MICs of a variety of antibiotics (13, 14, 15). In the current study, PAO4115 exhibited reduced killing by carbenicillin and ticarcillin compared to that of PAO1 (Fig. 2). Analysis of PAO4115 revealed decreased lysine decarboxylase activity at a neutral pH (Table 3). Since PA4115 is one of several genes believed to be involved in cadaverine synthesis by PAO1, it is not surprising that disruption of this allele did not drastically decrease the cadaverine content in

FIG. 3. Time-dependent killing and lysis assays for bacteria exposed to carboxypenicillins after growth in the presence or absence of cadaverine. Cells were exposed to 8 $\times$ the MIC of carbenicillin (A and C) or 64 $\times$ the MIC of ticarcillin (B and D) and were assessed for viability (A and B) and cell lysis (C and D). $\blacklozenge$, PAO1(pUCP22); $\blacksquare$, PAO4115(pUCP22); $\blacktriangle$, cadaverine-treated PAO1(pUCP22); $\bullet$, cadaverine-treated PAO4115(pUCP22).

these cells. In *E. coli*, cadaverine synthesis has been found to be pH dependent (17). The decrease in the lysine decarboxylase activity of PAO4115 was not observed at an acidic pH (5.6); however it was apparent at pH 6.8. Importantly, the pH of the medium used for the persistence assays, Mueller-Hinton broth, is 6.8. The addition of 0.5 mM cadaverine to PAO4115 cultures was able to partially complement the PA4115 mutation when the cultures were exposed to carbenicillin (Fig. 3A) and to fully complement the mutation when they were exposed to ticarcillin (Fig. 3B). The reason for the discrepancy in the effects of cadaverine on the two antibiotics is not clear at present.

The mechanism whereby polyamines increase susceptibility to β -lactams has not been fully elucidated; however, some studies have investigated the impacts of these biogenic amines on various mechanisms of resistance. In *E. coli*, polyamines bind to the inner channels of porin proteins OmpF and OmpC, thereby inhibiting the entry of β -lactams and quinolones into the cell (8). Since the addition of cadaverine increased the susceptibilities of PAO1 and PAO4115 to carbenicillin and ticarcillin (Table 4), it seems highly unlikely that the influx of carboxypenicillins is inhibited by cadaverine. Kwon and Lu reported that spermidine had no effect on the *P. aeruginosa* *ampC*-encoded β -lactamase at either the genetic or the protein level (15). Rather, these researchers postulated that the enhanced susceptibility to β -lactams demonstrated by *Staphylococcus aureus* in the presence of spermine may be due to increased β -lactam-mediated acylation of penicillin-binding proteins (PBPs) or decreased expression of PBP2a, the PBP implicated in methicillin resistance. Carbenicillin and ticarcillin preferentially bind to the same PBPs in *P. aeruginosa* (25). Therefore, a decrease either in the expression of PBPs with high affinity for these carboxypenicillins or in carboxypenicillin-mediated PBP acylation may account for the increase in PAO4115 persistence.

Shortly after β -lactam exposure, cells lose viability, followed by cell lysis that is dependent on the function of murein hydrolases (2) and the inhibition of cell division and subsequent filament formation (9). Thus, an impaired lytic system may also manifest in increased persistence in the presence of antibiotics. In this study, we found that PAO4115 lysed at a lower rate than the wild-type strain in the presence of carbenicillin and ticarcillin (Fig. 2C and D). Furthermore, the addition of exogenous cadaverine enhanced carbenicillin- and ticarcillin-induced lysis of PAO4115 (Fig. 3C and D). Since 0.5 mM cadaverine did not promote filament formation in PAO1 and its derivatives (data not shown), it is unlikely that cadaverine primes the cell for lysis through this mechanism. It appears more likely that cadaverine enhances murein hydrolase expression or activity, thereby enhancing carboxypenicillin-induced lysis.

The work presented here suggests that cadaverine increases the activity of β -lactams by overcoming persister formation. It should be noted, however, that a number of limitations are associated with this study. First, our initial screening method for mutants with altered persistence to carbenicillin likely resulted in the isolation of a "shallow/intermediate" persister mutant. Longer antibiotic exposure during enrichment may allow for the isolation of mutants with a stronger persister phenotype. Second, the concentrations of antibiotics used were above those considered clinically achievable. When we exposed PAO4115 to ticarcillin ($5\times$ MIC), the highest clinical dose

achievable in serum, this strain produced 3.6-fold and 7.7-fold more survivors than PAO1 after 10-h and 24-h exposures, respectively (data not shown). Therefore, PAO4115 persistence is observed at lower antibiotic concentrations; however, the persistence phenotype becomes more pronounced as the exposure time increases. Third, to more firmly establish that a lysine decarboxylase deficiency imparts a persister phenotype to *P. aeruginosa*, all of the genes involved in cadaverine synthesis should be systematically inactivated. The resulting mutant should then be tested for its ability to persist in the presence of carboxypenicillins as well as other antibiotics. Fourth, the use of cadaverine as a therapeutic agent has not been clinically tested, although animal studies suggest that it is a safe agent, even at high doses (36). Based on the assessment of the organ development, growth, and behavioral changes of rats treated with cadaverine, the no-observed-adverse-effect level (NOAEL) of this amine was determined to be 2,000 ppm (180 mg/kg of body weight/day) (36). In this study, we observed that *P. aeruginosa* grown in the presence of 20 mM (or 2.04 mg/ml) cadaverine showed increased susceptibility to carboxypenicillins. *In vivo* studies are required to determine if these high cadaverine concentrations are adequate for enhancing β -lactam potency with adequate safety. Finally, cadaverine has an unpleasant odor, which might make patient compliance difficult. Notwithstanding these limitations, the idea that polyamines could be used in combination with existing β -lactams for the treatment of *P. aeruginosa* infections is intriguing and warrants further investigation.

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