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Polyamines Induce Resistance to Cationic Peptide, Aminoglycoside, and Quinolone Antibiotics in *Pseudomonas aeruginosa* PAO1

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***Pseudomonas aeruginosa*, a gram-negative bacterium of human pathogens, is noted for its environmental versatility, enormous metabolic capacity, and resistance to antibiotics. Overexpression of the outer membrane protein OprH and increased resistance to polycationic peptide antibiotics (e.g., polymyxin B) mediated by the PhoPQ two-component system on induction of a putative lipopolysaccharide (LPS) modification operon (*PA3552-PA3559*) have been reported as part of the adaptive responses to magnesium limitation in *P. aeruginosa*. Induction of the *oprH-phoPQ* operon and the LPS modification operon by exogenous spermidine was revealed from GeneChip analysis during studies of polyamine metabolism and was confirmed by the *lacZ* fusions of affected promoters. From the results of MIC measurements, it was found that addition of spermidine or other polyamines to the growth medium increased the MIC values of multiple antibiotics, including polycationic antibiotics, aminoglycosides, quinolones, and fluorescent dyes. MIC values of these compounds in the transposon insertion mutants of *oprH*, *phoP*, *phoQ*, and *pmrB* were also determined in the presence and absence of spermidine. The results showed that the spermidine effect on cationic peptide antibiotic and quinolone resistance was diminished in the *phoP* mutant only. The spermidine effect on antibiotics was not influenced by magnesium concentrations, as demonstrated by MICs and *oprH*::*lacZ* fusion studies in the presence of 20 μM or 2 mM magnesium. Furthermore, in spermidine uptake mutants, MICs of cationic peptide antibiotics and fluorescent dyes, but not of aminoglycosides and quinolones, were increased by spermidine. These results suggested the presence of a complicated molecular mechanism for polyamine-mediated resistance to multiple antibiotics in *P. aeruginosa*.**

Pseudomonas aeruginosa is an important gram-negative human pathogenic bacterium that causes nosocomial infections. In particular, the organism is a predominant respiratory pathogen among cystic fibrosis patients producing chronic pulmonary infection and progressive deterioration in lung infection (23, 24, 25).

Currently, antibiotic regimens to treat *P. aeruginosa* infections include a two-drug combination of β-lactams and aminoglycosides or, alternatively, quinolones and cationic peptide antibiotics (7, 21). In the latest reports, the effectiveness of the β-lactam and aminoglycoside combination is 70% to 98% in the United States and the United Kingdom (11). Failure of the treatment is mostly due to intrinsic and acquired resistance to a single and/or multiple antibiotics (11). Unfortunately, the resistance rate is increasing annually as reported by National Surveillance of Antimicrobial Resistance in clinical isolates of *P. aeruginosa*, in which nationwide (United States) multidrug resistance to β-lactams, aminoglycosides, and quinolones has gradually increased, with a rate of 4% to 14% from 1993 to 2002 (22).

Antibiotic resistance of *P. aeruginosa* is caused by environmental factors such as exposure to subinhibitory concentrations of antibiotics or limiting concentrations of divalent cations (4, 11). It has long been reported that limiting concentrations of divalent Mg^{++} or Ca^{++} ions are involved in resistance to cationic peptide antibiotics (e.g., polymyxin B) in *Salmonella enterica* serovar Typhimurium and *P. aeruginosa* (4, 15). Molecular mechanisms of this resistance phenomenon in association with lipopolysaccharide (LPS) modification have been studied extensively in *S. enterica* serovar Typhimurium. The PhoPQ and PmrAB two-component regulatory systems have been reported to play roles in the induction of the LPS modification system *pmrHFIJKLM* operon, which is required for the biosynthesis of lipid A with 4-aminoarabinose to create a more positively charged LPS (4, 18). Homologues of serovar Typhimurium PhoPQ, PmrAB, and PmrHFIJKLM have also been reported in *P. aeruginosa* (15, 16).

Other possible environmental factors affecting antibiotic resistance in *P. aeruginosa* are polyamines. Polyamines (e.g., putrescine, spermidine, spermine, and cadaverine), derived from decarboxylation of arginine, ornithine, and lysine, are polycationic compounds found in all living organisms. Polyamines have also been known as a modulator in gene expression including DNA replication, transcription, translation, and protein activity in *Escherichia coli*, which directs optimal cell growth and viability as well as defense mechanisms against toxic environmental conditions (2, 3, 8, 26). *P. aeruginosa* can utilize many polyamines as sole carbon and/or nitrogen sources (13) and possibly as modulators to direct resistance to certain toxic environmental conditions. Although intensive research for polyamines has been done in recent decades, the actions of polyamines at the molecular levels in bacterial cells are largely unknown.

We have studied arginine metabolism and polyamine utilization in *P. aeruginosa* PAO1 (13) and have worked to understand the physiological roles of polyamines in this organism. In this report, we provide initial evidence from GeneChip exper-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant phenotype[a]	Source or reference
Strains		
P. aeruginosa		
PAO1	Wild type	5
MPAO1	Wild type	The University of Washington Genome Center (UWGC)
PAO5011	*spuF*::Tc	13
PAO5013	*spuH*::Tc	13
MPAO1	*oprH*::Tn-Tc (strain ID 32552)	UWGC[b]
MPAO1	*phoP*::Tn-Tc (strain ID 7863)	UWGC
MPAO1	*phoQ*::Tn-Tc (strain ID 37038)	UWGC
MPAO1	*pmrB*::Tn-Tc (strain ID 46521)	UWGC
MPAO1	*PA4774*::Tn-Tc (strain ID 50643)	UWGC
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR supE44* Δ(*lac*)*U169* (ϕ80Δ*lac*)	Bethesda Research Laboratories
Plasmids		
pBluescriptSK+	Ap^r, cloning vector	Stratagene
pQF50	Ap^r, *lacZ* transcriptional vector	13
pAU2	pQF50 derivative carrying the P_{PA4773}-*lacZ* fusion	This study
pAU6R	pQF50 derivative carrying the P_{oprH}-*lacZ* fusion	This study
pAU7	pQF50 derivative carrying the P_{PA3552}-*lacZ* fusion	This study

[a] Tc, tetracycline; Ap, ampicillin; ID, identification.
[b] UWGC (http://www.genome.washington.edu/UWGC/) mutants were generated by Tn-Tc insertions (9)

iments for the involvement of polyamines in antibiotic resistance. We substantiated this surprising finding and demonstrated that polyamines can trigger induction of resistance to cationic peptide antibiotics, aminoglycosides, quinolones, and dyes. Resistance to quinolones and cationic peptide antibiotics by exogenous spermidine appeared to be linked to the *oprH-phoPQ* operon.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. Strains of *P. aeruginosa* and *E. coli* were routinely grown on Luria-Bertani (LB) agar plate or in LB broth at 37°C as rich medium. When indicated, *P. aeruginosa* strains were also grown on minimal medium P [MMP; 30 mM Na_2HPO_4, 14 mM KH_2PO_4, 4 μM $FeSO_4$, 1 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$] containing 20 mM glutamate or other supplements when indicated as the carbon source (13). For the antibiotic susceptibility testing of *P. aeruginosa*, MMP or cation-adjusted Mueller-Hinton (Oxoid, Ogdensburg, New York) broth was used. Appropriate concentrations of antibiotics or polyamines were supplemented when needed. All antibiotics and polyamines were purchased from Sigma (Sigma, St. Louis, MO). Solutions of these compounds were prepared by dissolving the compounds in sterile double-distilled water or solvent, as suggested by the manufacturer, and sterilized by filtering through 0.4-μm-pore-size disposable membranes (Millipore, Billerica, Mass.).

RNA isolation, generation of cDNA, hybridization of GeneChip, and data analysis. *P. aeruginosa* PAO1 grown in MMP containing 20 mM glutamate as a carbon source and supplemented with spermidine or arginine (20 mM concentrations of each) was used to extract total RNA as described previously (12) and further purified by QIAGEN spin columns (Chatsworth, Calif.) after DNase I treatment. Preparation of cDNA, hybridization of GeneChip (Affymetrix, Santa Clara, CA 95051), and data analysis were performed as described previously (14).

Antibiotic susceptibility testing. Antibiotic susceptibility was tested by the standard broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS) (19, 20). Briefly, each stock solution of antibiotics was added to MMP-glucose broth to achieve serial twofold dilutions between 0.015 and 1,024 μg/ml and dispensed into sterile 17- by 100-mm snap-cap Falcon culture tubes (1 ml/tube; Fisher Scientific). Fresh overnight cultures of *P. aeruginosa* were diluted in saline to an optical density at 600 nm of 0.09 to 0.1 (approximately 1×10^8 viable cells per ml). A portion of the adjusted cell suspension (2 to 5 μl for $\sim 10^5$ cells) was inoculated to each MMP broth sample containing antibiotics as indicated. The cell cultures were then incubated overnight (12 to 14 h) at 37°C. The MIC was defined as the lowest concentration of each antibiotic that completely inhibited the growth of the inoculum. MICs were determined and confirmed by three independent experiments.

Construction of *lacZ* promoter fusions and β-galactosidase assays. Putative promoter regions for *oprH-phoPQ*, *PA4773-pmrAB*, and an operon for LPS modification (*PA3552-PA3559*) were amplified using the following pairs of PCR primers: 5′-CAGGCAGATCACGAGAAACAG-3′ and 5′-GCCGACGAAGTT GTCGGCGGC-3′ (380-bp fragment) for *oprH*; 5′-GATCGACTACCGCGGG GTGGT-3′ and 5′-GCAGTCGAACAGCTCGATGAC-3′ (469-bp fragment) for *PA4773*; and 5′-CGTCTACGGGCGCACCGCAGA-3′ and 5′-GGCGGCG ATTTCGTCCTCGCC-3′ (498-bp fragment) for *PA3552*. Genomic DNA extracted from *P. aeruginosa* PAO1 (10) was used as the template for PCR amplification. Each PCR fragment amplified by *Pfu* DNA polymerase (Stratagene) was purified by QIAGEN spin columns (Chatsworth, Calif.), ligated into a broad-host-range transcriptional fusion vector (pQF50) on the SmaI site, and introduced into *E. coli* DH5α by transformation. The orientation of each insert in the clone was confirmed by nucleotide sequencing at the Biotechnology Core Facility of Georgia State University. The resulting plasmids were introduced into appropriate *P. aeruginosa* strains by transformation (10). For measurements of β-galactosidase activities, cells were grown in MMP with supplements as indicated in the figures until the optical density at 600 nm reached 0.7 to 0.8. Cells were collected by centrifugation ($5{,}000 \times g$), and cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.0). After passage through a French pressure cell at 8,000 lb/in^2, soluble cellular extracts were used for the measurement of β-galactosidase activities with o-nitrophenyl-β-D-galactopyranoside as the substrate (17). The protein concentration of each sample was determined by the method of Bradford (1) with bovine serum albumin as the standard.

RESULTS

Induction of the *oprH-phoPQ* and *PA3552-PA3559* operons by exogenous spermidine. During our search for genes involved in polyamine utilization of *P. aeruginosa*, induction by spermidine of the *oprH-phoPQ* operon encoding an outer membrane porin and a two-component regulatory system and the *PA3552-PA3559* operon for LPS modification was found fortuitously from the GeneChip experiments. The expression profiles of these genes from cells grown in glutamate minimal medium were compared to those from cells grown in the presence of arginine or spermidine (Table 2). For the *PA3552-PA3559* operon, exogenous spermidine induced significantly expression levels of all genes in this operon, with the relative

TABLE 2. Gene expression profiles of *oprH-phoPQ*, *PA3552-PA3559*, and *pmrAB* in *P. aeruginosa* PAO1

Gene identification no. (name)[a]	Gene expression (relative ratio) in[b]:			Description of protein and its function[a]
	MMP	MMP + Arg	MMP + Spd	
PA1178 (*oprH*)	1,135 (1)	2,558 (2.3)	18,107 (16)	Outer membrane porin H1 precursor
PA1179 (*phoP*)	498 (1)	465 (0.9)	2,260 (4.5)	Two-component response regulator
PA1180 (*phoQ*)	363 (1)	448 (1.2)	1,223 (3.4)	Two-component signal sensor
PA3552	147 (1)	183 (1.2)	1,235 (8.4)	Hypothetical protein; LPS modification
PA3553	59 (1)	29 (0.5)	549 (9.3)	Probable glycosyltransferase; LPS modification
PA3554	168 (1)	275 (1.6)	1,624 (9.7)	Hypothetical protein; LPS modification
PA3555	54 (1)	75 (1.4)	338 (6.2)	Hypothetical protein; LPS modification
PA3556	264 (1)	272 (1.0)	1,650 (6.3)	Inner membrane L-Ara4N transferase ArnT
PA3557	48 (1)	91 (1.9)	463 (9.7)	Hypothetical protein; LPS modification
PA3558	94 (1)	92 (1.0)	870 (9.3)	Hypothetical protein; LPS modification
PA3559	109 (1)	115 (1.1)	1,115 (10.3)	Probable nucleotide sugar dehydrogenase
PA4776 (*pmrA*)	261 (1)	265 (1.0)	289 (1.1)	Two-component response regulator
PA4777 (*pmrB*)	206 (1)	236 (1.1)	244 (1.2)	Two-component signal sensor

[a] Gene number and the description of the protein and its function were cited from the *Pseudomonas* genome project (http://www.pseudomonas.com/) and J. B. McPhee et al. (16).
[b] Gene expression profiles were based on GeneChip experiments as described in Materials and Methods. Data represent raw signal intensities after analysis settings suggested by the manufacturer. Arg, 20 mM arginine; Spd, 20 mM spermidine.

increase in induction in the range of 6.2- to 10.3-fold. In comparison, exogenous arginine exhibited only 0.5- to 1.9-fold increase in induction. For the *oprH-phoPQ* operon, spermidine increased the induction levels of these genes by 16-, 4.5-, and 3.4-fold, and arginine only caused a marginal increase in induction (0.9- to 2.3-fold) of these genes. Since induction of these two operons has been reported as part of the resistance mechanism against cationic peptide antibiotics (e.g., polymyxin B) in *P. aeruginosa* (15, 16) and enteric bacteria (4, 18), data described above provided the first line of evidence to support the hypothesis that polyamines could be related to antibiotic susceptibility.

The other known transcriptional regulator for resistance to cationic peptide antibiotics is the PmrAB two-component system (16). No significant relative increase in the expression of *pmrA* and *pmrB* was found in the presence of arginine or spermidine (Table 2) or for the upstream *PA4773-PA4775* genes (data not shown).

Polyamines induced antibiotic resistance. To understand whether polyamines were involved in antibiotic susceptibility in *P. aeruginosa*, MICs of various antibiotics and dyes were determined and compared in the cells grown with or without polyamines (spermine, spermidine, putrescine, and cadaverine) or polyamine precursors (agmatine, ornithine, arginine, and lysine). As shown in Table 3, MICs of cationic peptide antibiotics (polymyxin B and colistin) increased eightfold in the cells grown with spermidine but were virtually unchanged in the cells grown with other compounds. For aminoglycosides (kanamycin and gentamicin), quinolones (ciprofloxacin and norfloxacin), and dyes (ethidium bromide and acridine orange), MICs increased up to 16-fold in the cells grown with all polyamines and agmatine. In contrast, the MIC of tetracycline was not affected by the presence of polyamines or polyamine precursors.

In comparison to spermidine, exogenous spermine exhibited a similar effect on the MICs of these antibiotics, but the rela-

TABLE 3. Antibiotic susceptibility of *P. aeruginosa* PAO1 and mutant strains grown with polyamines or their precursors

P. aeruginosa strain and growth conditions	MICs (μg/ml)[a]								
	Px-B	COL	KAN	GEN	CIP	NOR	TET	EtBr	ACO
PAO1 grown with									
MMP[b]	2	2	64	0.5	0.06	0.25	8	256	64
MMP (20 μM $MgSO_4$)[c]	**8**	**8**	**256**	**2**	0.06	0.25	8	256	64
MMP + spermidine	**16**	**16**	**256**	**2**	**1**	**2**	8	**>512**	**512**
MMP + spermine (1 mM)	**4**	**4**	**128**	**1**	**0.25**	**1**	8	**>512**	**256**
MMP + putrescine	2	2	**256**	**2**	**0.5**	**1**	8	**512**	**256**
MMP + cadaverine	2	2	**256**	**2**	**0.12**	**0.5**	8	**512**	**256**
MMP + agmatine	2	2	**256**	**2**	**0.12**	**0.5**	8	**512**	**256**
MMP + ornithine	2	2	64	0.5	0.06	0.25	8	256	64
MMP + arginine	2	2	64	0.5	0.06	0.25	8	256	64
MMP + lysine	2	2	64	0.5	0.06	0.25	8	256	64
PAO1 (*spuF*::Tc) grown with MMP + spermidine	**16**	**16**	64	0.5	0.06	0.25	>100	**>512**	**512**
PAO1 (*spuH*::Tc) grown with MMP + spermidine	**16**	**16**	64	0.5	0.06	0.25	>100	**>512**	**512**

[a] MIC measurements were repeated three times with identical results by broth dilution methods as described in Materials and Methods. Values in boldface indicate increases in the MICs of the tested antibiotics. Px-B, polymyxin B; COL, colistin; KAN, kanamycin; GEN, gentamicin; CIP, ciprofloxacin; NOR, norfloxacin; TET, tetracycline; EtBr, ethidium bromide; ACO, acridine orange.
[b] MMP contains 1 mM $MgSO_4$, and 20 mM concentrations of glutamate and other supplements unless otherwise indicated.
[c] MMP containing 20 μM $MgSO_4$ with incubation of 20 h.

TABLE 4. Effect of polyamine concentrations on MIC of polymyxin B against *P. aeruginosa* PAO1

Polyamine	MIC (μg/ml) of polymyxin B with polyamine (mM) at:[a]							
	0	0.31	0.62	1.25	2.5	5	10	20
Spermidine	0.5	1	2	2	4	4	4	8
Spermine	0.5	2	4	4	4	4	4	8

[a] MIC values were determined in Miller-Minton broth in the presence of the indicated concentrations of polyamines.

tive change in MICs was lower with spermine. This result could be due to the difference in polyamine concentrations applied in the study (1 mM spermine versus 20 mM spermidine). We have noticed that precipitates of unknown properties were formed when spermine and glutamate in higher concentrations were mixed in MMP.

To further substantiate the effects of polyamines on antibiotic resistance, the MIC of polymyxin B against PAO1 was determined in the presence of various concentrations of spermine and spermidine in Miller-Minton broth. As shown in Table 4, 0.6 mM spermidine can raise the MIC of polymyxin B by fourfold. Similarly, 0.3 mM spermine also caused a comparable increase in the MIC. A 16-fold increase in the MIC of polymyxin was observed with 20 mM spermidine or spermine.

Analysis of the effects of polyamines on expression of the *oprH-phoPQ*, *PA4773-pmrAB*, and *PA3552-PA3559* operons. The results of GeneChip experiments (Table 2) revealed that spermidine induced the *oprH-phoPQ* and *PA3552-PA3559* operons but showed no effect on the *PA4773-pmrAB* operon. To substantiate this finding and to test the possible effects of other polyamines and their precursor compounds on the regulation of these operons, *lacZ* fusions of the corresponding promoters were constructed as described in Materials and Methods. These fusions were introduced into the wild-type *P. aeruginosa* PAO1 strain, and the expression profiles of these promoters in response to different compounds were determined by monitoring the activities of β-galactosidase. T *oprH* promoter exhibited about 4.5-fold increase in induction in response to spermidine, and similar levels of induction were also observed in response to putrescine, cadaverine, and agmatine (Fig. 1). For The *PA3552* promoter showed a fourfold increase in induction in the presence of spermidine, while no significant effect was detected in the presence of putrescine, cadaverine, and agmatine (Fig. 1). In contrast, *PA4773* promoter activity was not affected by any polyamines or agmatine (data not shown).

As described above, only 1 mM spermine can be added to the MMP medium without forming precipitates. Under this condition, the *oprH* promoter was not induced by spermine (data not shown) while the *PA3552* promoter was induced by about fourfold. To analyze the possible effect of spermine on induction of the *oprH* promoter, experiments were carried out by growing cells in Miller-Hinton broth, to which 20 mM spermine and spermidine can be added without problem. As shown in Fig. 1, these two polyamines exhibited a comparable relative increase in induction of the *oprH* promoter.

Overall, these results were consistent with those of the GeneChip analysis (Table 2) regarding the induction effect of spermidine on the *oprH-phoPQ* and *PA3552-PA3559* operons. These data also suggested a differential effect of polyamines on the induction of the *oprH* and *PA3552* promoters.

The *oprH-phoPQ* knockout mutants exhibited different antibiotic susceptibility profiles. *P. aeruginosa* MPAO1 and its isogenic transposon insertion mutants of *oprH*, *phoP*, *phoQ*, *pmrB*, and *PA4773* were obtained from the University of Washington Genome Center (http://www.genome.washington.edu/UWGC/), and MICs of a variety of antibiotics against

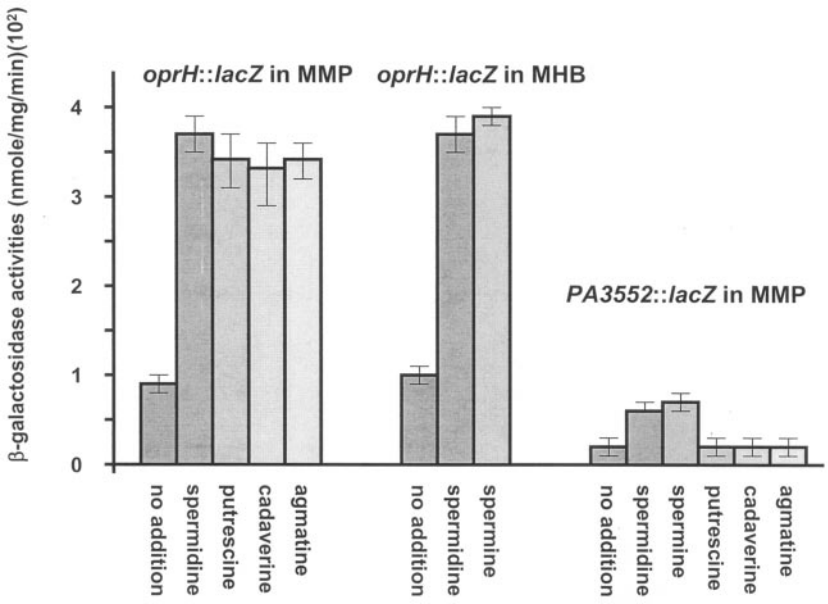

FIG. 1. Effects of polyamines on the induction of *oprH*::*lacZ* and *PA3552*::*lacZ* fusions. *P. aeruginosa* PAO1 containing each of the fusions was grown in either MMP or Miller-Hinton broth in the presence of exogenous supplements as indicated (final concentration, 20 mM), and β-galactosidase activities were measured as described in Materials and Methods.

TABLE 5. Antibiotic susceptibility of *P. aeruginosa* MPAO1 and its mutant strains grown with or without spermidine

P. aeruginosa strain and growth conditions	MIC (μg/ml)[a]								
	Px-B	COL	KAN	GEN	CIP	NOR	TET	EtBr	ACO
MPAO1 grown with									
MMP	2	2	16	0.25	1	4	8	256	64
MMP + spermidine[b]	16	16	64	1	4	16	8	>512	512
MPAO1 (*oprH*::Tc) grown with									
MMP	4	8	64	0.5	0.12	1	>100	256	64
MMP + spermidine	16	32	128	2	0.5	4	>100	>512	512
MPAO1 (*phoP*::Tc) grown with									
MMP	2	2	64	0.25	0.06	0.25	>100	256	64
MMP + spermidine	2	2	128	1	0.12	0.5	>100	>512	512
MPAO1 (*phoQ*::Tc) grown with									
MMP	16	32	64	0.5	0.06	0.25	>100	256	64
MMP + spermidine	≥128	≥128	128	4	0.25	1	>100	>512	512
MPAO1 (*PA3552*::Tc) grown with									
MMP	2	2	16	0.25	1	4	>100	256	64
MMP + spermidine	4	8	64	1	4	16	>100	>512	512

[a] MIC measurements were repeated three times by the broth dilution method as described in Materials and Methods.
[b] A 20 mM concentration of spermidine was added to MMP containing 20 mM glucose as a carbon source. Px-B, polymyxin B; COL, colistin; KAN, kanamycin; GEN, gentamicin; CIP, ciprofloxacin; NOR, norfloxacin; TET, tetracycline; EtBr, ethidium bromide; ACO, acridine orange.

these strains were determined in cultures with or without spermidine (Table 5). MICs of tetracycline for transposon insertion mutants were much higher than for the parent strain MPAO1 due to the presence of a tetracycline resistance cassette carried by the transposon. As revealed from these experiments, the *pmrB* and *PA4773* mutants had the same susceptibility profiles as strain MPAO1 (data not shown). Significant changes in antibiotic resistance profiles were observed in the *oprH*, *phoP*, and *phoQ* mutants as described below.

Consistent with what was observed in strain PAO1 (Table 3), spermidine also caused similar relative increases in the MICs of cationic peptide antibiotics (polymyxin B and colistin), aminoglycosides (kanamycin and gentamicin), quinolones (ciprofloxacin and norfloxacin), and dyes (ethidium bromide and acridine orange) for strain MPAO1 (Table 5). However, it was noted that for strain MPAO1, the MICs of kanamycin and quinolones in the absence of spermidine were 4-fold lower and 16-fold higher, respectively, than the MICs for PAO1.

The MICs of cationic peptide antibiotics for the *oprH* and *phoQ* mutants were two- to eightfold higher than for the *phoP* mutant and the parent strain MPAO1 when tested in the absence of spermidine. As with results for strain MPAO1, the addition of spermidine also increased the MICs of cationic peptide antibiotics for the *oprH* and *phoQ* mutants; however, this effect of spermidine was completely abolished for the *phoP* mutant. These results indicated that PhoP, the response regulator of the PhoPQ two-component system, is essential for resistance induced by spermidine to cationic peptide antibiotics.

The MICs of quinolones for the *oprH*, *phoP*, and *phoQ* mutants were significantly lower than for the parent strain MPAO1 in the absence of spermidine. However, exogenous spermidine still exerted its effect on increasing the MICs of quinolones for the *oprH* and *phoQ* mutants but not for the *phoP* mutant. Similar to what was concluded for cationic peptide antibiotics, these results indicated that PhoP is important for the spermidine effect on induced resistance to quinolones.

An increase in the MIC value in the presence of spermidine was also found for the MICs of aminoglycosides and fluorescent dyes for the *oprH*, *phoP*, and *phoQ* mutants. It should be noted that *P. aeruginosa* possesses intrinsic resistance to kanamycin due to the presence of an aminoglycoside 3′-phosphotransferase (6), which might be the reason that the spermidine effect was more obvious with gentamicin (four- to eightfold fold) than with kanamycin (twofold). Consistent with what was reported previously (15), we also observed that the MIC of kanamycin increased by fourfold for the *oprH*, *phoP*, and *phoQ* mutants.

Magnesium has no effect on polyamine-mediated antibiotic resistance. It has been reported that *P. aeruginosa* grown in low magnesium (e.g., 20 μM) medium exhibited increased resistance to cationic antibiotics and aminoglycosides, and expression of *oprH-phoPQ* and the *PA3552-PA3559* operon was also induced under this condition (22). To test whether magnesium limitation affects polyamine-mediated antibiotic resistance, the MICs of cationic peptide antibiotics, aminoglycosides, quinolones, tetracycline, and dyes were measured in spermidine-supplemented medium with either limiting (20 μM) or high (2 mM) concentrations of magnesium. Magnesium concentrations had no effect on spermidine-induced increases in MICs (data not shown). Magnesium limitation alone was able to increase the MICs of cationic peptides and aminoglycosides but showed no effect on other compounds tested (Table 3). These data suggested that the physiological responses triggered by magnesium limitation are different from those of polyamines.

The possible effect of magnesium on expression of the *oprH* promoter was analyzed by measurements of β-galactosidase activities from the *oprH*::*lacZ* transcriptional fusion. As shown in Fig. 2, spermidine induction of the *oprH* promoter activity was not affected by magnesium concentrations. In the absence of spermidine, the *oprH* promoter was significantly induced by magnesium limitation, as reported previously (20).

Roles of spermidine uptake in spermidine-mediated antibiotic resistance. Two mutant strains of *P. aeruginosa* PAO1 that were blocked in the polyamine transport system (*spuF*::Tc and *spuH*::Tc) were employed to determine whether spermidine

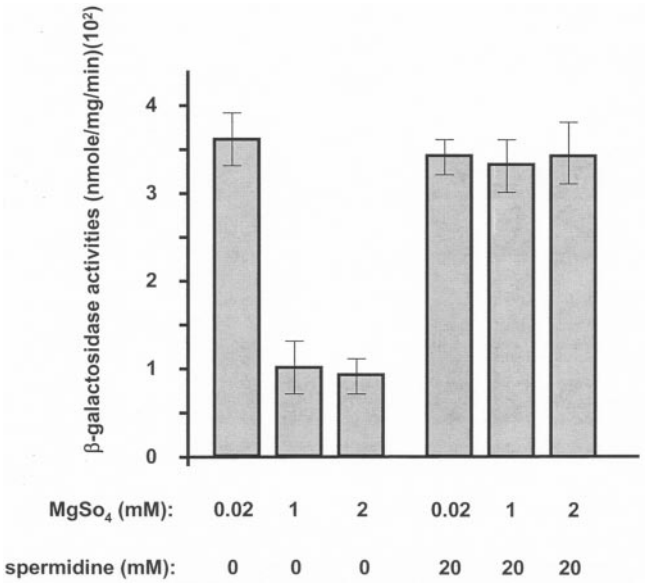

FIG. 2. Effects of magnesium concentrations on spermidine-dependent induction of the *oprH* promoter. The *oprH*::*lacZ* fusion was introduced into *P. aeruginosa* PAO1, and β-galactosidase activities from the cells grown under the indicated culture conditions were measured as described in Materials and Methods.

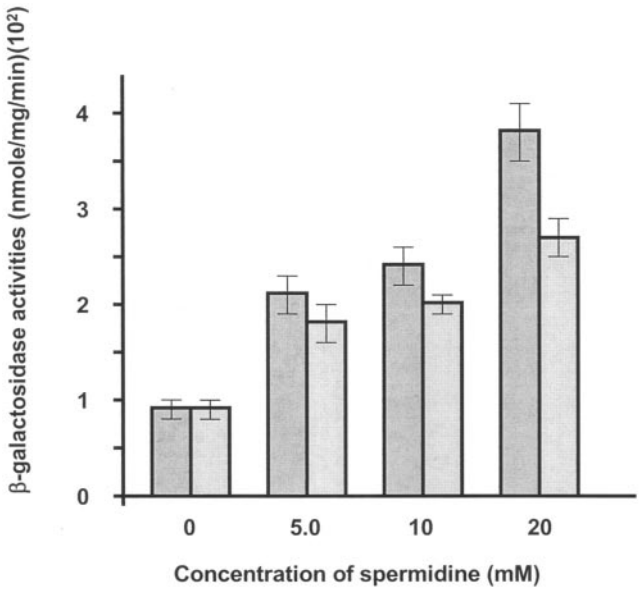

FIG. 3. Effects of spermidine uptake on induction of β-galactosidase activities. The *oprH*::*lacZ* fusion was expressed in *P. aeruginosa* PAO1 (dark gray bars) and its mutant strain (*spuF*::Tc) (light gray bars) in the presence of different concentrations of spermidine.

uptake is required for its effects on antibiotic resistance and induction of the *oprH* promoter. Utilization of spermidine as the sole source of carbon was abolished in these mutants (13). As shown in Table 3, the MICs of cationic peptide antibiotics and dyes for both mutant strains were fully increased by spermidine to the same levels as for the parental strain. In contrast, spermidine exerted no effect on the MICs of aminoglycosides and quinolones for the mutant strains.

Expression of the *oprH*::*lacZ* transcriptional fusion was monitored in cells grown under different concentrations of spermidine. As shown in Fig. 3, induction by spermidine in the wild-type strain PAO1 was concentration dependent. In the *spuF* mutant, exogenous spermidine still exerted an induction effect on the *oprH* promoter, but the level of induction by 20 mM spermidine was approximately two-thirds of that in the parental strain.

DISCUSSION

In this study, we presented solid evidence to demonstrate that polyamines (putrescine, spermidine, spermine, and cadaverine) and agmatine (the precursor compound of putrescine) represent a new group of environmental factors affecting antibiotic resistance in *P. aeruginosa*. In general, very little information is currently available regarding the effects of environmental factors on antibiotic resistance in bacteria, contrary to the enormous number of reports on mechanisms of drug inactivation, target modifications, uptake, or efflux. The magnesium limitation effect probably was the only case in this category that is being studied extensively at the molecular level (4, 15, 16, 18). Although polyamine supplementation and magnesium limitation exhibited similar phenotypes of antibiotic resistance, these two factors exerted their effects on cells through different routes, as indicated by the resistance spectrum, induction patterns, and genetic responses.

The resistance spectrum triggered by polyamine supplementation was significantly broader than that of magnesium limitation. Magnesium-mediated resistance included cationic peptide antibiotics and aminoglycosides (15). We demonstrated (Table 3) that the MICs of aminoglycosides, quinolones, and fluorescent dyes were increased to different levels by all polyamines and that the MICs of cationic peptide antibiotics were increased significantly by spermidine and spermine. These results also suggested that the spermidine- and/or spermine-responsive molecular mechanisms may be different from those of other polyamines.

Spermidine-mediated resistance was independent of magnesium concentration. It has been reported that the magnesium-mediated response was only induced by low concentrations of this divalent cation. In contrast, spermidine-mediated resistance required 5 mM to 20 mM concentrations this polyamine, and this response was not affected by the concentrations of magnesium (20 μM versus 2 mM). The concentration required for the induction of resistance may be clinically relevant to the efficiency of antibiotic treatment. The physiological concentrations of magnesium and polyamines are in the millimolar ranges in eukaryotic cells and tissues (3). These levels of polyamines, but not magnesium, would be enough to induce resistance in *P. aeruginosa* against a variety of antibiotics.

The genetic responses to spermidine and magnesium are different but overlapping. Spermidine supplementation or magnesium limitation (15, 16) caused cationic peptide resistance in *P. aeruginosa*. Cationic peptide antibiotics (e.g., polymyxin B) were reported to disrupt the structural organization of LPS, which increases permeability of the cationic peptide antibiotics and kills bacterial cells. For cell survival, a cascade of signal transduction was triggered to add 4-aminoarabinose

residues to the lipid A portion of LPS. As a result, this modification makes LPS more positively charged, reducing the binding potential of cationic peptide antibiotics. In the wild-type strain of *P. aeruginosa*, this regulatory response worked only under very low magnesium conditions, mediated by the PhoPQ two-component system to transmit signals to the *PA3552-PA3559* operon of LPS modification. It has been proposed that the PhoP response regulator by itself was active and phosphorylated by unknown mechanisms and that the membrane-anchored PhoQ sensor acts as a phosphatase to deactivate PhoP by the presence of magnesium (15, 16). In the case of spermidine, the results of GeneChip experiments and promoter fusion studies have indicated that this compound induced the expression of the *oprH-phoPQ* and *PA3552-PA3559* operons regardless of the magnesium concentrations. In addition, the results of knockout mutant analyses showed that only PhoP but not PhoQ was essential for cationic resistance in the presence of spermidine. This observed delineation of PhoP and PhoQ was consistent with what has been reported by Hancock and coworkers (15, 16) and suggested interactions between the PhoPQ system and the spermidine-responsive regulatory elements yet to be identified. The PmrAB two-component system was excluded from this study as possible candidates for spermidine- or polyamine-responsive regulatory elements based on the results of GeneChip analysis, promoter fusions, and phenotypes of knockout mutants.

While the involvement of PhoPQ in causing resistance to cationic peptide antibiotics has been reported (15), we were surprised that PhoPQ also played a role in quinolone susceptibility (Table 4). In addition, we also observed that inactivation of PhoPQ can increase the MIC of kanamycin, which was consistent with what was reported previously (15), but produced no change in the MIC of another aminoglycoside, gentamicin. More work is required to elucidate the diverse molecular mechanisms of PhoPQ in the control of antibiotic susceptibility.

The complexity of polyamine-mediated antibiotic resistance was further reflected by the results of spermidine uptake mutants. Spermidine uptake was greatly diminished in these mutants as reported previously (13). Resistance in the presence of spermidine to aminoglycosides and quinolones was lost in these mutants (Table 3), suggesting that an increased intracellular concentration of spermidine, and perhaps polyamines in general, is required for the induction of resistance mechanisms for these antibiotics. Contrarily, resistance to cationic peptide antibiotics and dyes in the presence of spermidine was retained in the uptake-deficient mutants, and a much lower concentration of spermidine or spermine was sufficient to increase the MIC of polymyxin B significantly (Table 4). These results would suggest the presence of a spermidine-responsive sensor residing on the cytoplasmic membrane in control of the phosphorylation status of PhoP.

The presence of multiple efflux pumps is known to account for high levels of intrinsic antibiotic resistance in *P. aeruginosa*. It is reasonable to speculate that spermidine and other polyamines may be able to induce the expression of efflux pumps. We considered this hypothesis unlikely as no significant induction of any known efflux systems by spermidine can be identified from DNA microarray analysis (data not shown). However, polyamines might have a positive effect on the synthesis or the activity of efflux pumps by posttranscriptional regulation. In summary, the polyamine-mediated antibiotic resistance reported here warrants further studies to elucidate the molecular mechanisms of drug resistance of these potent compounds in *P. aeruginosa*.

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