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Polyamine Effects on Antibiotic Susceptibility in Bacteria▽

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Biogenic polyamines (e.g., spermidine and spermine) are a group of essential polycationic compounds found in all living cells. The effects of spermine and spermidine on antibiotic susceptibility were examined with gram-negative *Escherichia coli* and *Salmonella enterica* serovar Typhimurium bacteria and clinical isolates of *Pseudomonas aeruginosa* and with gram-positive *Staphylococcus aureus* bacteria, including methicillin-resistant *S. aureus* (MRSA). Exogenous spermine exerted a dose-dependent inhibition effect on the growth of *E. coli*, *S. enterica* serovar Typhimurium, and *S. aureus* but not *P. aeruginosa*, as depicted by MIC and growth curve measurements. While the MICs of polymyxin and ciprofloxacin were in general increased by exogenous spermine and spermidine in *P. aeruginosa*, this adverse effect was not observed in enteric bacteria and *S. aureus*. It was found that spermine and spermidine can decrease the MICs of β-lactam antibiotics in all strains as well as other types of antibiotics in a strain-dependent manner. Significantly, the MICs of oxacillin for MRSA Mu50 and N315 were decreased more than 200-fold in the presence of spermine, and this effect of spermine was retained when assessed in the presence of divalent ions (magnesium or calcium; 3 mM) or sodium chloride (150 mM). The effect of spermine on the sensitization of *P. aeruginosa* and MRSA to antibiotics was further demonstrated by population analysis and time-killing assays. The results of checkerboard assays with *E. coli* and *S. aureus* indicated a strong synergistic effect of spermine in combination with β-lactams and chloramphenicol. The decreased MICs of β-lactams implied that the possible blockage of outer membrane porins by exogenous spermine or spermidine did not play a crucial role in most cases. In contrast, only the MIC of imipenem against *P. aeruginosa* was increased by exogenous spermine and spermidine, and this resistance effect was abolished in a mutant strain devoid of the outer membrane porin OprD. In *E. coli*, the MICs of carbenicillin, chloramphenicol, and tetracycline were decreased in two *acrA* mutants devoid of a major efflux pump, AcrAB. However, retention of the spermine effect on antibiotic susceptibility in two *acrA* mutants of *E. coli* suggested that the AcrAB efflux pump was not the target for a synergistic effect by spermine and antibiotics and ruled out the hypothesis of spermine serving as an efflux pump inhibitor in this organism. In summary, this interesting finding of the effect of spermine on antibiotic susceptibility provides the basis for a new potential approach against drug-resistant pathogens by use of existing β-lactam antibiotics.

Biogenic polyamines (e.g., putrescine, spermidine, and spermine) are essential cationic compounds in all living organisms (1, 11). Inside the cells, these positively charged molecules are mostly associated with acidic macromolecules, such as genomic DNA and rRNA. The involvement of polyamine in numerous cellular functions has been extensively studied in eukaryotes and bacteria (3, 11, 13–15).

Recently, we reported that exogenous polyamine can affect the antibiotic susceptibility of *Pseudomonas aeruginosa* PAO1 in two opposite ways (11, 18, 19). Specifically, exogenous polyamine increased the MICs of cationic peptides, aminoglycosides, and quinolone antibiotics. At the genetic level, exogenous polyamine induced the expression of the *oprH-phoPQ* operon and the *pmrHFIJKLM* operon, both of which participate in the resistance mechanisms for polymyxin B and other cationic peptide antibiotics (25, 26). On the other hand, the MICs of β-lactams and chloramphenicol were decreased by the presence of exogenous polyamine. While the possible molecular mechanism for this observed sensitization effect is not known, we have ruled out the possibility of polyamine effects on the expression or specific activity of β-lactamase and on the disruption of outer membrane permeability (18).

In *Escherichia coli*, polyamine was reported to block the flow of certain β-lactams through the OmpF and OmpC porins, which serve as channels for the entrance of these antibiotics through the outer membrane (5). Similarly, the outer membrane porin OprD of *P. aeruginosa* was reported as the channel for a specific type of β-lactam antibiotics including imipenem and meropenem (22, 33). Once inside the cells, these antibiotics can still be extruded by the operation of efflux pumps, e.g., the AcrAB complex of *E. coli* (20, 31). An increased level of efflux pump activity is one of the major mechanisms for multidrug resistance in clinical isolates (32). Potentially, this adverse effect of induced efflux can be overcome by efflux pump inhibitors, which are compounds that physically block the pumps (16, 23).

Without an outer membrane, gram-positive bacteria are highly subject to the antibacterial activities of β-lactams. However, the emergence and spread of strains resistant to β-lactams have been a major problem in clinical treatments, e.g., methicillin-resistant *Staphylococcus aureus* (MRSA) in the health care setting. Even more worryingly, MRSA is now emerging in community clinical treatments. Vancomycin and teicoplanin have been the last line of antibiotics against MRSA, but overuse has led to the emergence of vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and

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VRSA, respectively). The treatment options for these infections are severely compromised, and thus, new antimicrobial remedies against MRSA, VISA, and VRSA are urgently required (4, 7, 17, 28).

In this study, we extended the initial observation of polyamine effects on antibiotic susceptibility to clinical isolates of *P. aeruginosa* and strains of *E. coli*, *Salmonella enterica* serovar Typhimurium, and *S. aureus*, including MRSA and VISA. The possible interaction of polyamine with the outer membrane porin OprD and its subsequent effect on the efficacies of imipenem and other β-lactams were characterized in an *oprD* knockout mutant of *P. aeruginosa* PAO1. We also analyzed the potential effect of spermine on drug efflux by employing the AcrA mutant strains of *E. coli*. In addition, the results indicated that exogenous spermine enhances killing of these bacteria by β-lactams with a strong synergistic effect.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. aureus* strains Mu50 and N315 were kindly provided by Mark Smeltzer of the University of Arkansas, Little Rock, and *S. aureus* strain ATCC 35556 was from the American Type Culture Collection. *E. coli* type strains K-10 and K-12 and an enterotoxigenic strain, C912-61, were kindly provided by Phang C. Tai of Georgia State University, and 10 clinical isolates of *P. aeruginosa* were gifts from James Versalovick of the Baylor College of Medicine. *P. aeruginosa* PAO1 and *Salmonella enterica* serovar Typhimurium LT2 were from the stock cultures of this laboratory. Other strains used in this study included *E. coli* N43 (*acrA*) and N818 (Δ*acrA*) and their parent strain, W4573 (24). All bacterial strains were routinely grown on Luria-Bertani (LB) agar plates or broth at 37°C. When mentioned, *P. aeruginosa* strains were also grown in minimal medium P with supplements as indicated (19). Divalent, cation-adjusted Mueller-Hinton (MH) (Oxoid, Ogdensburg, NY) broth was used for the antibiotic susceptibility tests. Appropriate concentrations of antibiotics, polyamines, or other chemicals were supplemented when needed. All antibiotics, polyamines (spermidine trihydrochloride and spermine tetrahydrochloride), and other chemicals used in this study were purchased from Sigma (Sigma, St. Louis, MO). Solutions of these compounds were prepared by dissolving in sterile double-distilled water or solvent suggested by the manufacturer and sterilized by filtering through the 0.4-μm disposable membranes (Millipore, Billerica, MA).

Polyamines and antibiotic susceptibility tests. The MICs of polyamines (spermidine and spermine) and antibiotics were determined by the standard broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS) (29, 30). Serial twofold dilutions of tested compounds were done in the MH broth, and additional compounds, such as $MgSO_4$, $CaCl_2$, and NaCl, were added when needed to the MH broth before dilutions. Fresh overnight cultures of each bacterial strain were diluted in saline to an optical density at 600 nm (OD_{600}) of 0.1 to 0.12 (approximately 1×10^8 to 5×10^8 CFU/ml), and a portion of the adjusted cell suspension (1 to 5 μl for ~10^5 CFU) was inoculated. The cell cultures were incubated overnight (14 to 16 h) at 37°C. MIC measurements were repeated and confirmed by three independent experiments.

Growth curve measurements. The overnight culture was diluted 100-fold in 50 ml of the fresh MH broth, and bacterial growth was continued at 37°C with shaking (350 rpm) until the OD_{600} reached around 0.6. The fresh culture was then diluted 10-fold in 50 ml of prewarmed MH broth supplemented with an indicated concentration of spermine or spermidine, and the growth rate of the cells was monitored by OD_{600} measurements.

Checkerboard assays. An array of combinations was made between 0 and 8 mM of spermine and 0 and 1,024 μg/ml of different antibiotics. The bacterial inoculums and incubation conditions of each combination were the same as those used in the MIC measurements by the standard broth dilution method described above. The fractional inhibitory concentration (FIC) was calculated as described by White et al. (36). Synergy was defined as an FIC index of ≤0.5, indifference as an FIC index of >0.5 but ≤4, and antagonism as an FIC index of >4 (36).

Time-killing assays. The bacterial culture grown overnight in the MH broth was diluted in saline to an OD_{600} reading of 0.08 to 0.12, and an aliquot of the diluted cells (1×10^5 to 5×10^5 CFU) was inoculated into 1 ml of the MH broth in the presence or absence of the indicated compounds. The culture was incubated at 37°C without shaking, and aliquots (100 μl) were withdrawn at specific time intervals as indicated and spread on LB plates undiluted or after 10-fold serial dilutions. Bacterial colonies on the plates were counted after 18 to 24 h of incubation at 37°C. Synergy was defined as a ≥2-$\log_{10}$ decrease in the number of CFU/ml below the starting inoculum level at 24 h with the combination of spermine and the tested antibiotic compared with the result for spermine or the antibiotic alone (36).

Population analysis. Population analysis of bacterial strains was essentially done as described previously (10). Briefly, bacterial cultures were grown overnight at 37°C and cell numbers were adjusted to 1×10^8 to 5×10^8 CFU/ml. Aliquots of the cells (100 μl) after appropriate dilutions were spread on three types of plates: LB alone and LB containing twofold serial dilutions of the tested antibiotic with or without 1 mM of spermine. The bacterial colonies were counted after incubation of the plates at 37°C for 48 h.

Cloning of *oprD* from *P. aeruginosa* PAO1. The *oprD* gene, encoding an outer membrane porin, was subcloned from cosmid pMO012508 (*Pseudomonas* Genetic Stock Center, East Carolina University) encompassing chromosome nucleotides 1022347 to 1047543. A 2,154-bp BamHI-KpnI fragment containing the intact *oprD* gene was inserted into pAU47, an *E. coli-P. aeruginosa* shuttle vector, and the resulting plasmid was designated pAU48. The pAU47 shuttle vector was derived from pQF50 (6), in which the *lacZ* gene was deleted after EcoRI digestion and self-ligation and the tetracycline resistance marker was inserted into the ScaI site on the *bla* gene to switch the antibiotic resistance marker from ampicillin to tetracycline.

Construction of the *oprD* knockout mutant DK101. A 2,154-bp BamHI-KpnI fragment containing the intact *oprD* gene as described above was inserted into pBluescriptSK+ (Stratagene, La Jolla, CA), followed by insertion of a gentamicin resistance cassette (Gm) into the EcoRI site of *oprD* (34). The BamHI-KpnI fragment containing *oprD*::Gm was then isolated and inserted into the SmaI site of pRTP1 (35). The resulting plasmid, pAU54, was introduced into *E. coli* SM10 and mobilized into a spontaneous streptomycin-resistant *P. aeruginosa* strain, DK100, by biparental plate mating as described previously (9). Following incubation at 37°C for 16 h, transconjugants were selected on LB plates supplemented with gentamicin (250 μg/ml) and streptomycin (500 μg/ml), and one of the resulting mutants, DK101, was subjected to further analysis.

RESULTS

Polyamine effects on bacterial growth. The effects of spermidine and spermine on bacterial growth were first assessed by MIC measurements. Spermidine up to 16 mM showed no growth inhibition on all bacterial strains tested. In contrast, diverse responses were observed in the presence of exogenous spermine. While *P. aeruginosa* PAO1 and clinical isolates of *E. coli* K-12 and *E. coli* C921-16 exhibited no apparent growth inhibition by spermine up to 16 mM, the MICs of spermine for *E. coli* K-10 and *Salmonella enterica* serovar Typhimurium LT2 were determined to be 1 to 2 mM (Table 1) by the standard twofold broth dilution method.

Similarly, with an estimated MIC at 4 mM, spermine also exerted an apparent growth inhibition effect on three strains of *S. aureus*: ATCC 35556, sensitive to both methicillin and vancomycin (MSSA/VSSA); N315, resistant to methicillin (MRSA/VSSA); and Mu50, resistant to high levels of methicillin and intermediate levels of vancomycin (MRSA/VISA).

The growth inhibition effect of spermine was also checked by the agar dilution method. It was found that the estimated numbers of CFU on the agar plates were not affected by the presence of exogenous spermine, but the colony sizes did get smaller with increasing concentrations of spermine (0 to 16 mM).

To further characterize the growth inhibition effect of spermine, cell growth in the MH broth with shaking was monitored by OD_{600} measurements. Cells growing in the logarithmic phase were diluted 10-fold in a prewarmed broth with or without exogenous spermine. Growth of *P. aeruginosa* PAO1

TABLE 1. Polyamine effects on antibiotic susceptibility of *E. coli*, *S. enterica* serovar Typhimurium, and *S. aureus*

Antibiotic	MIC (μg/ml)[a] for indicated strain and treatment													
	*E. coli*K-10		*E. coli*K-12		*E. coli* C921-61		*S. enterica* serovar Typhimurium LT2		*S. aureus* N315		*S. aureus* Mu50		*S. aureus* ATCC 35556	
	None	SpN	None	SpN	None	SpN	None	SpN	None	SpN	None	SpN	None	SpN
Ampicillin	8	0.5	4	2	4	2	4	1	128	4	64	2	2	≤0.06
Amoxicillin	ND	ND	ND	ND	ND	ND	ND	ND	16	0.25	16	0.5	1	≤0.06
Azlocillin	16	0.25	16	2	8	2	16	4	16	1	16	1	0.5	0.12
Carbenicillin	32	2	16	4	16	2	8	1	32	0.5	≥512	4	1	≤0.06
Cefoperazone	ND	ND	ND	ND	ND	ND	ND	ND	4	0.5	>1,024	16	1	0.25
Cefotaxime	ND	ND	ND	ND	ND	ND	ND	ND	2	0.5	≥1,024	4	0.5	0.25
Ceftazidime	ND	ND	ND	ND	ND	ND	ND	ND	32	4	512	16	8	≤0.06
Ceftriaxone	ND	ND	ND	ND	ND	ND	ND	ND	4	1	>1,024	16	2	1
Cephaloridine	8	0.5	4	2	4	2	0.015	0.015	1	≤0.06	16	0.25	≤0.06	≤0.06
Cephalothin	ND	ND	ND	ND	ND	ND	ND	ND	1	≤0.06	64	1	0.5	≤0.06
Cloxacillin	ND	ND	ND	ND	ND	ND	ND	ND	8	≤0.06	≥512	1	0.12	≤0.06
Oxacillin	1,024	64	512	128	512	128	256	128	16	≤0.06	512	1	0.12	≤0.06
Penicillin G	64	2	64	16	32	8	16	2	32	0.12	32	0.25	≤0.06	≤0.06
Piperacillin	2	0.25	2	0.5	2	0.5	4	0.5	32	0.5	128	2	0.5	0.12
Ticarcillin	8	0.5	8	2	4	1	4	0.5	32	0.25	512	4	1	0.25
Chloramphenicol	16	1	32	2	4	2	2	1	8	0.25	8	2	8	0.25
Ciprofloxacin	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	1	0.5	64	32	0.25	0.25
Erythromycin	64	8	64	16	64	64	64	32	ND	ND	ND	ND	ND	ND
Fusidic acid	>1,024	64	1,024	512	1,024	1,024	>1,024	1,024	ND	ND	ND	ND	ND	ND
Kanamycin	2	0.25	4	1	4	4	4	2	ND	ND	ND	ND	ND	ND
Novobiocin	>1,024	512	>1,024	1,024	512	256	>1,024	1,024	ND	ND	ND	ND	ND	ND
Polymyxin B	0.5	0.25	0.25	0.25	0.5	0.5	0.5	0.5	64	16	128	32	64	4
Spectinomycin	16	1	16	2	16	16	32	32	ND	ND	ND	ND	ND	ND
Tetracycline	8	0.5	8	2	2	1	2	2	0.12	≤0.06	32	4	0.12	≤0.06
Vancomycin	ND	ND	ND	ND	ND	ND	ND	ND	0.5	0.25	≥4	2	1	0.5
Spermine[b]	1	ND	>16	ND	>16	ND	2	ND	4	ND	4	ND	4	ND

[a] The MICs of antibiotics were determined in the absence (none) or presence of 1 mM spermine (SpN); only 0.5 mM of SpN was applied to *E. coli* K-10. ND, not determined.

[b] The MICs of spermine are expressed in mM.

was not affected by 10 mM of spermine, and the doubling time of *S. aureus* Mu50 was increased from 39 min to 62 min by the presence of 1 mM of spermine (data not shown). Contrarily, growth of *E. coli* K-12 was sensitive to exogenous spermine (Fig. 1); its growth rate was reduced significantly even by 1 mM of spermine. When a higher concentration of spermine (2 to 4 mM) was added, growth was stopped for several hours before recovery. Similar patterns of growth inhibition by spermine were also obtained for *E. coli* K-10 and *S. enterica* serovar Typhimurium LT2.

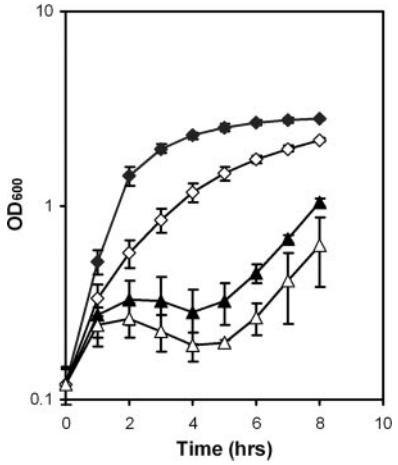

FIG. 1. Spermine effect on growth of *E. coli* K-12. Growth of *E. coli* K-12 in the presence or absence of exogenous spermine was monitored by OD measurements as described in Materials and Methods. Filled diamonds, no spermine; open diamonds, 1 mM spermine; filled triangles, 2 mM spermine; open triangles, 4 mM spermine. Also shown are error bars for each data point, representing triplicate experiments.

The results described above indicated that the growth inhibition effect of spermine is bacterial species dependent and could be affected by the growth environment. This observed effect was neither bactericidal nor bacteriostatic in essence, as growth can be recovered after several hours of adaptation to the presence of spermine. On the basis of these results, the spermine concentrations as indicated for the MIC measurements of antibiotics were carefully determined for each bacterial strain.

Spermine effects on antibiotic susceptibility of *S. aureus*. The MICs of a variety of β-lactams and other types of antibiotics in the absence and presence of spermine (1 mM) were determined for the three representative strains of *S. aureus* as described above.

As shown in Table 1, the MICs of β-lactams were decreased to different levels by the presence of spermine in all three strains. Significantly, the MIC of oxacillin, which is commonly used by physicians to replace methicillin, was reduced over 500-fold in Mu50 and over 250-fold in N315. The data suggest that MRSA resistant to β-lactam antibiotics might be eradicated efficiently by β-lactams in the presence of spermine.

While spermine exerted a much stronger effect on β-lactam susceptibility, it also worked well with chloramphenicol, polymyxin B, and tetracycline but showed no effect or marginal effects on vancomycin, ciprofloxacin, and gentamicin.

The effect of spermine on the MIC of oxacillin against Mu50 was also evaluated in the presence of divalent and monovalent positively charged ions. It was found that the effect of spermine on the MIC of oxacillin was retained in the presence of 3 mM

TABLE 2. Polyamine effects on MICs of antibiotics in clinical isolates of *P. aeruginosa*[a]

Strain no.	MIC (μg/ml) for indicated antibiotic and treatment											
	Carbenicillin			Chloramphenicol			Ciprofloxacin			Polymyxin B		
	None	SpN	SpD	None	SpN	SpD	None	SpN	SpD	None	SpN	SpD
PAO1	64	4	4	128	32	32	0.125	0.25	1	1	4	16
F4980	256	8	16	32	2	2	4	4	2	0.25	0.5	2
F66336	256	8	16	8	0.5	2	0.5	2	1	0.125	0.5	0.5
H4563	>1,024	64	32	16	4	2	128	4	2	0.125	0.5	0.5
M22152	>1,024	64	64	16	2	2	4	4	2	0.5	1	2
M37310	1,024	32	32	16	4	4	2	4	2	0.125	0.5	0.5
M38100A	16	64	32	4	8	4	2	4	8	<0.06	<0.06	<0.06
M38100B	1,024	32	32	16	8	8	32	8	4	16	4	1
T2095	256	8	16	32	4	4	2	4	4	0.125	0.5	0.5
T5177	>1,024	64	64	16	2	2	2	4	2	0.5	1	2
T6268	16	2	4	16	2	2	4	4	4	1	4	4
T15464	>1,024	64	32	32	8	2	2	4	4	0.5	1	2

[a] Spermine (SpN; 1 mM) and spermidine (SpD; 20 mM) were used for MIC measurements. These concentrations of polyamines did not affect the growth of clinical isolates.

of magnesium sulfate or calcium chloride (3 mM) and 150 mM of sodium chloride (data not shown).

Clinical isolates of *Pseudomonas aeruginosa*. In our previous studies of polyamine effects on antibiotic susceptibility, all strains were derived from the same lineage of *P. aeruginosa* PAO1. To determine whether these initial observations were common phenomena in this bacterial species, 10 clinical isolates of *P. aeruginosa* were tested for the effects of spermine (1 mM) and spermidine (20 mM) on the MICs of four types of antibiotics: carbenicillin, chloramphenicol, ciprofloxacin, and polymyxin B. These clinical isolates, except M38100A, did not grow in minimal medium with glucose and ammonium as the source of carbon and nitrogen, and hence, all measurements were done with the MH medium. Consistent with our previous reports (18, 19) for *P. aeruginosa* PAO1, the presence of either spermine or spermidine decreased the MICs of carbenicillin and chloramphenicol and increased the MICs of ciprofloxacin and polymyxin B in these clinical isolates in general (Table 2).

It was found that most of these clinical isolates had much higher MICs for carbenicillin than the reference strain *P. aeruginosa* PAO1. All strains exhibited sensitization to carbenicillin by polyamine treatment except strain M38100A, which instead became more resistant to carbenicillin in the presence of polyamines. Exceptions were also found in strain H4563 for ciprofloxacin and in strain M38100B for ciprofloxacin and polymyxin B. The MICs of these antibiotics in these two strains were significantly higher than those in other strains (Table 2); however, the MICs in these exceptional cases were decreased by the presence of polyamines, as opposed to being increased as in most cases.

It is very likely that the distinct antibiotic susceptibility profile of each strain is a reflection of mutations accumulated during treatments. This supports the hypothesis that the effects of polyamines on antibiotic susceptibility can be reversed or abolished by changes at the genetic level.

Effect of exogenous polyamines on sensitization of *E. coli* and *S. enterica* serovar Typhimurium to antibiotics. Interactions of spermine and other biogenic polyamines with porins could cause a reduced antibiotic flux in enteric bacteria (5). Decreases in β-lactam and quinolone antibiotic flux rates in the presence of polyamines have been previously reported (5). As a result, increased MICs for affected antibiotics in the presence of exogenous polyamines would be expected in enteric bacteria. This expectation would be contradictory to what we have observed in *P. aeruginosa*, and surprisingly, it has never been tested and reported to occur in enteric bacteria. Accordingly, we performed MIC measurements for a variety of antibiotics against several strains of *E. coli* and *S. enterica* serovar Typhimurium in the presence and absence of spermine or spermidine.

As shown in Table 1, exogenous spermine exerted different degrees of sensitization to chloramphenicol and β-lactams (ampicillin, azlocillin, carbenicillin, cephaloridine, oxacillin, penicillin G, piperacillin, and ticarcillin) on all strains and to other tested antibiotics in a strain-specific pattern. Similar results were also obtained with exogenous spermidine (20 mM; data not shown). One very significant finding was that in no case did polyamines seem to cause any noticeable increase in the MICs of tested antibiotics in these enteric bacteria, in contrast to the increased MICs of polymyxin B and ciprofloxacin in *P. aeruginosa* (19).

Checkerboard assays of *E. coli* and *S. aureus*. Spermine was more efficient than spermidine in exerting its effect on antibiotic susceptibility. However, as described above, spermine can potentially inhibit growth by an unknown mechanism in enteric bacteria. Therefore, the observed sensitization effect of spermine on antibiotic susceptibility could be the result of a synergistic effect by spermine and antibiotics. To assess this possibility, checkerboard assays were conducted using *E. coli* K-12 and *S. aureus* Mu50 with spermine and antibiotics. The FIC index results as shown in Table 3 suggested a strong synergistic interaction between spermine and the tested antibiotics (Table 3).

Population analysis profiles. To further substantiate the effect of spermine on antibiotic susceptibility, population analysis was performed using *P. aeruginosa* PAO1 with carbenicillin. As shown in Fig. 2A, the concentrations of carbenicillin required to completely inhibit the growth of the inoculated cells were 8 and 64 μg/ml, respectively, in the presence and absence of 1 mM spermine. This result indicated an eightfold sensiti-

TABLE 3. FIC indexes for spermine in combination with antibiotics in *E. coli* and *S. aureus*

Organism	Antibiotic in combination with spermine	FIC index
E. coli K-10	Carbenicillin	0.375
	Chloramphenicol	0.375
	Penicillin G	0.25
S. aureus Mu50	Ampicillin	0.25
	Oxacillin	0.188
	Penicillin G	0.25

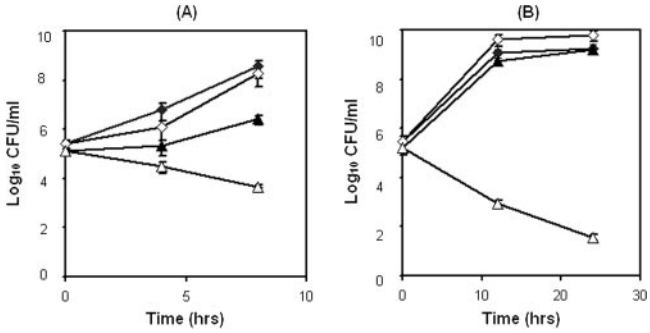

FIG. 3. Time-killing curves for *P. aeruginosa* PAO1 (A) and *S. aureus* Mu50 (B). Time-killing curves were performed as described in Materials and Methods in the MH broth alone (filled diamonds) or with 1 mM spermine (open diamonds), 8 μg/ml carbenicillin or oxacillin (filled triangles), or a combination of spermine and carbenicillin or oxacillin (open triangles). Also shown are error bars for each data point, representing triplicate experiments.

zation effect in the presence of spermine, consistent with the results of MIC measurements.

Population analysis was also conducted using *S. aureus* Mu50 treated with oxacillin in the presence and absence of spermine, and the results are shown in Fig. 2B. In the absence of spermine, oxacillin at 128 μg/ml basically showed no effect on growth inhibition of Mu50, and >512 μg/ml was needed to abolish cell growth. In the presence of spermine, oxacillin at 8 μg/ml was sufficient to stop the growth of all inoculants.

Time-killing assays. The sensitization effect of spermine was also assessed by time-killing assays. As shown in Fig. 3A, the growth of *P. aeruginosa* PAO1 in MH broth was continuous for at least 8 h in the presence of spermine (1 mM) or carbenicillin (16 μg/ml). In contrast, a combination of spermine and carbenicillin exerted a killing effect on PAO1; more than 95% of the inoculated cell population was killed in 8 h. Essentially identical results were also obtained with 10 mM of spermidine in place of 1 mM spermine in these assays (data not shown).

S. aureus Mu50 was also subjected to time-killing assays with spermine and oxacillin, and the results are shown in Fig. 3B. The MIC of oxacillin in Mu50 was 512 μg/ml in the MH broth (Table 1), and as expected, Mu50 treated with 20 μg/ml of oxacillin alone showed a viability count similar to that for the control group after 12 h of growth in the broth. With a combination of spermine and oxacillin at these concentrations, cell viability counts were dramatically decreased for 24 h, with more than 99% of the inoculated cells killed. A similar result was also obtained for *S. aureus* N315 (data not shown).

The increase in resistance to imipenem by polyamines was abolished in the *oprD* mutant of *P. aeruginosa*. In the previous report, we demonstrated that polyamines can sensitize *P. aeruginosa* PAO1 to a variety of β-lactam antibiotics. However, we were surprised to note that the only exception was imipenem. As shown in Table 4, the MICs of imipenem were increased up to four- to eightfold by exogenous spermidine or spermine in a dose-dependent manner but showed no change by arginine, lysine, agmatine, putrescine, or cadaverine (data not shown).

It has been reported that the OprD porin facilitates the penetration of imipenem through the outer membrane and that an *oprD* mutant is resistant to imipenem (12). For *E. coli*, it has been demonstrated that polyamines promote channel closures of the OmpF and OmpC porins (5). It is possible that spermine or spermidine can block the channel of OprD porin and thus increase resistance to imipenem. To test this hypoth-

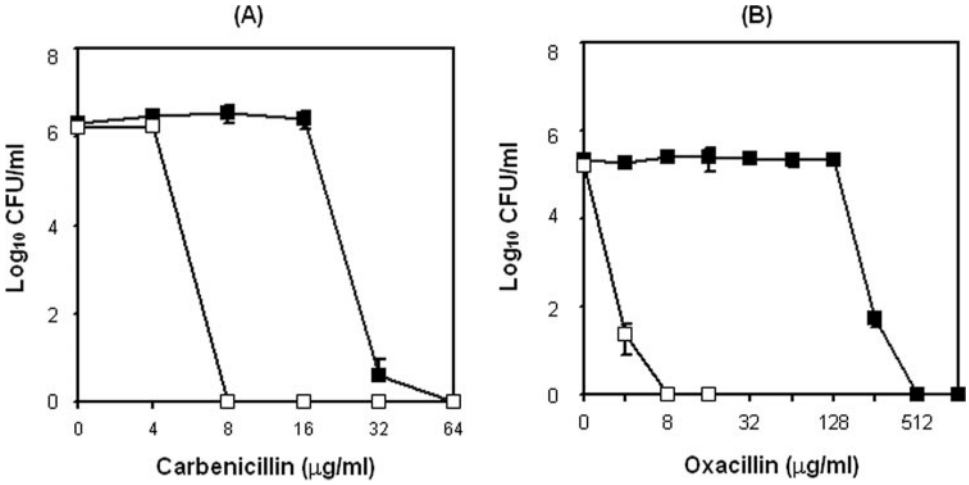

FIG. 2. Population analysis profiles of *P. aeruginosa* PAO1 (A) and *S. aureus* Mu50 (B). The numbers of viable cells were determined as CFU on MH plates with carbenicillin for *P. aeruginosa* and oxacillin for *S. aureus* at the indicated concentrations in the presence (open squares) or absence (filled squares) of 1 mM spermine. Also shown are error bars for each data point, representing triplicate experiments.

TABLE 4. Polyamine-dependent imipenem resistance in *P. aeruginosa* PAO1

Polyamine	MIC (μg/ml) of impenem in combination with polyamine at indicated concn (mM)				
	0	0.5	1	5	10
Spermidine	4	4	4	8	16
Spermine	4	8	8	16	32

esis, the MICs of imipenem in the presence and absence of spermine were determined with an *oprD* knockout mutant. As shown in Table 5, in comparison to that in the parent strain PAO1, the MIC of imipenem in the *oprD* mutant was increased eightfold and was insensitive to the presence of spermine. When the *oprD* mutant was complemented with plasmid pAU48, carrying *oprD*, the recombinant strain exhibited a 64-fold reduction in imipenem MIC, and the resistance effect by spermine was restored, as demonstrated by a MIC increase to the same level as that in the wild-type strain PAO1.

Although the spermine-induced increase in imipenem MIC was completely abolished, the *oprD* mutant still retained the polyamine-mediated sensitization to carbenicillin and resistance to polymyxin B, as in the parent strain PAO1 (Table 5).

The multidrug sensitization effect by polyamines was not affected by the AcrAB efflux pump in *E. coli*. It has been demonstrated in many cases that an increased level of efflux pump activity can result in multidrug resistance in bacteria. As exogenous spermine and spermidine caused the exposed bacteria to be more sensitive to multiple antibiotics, one hypothesis was that these polyamines can serve as the efflux pump inhibitors. To test this hypothesis, the effects of spermine (1 mM) on the MICs of carbenicillin, chloramphenicol, and tetracycline were assessed with two *acrA* mutants (N43 and N818) and their parent strain, W4573 of *E. coli*.

As shown in Table 6, two- to fourfold reductions in the MICs of the three tested antibiotics were observed in the parent strain W4573 in the presence of spermine. When the AcrAB efflux pump was destroyed by an *acrA* deletion in strain N818, the MICs of these three antibiotics were decreased fourfold in the absence of spermine. Addition of spermine to the medium caused further 4- to 16-fold reductions of MICs. Similar patterns of MIC reduction were also observed in another *acrA* mutant strain, N43. These results supported that AcrAB is an efflux pump for carbenicillin, chloramphenicol, and tetracycline and that the sensitization effect of spermine on these antibiotics was retained in the *acrA* mutants.

TABLE 5. Effects of OprD on spermine-dependent antibiotic susceptibility in *P. aeruginosa*

Strain (description)[a]	MIC (μg/ml)[b] for indicated antibiotic and treatment					
	Imipenem		Carbenicillin		Polymyxin B	
	None	SpN	None	SpN	None	SpN
DK100 (Sm^r)	4	32	64	4	0.5	4
DK101 (Sm^r; *oprD*::Gm)	32	32	64	2	0.5	4
DK101/pAU48	0.5	32	64	2	0.5	4

[a] Sm^r, streptomycin resistance; Gm, gentamicin resistance cassette. Plasmid pAU48 carries an intact *oprD* gene.
[b] MIC measurements were done in the absence (none) or presence of 10 mM spermine (SpN).

TABLE 6. Effect of AcrAB efflux pump on spermine-dependent antibiotic susceptibility in *E. coli*

Antibiotic	MIC (μg/ml)[a] for indicated strain and treatment					
	W4573 (parent)		N43 (*acrA*)		N818 (Δ*acrA*)	
	None	SpN	None	SpN	None	SpN
Carbenicillin	16	4	4	0.25	2	0.25
Chloramphenicol	8	2	2	<0.06	2	0.12
Tetracycline	2	1	0.5	<0.06	0.5	0.12

[a] MIC measurements were done in the absence (none) or presence of 10 mM spermine (SpN).

DISCUSSION

The results of this study support the conclusion that exogenous polyamine can increase antibiotic susceptibility in gram-negative *P. aeruginosa*, *E. coli*, and *S. enterica* serovar Typhimurium bacteria and in gram-positive *S. aureus* bacteria, including MRSA. All these data strongly suggested that the sensitization or synergy effect of exogenous polyamine on most β-lactams and on some other specific types of antibiotics is common among many bacterial pathogens. In particular, the effect of spermine on β-lactams was fascinating. Regardless of the causes for resistance in the clinical isolates of *P. aeruginosa* or *S. aureus*, the MICs of the affected β-lactam antibiotics were greatly decreased by exogenous spermine. This finding could be clinically significant, as it holds a promise to extend the application of existing β-lactam antibiotics and to provide an alternative approach for eradicating resistant pathogens.

Although the possible working mechanism of this interesting effect of polyamine is still unknown, considering its multilevel and species-dependent effects, it is very likely that more than one mechanism is involved. At least in the case of β-lactam antibiotics in *S. aureus*, the results from strains of MRSA presented in this study strongly suggested a group of possible candidates: penicillin-binding proteins (PBPs). It is generally accepted that expression of *mecA*, encoding PBP2A, in strains of MRSA is the major determinant of resistance to β-lactams (2, 21). When exposed to β-lactam antibiotics, the four native PBPs become inactivated and their transpeptidase function is taken over by PBP2A, which has very low affinity for most members of this family of antimicrobial agents (8, 37). Along this line, it was tempting to speculate that spermine and other polyamines might exert their effects by increasing PBP acylation by β-lactam antibiotics or by decreasing the expression levels of PBPs.

Drug uptake is one of the many determinants for antibiotic susceptibility. For the gram-negative bacteria, the outer membrane provides the first line of barricade, and the outer membrane porins are channels for passage of selectable compounds. In *E. coli*, although OmpF and OmpC porins might be blocked by polyamines from the inner side of the channel (5), our results for MIC measurements seemed to suggest that this interaction plays no role in the synergistic effect of exogenous polyamines and antibiotics. The only exception was the OprD

porin for imipenem in *P. aeruginosa*. The OprD porin is known to be important for imipenem uptake and resistance (12); the increased MIC of imipenem in the presence of spermine supported the hypothesis of OprD blockage by spermine.

In this study, we also tested the hypothesis of whether polyamines could serve as efflux pump inhibitors, which potentially would exert a synergistic effect on antibiotic susceptibility. Using strains of *E. coli* deficient in the AcrAB efflux pump, we were still able to detect the polyamine effect in these mutants. Although these results did not seem to support the hypothesis, it was also noted that the changes (*n*-fold) in MICs by spermine were relatively higher in the *acrA* mutants than in the parent strain (Table 6). This might be related to the synergistic effect and an increased working concentration of antibiotics due to a diminished major efflux pump in *E. coli*.

Although exogenous polyamines can increase the levels of resistance to polymyxin B and ciprofloxacin in *P. aeruginosa*, this did not seem to apply in *E. coli* and *S. enterica* serovar Typhimurium (Table 1). In *P. aeruginosa* (19) and enteric bacteria, resistance to polymyxin B is associated with lipopolysaccharide modifications by enzymes encoded by the *pmrHFIJKLM* operon, which is regulated by multiple genetic factors, including the *phoPQ* and *pmrAB* two-component systems (25–27). With such a high degree of similarity in the genetic contents, it is intriguing that *P. aeruginosa* responded differently from enteric bacteria to exogenous polyamines with respect to polymyxin B susceptibility. We demonstrated in a previous report (19) that polyamines induced the expression of *phoPQ* and that the increase in resistance to polymyxin B by polyamines was abolished in the *phoP* knockout mutant of the response regulator but not in the *phoQ* mutant of the sensor kinase. This might be the result of cross talk between PhoPQ and a polyamine-specific signal transduction pathway yet to be identified in *P. aeruginosa*.

In summary, while we have excluded the involvement of some possible candidates, more work is required to elucidate the molecular mechanism(s) of this fascinating effect of polyamines on antibiotic susceptibility. Regardless of the possible working mechanism yet to be identified, this study provided evidence for a synergistic effect of spermine in combination with β-lactam antibiotics as a potential treatment of MRSA and VISA.

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