

# Surface-Localized Spermidine Protects the *Pseudomonas aeruginosa* Outer Membrane from Antibiotic Treatment and Oxidative Stress

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**Extracellular DNA acts as a cation chelator and induces the expression of antibiotic resistance genes regulated by  $Mg^{2+}$  levels. Here we report the characterization of novel DNA-induced genes in *Pseudomonas aeruginosa* that are annotated as homologs of the spermidine synthesis genes *speD* (PA4773) and *speE* (PA4774). The addition of sublethal concentrations of DNA and membrane-damaging antibiotics induced expression of the genes PA4773 to PA4775, as shown using transcriptional *lux* fusions and quantitative RT-PCR. Exogenous polyamine addition prevented DNA- and peptide-mediated gene induction. Mutation of PA4774 resulted in an increased outer membrane (OM) susceptibility phenotype upon polymyxin B, CP10A, and gentamicin treatment. When the membrane-localized fluorescent probe  $C_{11}$ -BODIPY<sup>581/591</sup> was used as an indicator of peroxidation of membrane lipids, the PA4774::*lux* mutant demonstrated an increased susceptibility to oxidative membrane damage from  $H_2O_2$  treatment. Addition of exogenous polyamines protected the membranes of the PA4774::*lux* mutant from polymyxin B and  $H_2O_2$  treatment. Polyamines from the outer surface were isolated and shown to contain putrescine and spermidine by using high-performance liquid chromatography and mass spectrometry. The PA4774::*lux* mutant did not produce spermidine on the cell surface, but genetic complementation restored surface spermidine production as well as the antibiotic and oxidative stress resistance phenotypes of the membrane. We have identified new functions for spermidine on the cell surface and propose that polyamines are produced under  $Mg^{2+}$ -limiting conditions as an organic polycation to bind lipopolysaccharide (LPS) and to stabilize and protect the outer membrane against antibiotic and oxidative damage.**

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that is able to cause chronic infections, including wound infections, otitis media, chronic sinusitis, and lung infections in persons with cystic fibrosis (CF) (29, 53, 56). Chronic infections are proposed to be caused by bacterial biofilms, and there is strong evidence to suggest that *P. aeruginosa* grows as a biofilm in the lungs during chronic infections in CF patients (11, 46, 53). *P. aeruginosa* is also a major cause of hospital-acquired infections, many of which are due to biofilm colonization of medical implant devices or respirators (14). This opportunistic pathogen possesses high levels of intrinsic antibiotic resistance due to reduced outer membrane permeability, drug inactivation, drug efflux, and mechanisms involving target site mutation (17, 20). However, *P. aeruginosa* surface-adherent biofilms are 4- to 2,500-fold more resistant to various antimicrobial agents than free-floating planktonic cultures (1, 7, 49, 51). Biofilms are multicellular, surface-associated, microbial communities encased in an extracellular matrix that displays a complex three-dimensional structure and has increased resistance to antimicrobials, environmental stresses, and the host immune response (3, 12, 25). Bacteria growing in a biofilm can evade the host immune response and antibiotic therapy, complicating the treatment process and leading to the development of chronic infections (11, 46, 53).

Gram-negative bacteria possess an outer membrane (OM), which functions as a permeability barrier to extracellular compounds. The asymmetric outer membrane is composed of lipopolysaccharides (LPS) in the outer leaflet and phospholipids in the inner leaflet. LPS is stabilized by the binding of divalent cations such as  $Mg^{2+}$  and  $Ca^{2+}$ , which bind neighboring LPS molecules such that displacement of the cations results in destabilization and major disruptions of the cell membrane integrity, ultimately leading to cell death (19). One mechanism of antibiotic resistance involves modifications of LPS that reduce the binding and pene-

tration of cationic antimicrobial peptides. The aminoarabinose LPS modification operon *arnBCADTEF-ugd* is controlled by the PhoPQ and PmrAB cation-sensing two-component systems and is regulated in response to  $Mg^{2+}$  levels in planktonic cultures (43, 44). Under  $Mg^{2+}$ -limiting conditions, this operon is required for antimicrobial peptide resistance (37). Strains with spontaneous polymyxin B resistance mutations in *pmrB* that constitutively produced aminoarabinose-modified LPS were isolated (47), and in these strains, aminoarabinose addition to the phosphates of the lipid A core masks the negative charges and reduces the binding of cationic antimicrobial peptides to the outer membrane. More recently, polymyxin B-resistant clinical isolates of *P. aeruginosa* with specific regulatory mutations that demonstrated increased expression of PhoPQ and PmrAB-controlled genes, including the *arn* operon, were described (59).

In a study to identify biofilm-specific antibiotic resistance determinants in *P. aeruginosa*, Mah et al. described the *ndvB* gene that was upregulated in biofilms and was required for the production of periplasmic cyclic glucans, which bind aminoglycosides and prevent their entry into the cytoplasm (41). We recently reported a novel property of DNA as a divalent metal cation chelator, likely due to its highly anionic charge from phosphates in the deoxyribose backbone (49). At high concentrations, DNA is toxic and chelates cations from the OM, leading to major disruptions of

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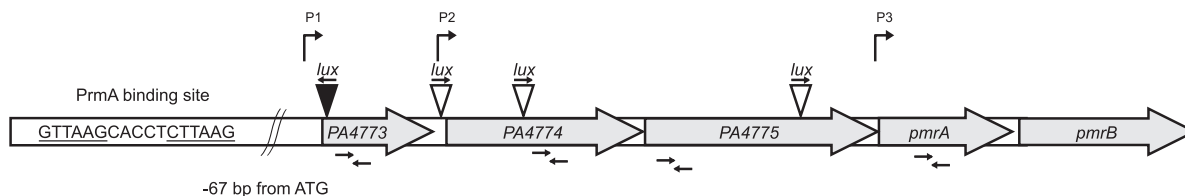
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**FIG 1** Schematic view of *PA4773* to *PA4775*. *PA4773* to *PA4775* are located immediately upstream of *pmrAB*. The locations of the mini-Tn5-*lux* transposon mutants are indicated (the black triangle indicates a nontranscriptional *lux* fusion; white triangles indicate transcriptional *lux* fusions), along with the locations of the promoters and the PmrA binding site, located 67 bp upstream of the start codon of *PA4773*. Primer pair locations used for qRT-PCR are indicated by the small arrows below the diagram. The putative promoters (P1, P2, and P3) were determined on the basis of transcript length analysis, as shown in Fig. S1 in the supplemental material.

both inner and outer membranes and death, similar to the cation-chelating activity of EDTA (49). However, the addition of sublethal levels of DNA to planktonic cells mimics a DNA-rich biofilm and induces the *arn* operon, resulting in high levels of resistance to antimicrobial peptides and aminoglycosides (49). As extracellular DNA accumulates in the biofilm matrix, we proposed that this outer membrane permeability mechanism of resistance is another example of a biofilm-induced resistance mechanism (49).

In previous studies examining the PhoPQ and PmrAB regulons, we identified the *PA4773-PA4775* gene cluster, located immediately upstream of *pmrAB*, which is induced under  $Mg^{2+}$ -limiting conditions (Fig. 1) and is regulated primarily by PmrAB (37, 43, 44). The genes *PA4773* and *PA4774* show significant similarity to the polyamine synthesis genes *speD* (52%) and *speE* (56%) of *Escherichia coli*. SpeD converts S-adenosylmethionine (SAM) to decarboxylated SAM (dSAM), which is used as a substrate with putrescine to synthesize spermidine by SpeE (40, 62). *P. aeruginosa* contains *speD* (*PA0654*) and *speE* (*PA1687*), in addition to their respective homologs *PA4773* and *PA4774*. *PA4775* is annotated as a hypothetical protein of unknown function with a PSORT-predicted signal peptide and one predicted transmembrane domain.

Polyamines are hydrocarbons containing two or more amine groups and are therefore polycationic. In bacteria, spermidine and putrescine are the predominant cytoplasmic polyamines; cadaverine is found in lesser quantities, and the presence of spermine is controversial (60). These molecules are universal, being found in some combination in all cellular life forms (60). Their ubiquitous nature has made defining their exact role within cells difficult. Research is widely available to suggest that they are essential for optimal cell growth and viability, and additionally, they have been linked to microbial pathogenesis, biofilm formation, escape from phagolysosomes, bacteriocin production, toxin activity, and antibiotic resistance (60, 66). Polyamines are frequently associated with the negatively charged nucleic acids (and proteins) in the cytoplasm and have been shown to protect DNA from oxidative damage (16).

Here, we report that *PA4773* to *PA4775* are induced by the cation-chelating activity of extracellular DNA. We provide evidence that *PA4773* and *PA4774* constitute an inducible pathway of spermidine synthesis in *P. aeruginosa* as a replacement for divalent metal cations and have identified novel functions of polyamines in protecting bacterial membranes from antibiotic and oxidative damage.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All strains and plasmids used are listed in Table 1. *P. aeruginosa* PAO1 was used as the wild-type strain. The mini-Tn5-*lux* transposon mutants in *PA4773* to *PA4775* were previously isolated and shown not to have polar effects on the downstream regulatory *pmrAB* genes, as previously published (see Fig. S1 in the supplemental material) (37, 43). Unless otherwise stated, cultures were routinely grown at 37°C in BM2 defined minimal medium with either high (2 mM) or low (0.02 mM)  $MgSO_4$ . BM2 growth medium includes the following components: 0.1 M HEPES (pH 7), 7 mM ammonium sulfate, 20 mM sodium succinate (pH 6.7), 10  $\mu$ M iron sulfate, 1,600  $\mu$ M phosphate buffer (pH 7.2), 1.62  $\mu$ M manganese sulfate, 2.45  $\mu$ M calcium chloride, 13.91  $\mu$ M zinc chloride, 4.69  $\mu$ M boric acid, and 0.67  $\mu$ M cobalt chloride (48). When added, the source of extracellular DNA was fish sperm DNA (USB).

**Gene expression assays.** Gene expression was performed in a high-throughput format using 96-well microplates as previously described (49). Briefly, overnight cultures were grown in BM2 defined medium supplemented with 2 mM or 0.02 mM  $Mg^{2+}$  and extracellular DNA as indicated, diluted 1/1,000 into 150  $\mu$ l of culture medium in 96-well black plates with a transparent bottom (9520 Costar; Corning Inc.), and overlaid with 50  $\mu$ l of mineral oil to prevent evaporation. Microplate planktonic cultures were incubated at 37°C in a Wallac Victor<sup>3</sup> luminescence plate reader (Perkin-Elmer), and optical density at 600 nm ( $OD_{600}$ ) (to assess growth) and luminescence (counts per second [CPS]) (to assess gene expression) readings were taken every 20 min throughout 18 h of growth.

**Preparation of surface polyamines.** Mid-log-phase cultures (100 ml) were grown under inducing conditions (0.02 mM  $Mg^{2+}$ ). Cells were collected by centrifugation (10 min, room temperature [RT], 5,000  $\times$  g) and normalized to equivalent cell numbers for all strains. Cells were resuspended in 1 ml of 1 M NaCl dissolved in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.4) and incubated for 10 min at 37°C shaking. After incubation, cells were centrifuged three times (10 min, RT, 5,000  $\times$  g) to remove whole cells, and the supernatant, which now contained the surface-washed polyamines as previously described (31), was collected.

**Measurement of XylE activity.** XylE assays were performed as previously described with minor modifications (43). Twenty-five-milliliter cultures of *P. aeruginosa* *phoQ::xylE* (43) were grown to mid-log phase. Cells were collected and subjected to a 1 M NaCl wash, a 50 mM  $KH_2PO_4$  (pH 7.5) buffer wash, or a 50 mM  $KH_2PO_4$  (pH 7.5) buffer wash containing 10% acetone followed by sonication, which lysed cells and released the enzyme XylE (catechol 2,3-dioxygenase) from the cytoplasm. XylE assays were performed after the addition of 0.3 mM catechol in 50 mM potassium phosphate buffer to 100  $\mu$ l of lysates or culture washes. The conversion of catechol to 2-hydroxymuconic semialdehyde was measured every 5 min over the course of 1 h by measuring  $A_{405}$  with a Wallac Victor<sup>3</sup> luminescence plate reader (Perkin-Elmer), and the XylE activity was measured as a change in absorbance.

TABLE 1 Bacterial strains used in this study<sup>a</sup>

Strain or mutation	Genotype	Reference or source
<b>Strains</b>		
PAO1	Wild-type <i>P. aeruginosa</i>	R. E. Hancock
PA4773	PA4773 transposon mutant, nontranscriptional fusion, 98_E1	37
PA4773-4::lux	Intergenic PA4773-PA4774 transposon mutant, and transcriptional fusion, 11_A9	43
PA4774::lux	PA4774::lux transposon mutant and transcriptional fusion, 38_F9	37
PA4775::lux	PA4775::lux transposon mutant and transcriptional fusion, 50_F5	37
PA4774::lux <sub>CC</sub>	PA4774::lux chromosomally complemented with PA4773-PA4775 integrated into attTn7 site	This study
PA4773-4::lux <i>phoQ</i> :: <i>xylE</i>	Double mutant; transcriptional fusion PA4773-4::lux in a <i>phoQ</i> :: <i>xylE</i> mutant background	43
PA4773-4::lux <i>pmrB</i> :: <i>xylE</i>	Double mutant; transcriptional fusion PA4773-4::lux in a <i>pmrB</i> :: <i>xylE</i> mutant background	43
PA3553::lux	PA3553::lux transposon mutant and transcriptional fusion, 53_D10	37
<b>Plasmids</b>		
pUCP22	Amp <sup>r</sup> , multicopy broad-host-range expression vector	64a
pCR2.1-TOPO	PCR cloning vector, Amp <sup>r</sup>	Invitrogen
pUC18T-mini-Tn7T-Gm	Gm <sup>r</sup> on mini-Tn7T; mobilizable; for gene insertion in Gm <sup>s</sup> bacteria	10
pTNS2	Helper plasmid for integration into the att site	10
pUC18T-mini-Tn7T-PA4773-PA4775	pUC18T-mini-Tn7 containing PA4773-PA4775 genes, including native upstream promoter	This study

<sup>a</sup> Abbreviations: Amp<sup>r</sup>, ampicillin resistance; Gm<sup>r</sup>, gentamicin resistance; Gm<sup>s</sup>, gentamicin sensitive.

**HPLC-UV-mass spectrometry (MS) analysis of polyamines.** Polyamines were analyzed as their dansyl chloride derivatives in reversed-phase high-performance liquid chromatography (HPLC) with modifications of previously established methods (15, 31, 32). The dansylation reaction mixture consisted of 100  $\mu$ l of NaCl-washed cell surface extract, 100  $\mu$ l of 0.5 M NaHCO<sub>3</sub>-Na<sub>2</sub>CO<sub>3</sub> buffer (pH 9.4), and 100  $\mu$ l of 20 mg/ml dansyl chloride. After incubation for 60 min at 60°C with shaking, methylamine (30  $\mu$ l of 0.5 mol/liter) was added to the reaction mixture to consume the excess dansyl chloride. The solutions were vortexed, incubated for an additional 30 min at 60°C, centrifuged for 10 min at 13,400  $\times$  g, and finally transferred into new vials.

HPLC separation was performed with an Agilent 1200 SL HPLC system with a Luna C<sub>18</sub> (2) reverse-phase column (50 by 2 mm) (Phenomenex, Torrance, CA), thermostated at 30°C, with a buffer gradient system composed of 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B at a flow rate of 0.20 ml/min. To 2  $\mu$ l of sample solution, 9  $\mu$ l of 0.1% formic acid in water was added, and 10  $\mu$ l of this solution was injected onto the column. After injection, the column was washed with 95% mobile phase A and 5% mobile phase B for 2 min to effectively remove salts. Elution of the analytes was done by using a linear gradient from 5% to 95% mobile phase B over a period of 25 min. UV absorbance was monitored at 340 nm to detect dansyl-labeled compounds. Mass spectra were acquired in positive-mode ionization using an Agilent 6220 accurate-mass time-of-flight (TOF) HPLC/MS system (Santa Clara, CA) equipped with a dual sprayer electrospray ionization source, with the second sprayer providing a reference mass solution. Mass spectrometric conditions were as follows: drying gas, 10 liters/min at 325°C; nebulizer, 20 lb/in<sup>2</sup>; mass range, 100 to 1,000 Da; acquisition rate, ~1.03 spectra/s; fragmentor, 120 V; skimmer, 60 V; capillary, 3,200 V; and instrument state, 2-GHz extended dynamic range. Mass correction was performed for every individual spectrum using peaks at *m/z* 121.05087 and 922.00979 from the reference solution. Data acquisition was performed using the Mass Hunter software package (version B.02.01.) HPLC-UV-MS data were analyzed by using Agilent Mass Hunter qualitative analysis software (version B.03.01) using a targeted approach.

**Construction of chromosomally complemented strain.** Initial attempts to express single genes from pUCP plasmids were unsuccessful due to increased toxicity, as determined by live/dead staining (data not shown), when these genes were expressed individually in high-copy plas-

mids. Therefore, we cloned PA4773, PA4774, and PA4775 with their native upstream promoter and integrated them into the chromosome at the neutral attTn7 sites using the mini-Tn7 integration system as previously described (9). The genes were PCR amplified using the primers P4773FBamHI and P4775RBamHI and cloned into the TA cloning vector pCR2.1 (Invitrogen). The insert was BamHI excised and subcloned into the BamHI-digested pUC18T-mini-Tn7T-Gm plasmid (9). The *P. aeruginosa* PA4774::lux mutant strain was transformed simultaneously with pUC18T-mini-Tn7-PA4773-PA4775 and the helper plasmid pTNS2 (10) via electroporation. Transformants were selected on LB agar containing 50  $\mu$ g/ml gentamicin, and positive clones were confirmed by PCR. The gentamicin resistance cassette was subsequently removed following the protocol by Choi and Schweizer, using Flp-mediated excision (9).

**Outer membrane permeability assay.** Outer membrane permeability in response to polymyxin B treatment was assessed as previously described, using a PTI Quantamaster spectrofluorimeter (39). Briefly, *P. aeruginosa* strains were grown in BM2 with 0.02 mM Mg<sup>2+</sup> to mid-log phase (OD<sub>600</sub> = 0.5), washed and resuspended in 5 mM HEPES buffer (pH 7.2) containing 5 mM glucose and 0.5 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and held at room temperature until testing was completed, which was within 2 h. Twenty microliters of 1-*N*-phenyl-naphthylamine (NPN; 0.5 mM stock) was added to one milliliter of culture (OD<sub>600</sub> = 0.5), and baseline NPN fluorescence was recorded for up to 50 s before the addition of 6.4  $\mu$ g/ml (final concentration) polymyxin B or 20  $\mu$ g/ml gentamicin. Fluorescence measurements were taken until the counts/s reached a steady state (generally within 150 s). To monitor NPN fluorescence, the excitation/emission wavelengths were set to 350 nm and 420 nm, respectively, both with 5-nm slit widths. To complement the OM permeability phenotypes, control polyamines (Sigma) were exogenously added to the cultures at various concentrations, and cultures were incubated for 10 min prior to challenge with antibiotics.

**Quantitative reverse transcription (RT)-PCR.** Bacterial cultures of *P. aeruginosa* PAO1 and the PA4773, PA4773-4::lux, and PA4774::lux mutants were grown in BM2 medium containing 2 mM or 0.02 mM Mg<sup>2+</sup> or 2 mM Mg<sup>2+</sup> supplemented with 0.75% DNA to an OD<sub>600</sub> of 0.25 to 0.30. Cells were then treated with RNAprotect bacterial reagent (Qiagen) according to the manufacturers' instructions and harvested by centrifugation prior to storage at -80°C. Total RNA was extracted using an RNeasy RNA isolation minikit (Qiagen) and treated with DNase using DNasefree

TABLE 2 Primer sequences

Primer	5'–3' sequence <sup>a</sup>
P4773FBamHI	CCGGATCCGTATCCACCAGCCGTACCTG
P4775RBamHI	CCGGATCCCTCAGTGATTCCACAATTCCC
PA3552rtF	GTAGCGGCATCCATTTCATC
PA3552rtR	CATCGACGTTTCTCCAGGAT
PA4773rtF	CAGTGGATCGAGGAAAGCAT
PA4773rtR	GTAATCCGGCCAGGTATGG
PA4774rtF	TTCTACGAGCTGCTGCATTC
PA4774rtR	GACCTGGGAGAAGACACTGC
PA4775rtF	GTACCGCTGGCCGTCACAC
PA4775rtR	GTCTTCGCTCAGCTCGATG
pmrArtF	CACCAGGTGACCCTGTCC
pmrArtR	CGTAGAGGCTCTGCTCCAGT
proCrtF	CAGGCCGGGCGAGTTGCTGTC
proCrtR	GGTCAGGCGGAGGCTGTCT

<sup>a</sup> Restriction sites are underlined.

(Ambion), and cDNA was synthesized with a high-capacity cDNA synthesis kit (ABI Biosystems). Prokaryotic gene expression was measured by using iQ SYBR green supermix (Bio-Rad) and bacterial primers specific to *PA3553*, *PA4773*, *PA4774*, *PA4775*, *pmrA*, and the *proC* housekeeping gene (58). Primer sequences are given in Table 2. For quantitative RT-PCR (qRT-PCR), quantification and melting curve analyses were performed with an iQ5 real-time pcr system (Bio-Rad) according to the manufacturer's instructions. Each reaction was done in triplicate, and standard deviations were used to calculate a range of activation (fold) using the  $2^{-\Delta\Delta CT}$  method (38).

**BODIPY<sup>581/591</sup> microscopy.** Overnight cultures were subcultured and grown to mid-log phase in low-Mg<sup>2+</sup> (0.02 mM) BM2 medium at 37°C. Cells were then stained with 2  $\mu$ M C<sub>11</sub>-BODIPY<sup>581/591</sup> for 10 min prior to treatment with 5 mM H<sub>2</sub>O<sub>2</sub> for 10 min. Cells were visualized on 1% agarose beds with a Leica DMI4000 B inverted microscope equipped with an ORCA R2 digital camera and Metamorph software for image acquisition. The excitation and emission filters were 555/25 and 605/52 for red fluorescence and 490/20 and 525/36 for green fluorescence.

**Membrane lipid peroxidation assay.** Overnight cultures were subcultured and grown to mid-log phase in low-Mg<sup>2+</sup> (0.02 mM) BM2 medium at 37°C. Cells were concentrated to 1  $\times$  10<sup>9</sup> CFU/ml and stained with 2  $\mu$ M C<sub>11</sub>-BODIPY<sup>581/591</sup>, which was incubated at room temperature to allow membrane incorporation. Cells (1  $\times$  10<sup>8</sup> CFU/well) were added to 96-well black microplates with transparent bottoms (9520 Costar; Corning Inc.), and an initial fluorescence reading taken using a Wallac Victor<sup>3</sup> plate reader (Perkin-Elmer) with emission filters set to 632 nm for red and 535 nm for green. After time zero readings, H<sub>2</sub>O<sub>2</sub> was added (5 to 25 mM), and red and green fluorescence readings were taken every 5 min over the course of 2 h.

**Statistical analysis.** Statistical analysis was performed on the data using the unpaired Student's *t* test to calculate significant differences between PAO1 and mutant strains.

## RESULTS

**The Mg<sup>2+</sup>-regulated genes *PA4773* to *PA4775* are induced by the cation-chelating activity of extracellular DNA.** Previous work has shown *PA4773* to *PA4775* to be strongly induced by limiting (0.02 mM) Mg<sup>2+</sup> and repressed under high (2 mM)-Mg<sup>2+</sup> conditions (37, 43, 44). This result was confirmed using the *PA4773-4::lux*, *PA4774::lux*, and *PA4775::lux* strains, which are transcriptional *lux* fusions and transposon insertion mutants (Fig. 2A). Purified PmrA-His<sub>6</sub> has been shown to bind the *PA4773* promoter, which contains a PmrA binding site (Fig. 1) (44). *PA4773* to *PA4775* are also induced by the addition of sublethal doses of

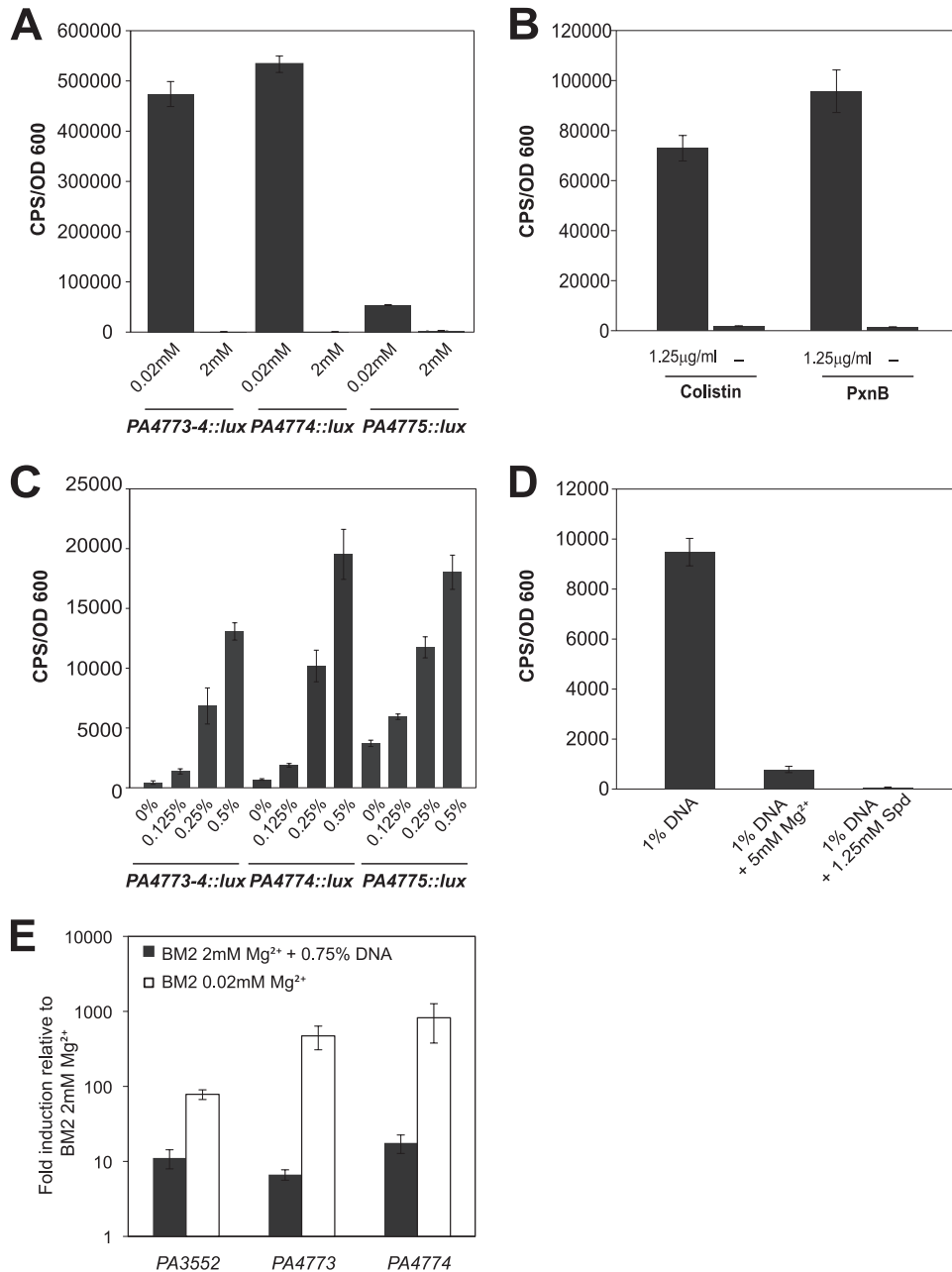
antimicrobial peptides under high-Mg<sup>2+</sup> conditions, as shown with the *PA4774::lux* strain (Fig. 2B) (43).

We recently showed that DNA is a cation chelator and induces the expression of the *arnBCADTEF-ugd* antimicrobial peptide resistance operon (49). To determine if *PA4773* to *PA4775* are also induced by the presence of extracellular DNA, we measured the expression of *lux* transcriptional fusions to each gene using a system where extracellular DNA was added to BM2 medium containing high levels of Mg<sup>2+</sup> (2 mM). The addition of exogenous DNA to planktonic cultures induced expression of each of the transcriptional reporters *PA4773-4::lux*, *PA4774::lux*, and *PA4775::lux*, in a concentration-dependent manner (Fig. 2C). In this experiment, there are very low levels of expression without exogenous DNA, as the high concentration of Mg<sup>2+</sup> (2 mM) represses expression of *PA4773* to *PA4775* (Fig. 2A). The DNA-mediated induction can be prevented by the addition of excess 5 mM Mg<sup>2+</sup>, indicating that it is the cation-chelating activity of extracellular DNA that induces expression of these genes (Fig. 2D). DNA-mediated induction can also be prevented by addition of excess spermidine (Fig. 2D).

As the *lux* reporter strains used to measure promoter activity also had mutations in these genes, quantitative RT-PCR was performed with wild-type PAO1 to show that limiting Mg<sup>2+</sup> and exogenous DNA both induced the expression of *PA4773* and *PA4774*. The expression of *PA4773* and *PA4774* was induced 6- to 17-fold by extracellular DNA and induced up to 800-fold by limiting Mg<sup>2+</sup> (Fig. 2E). *PA3552* is the first gene in the *arn* operon and was included as a positive control, as it is known to be both low-Mg<sup>2+</sup> and DNA regulated (Fig. 2E) (49). Induction of the *arn* genes and *PA4773* to *PA4775* is highest in the presence of 20  $\mu$ M Mg<sup>2+</sup>, and the addition of high concentrations of DNA (0.5 to 1%) to medium containing 2 mM Mg<sup>2+</sup> cannot mimic the effects of 20  $\mu$ M Mg<sup>2+</sup>. The highest level of DNA induction is comparable to gene expression in 500  $\mu$ M Mg<sup>2+</sup>, indicating that DNA can chelate up to 75% of the Mg<sup>2+</sup> in the medium (data not shown).

Expression of *PA4773-4::lux* was monitored under limiting Mg<sup>2+</sup> conditions in a single *PA4773-4::lux* mutant, as well as in a double *PA4773-4::lux phoQ::xylE* mutant and a double *PA4773-4::lux pmrB::xylE* mutant. *PA4773-4::lux* expression was highly induced even when *phoQ* was inactivated, but no induction was seen when *pmrB* was inactivated in the double mutant (Fig. 3A). These data indicate that the PmrAB two-component system is required for the expression of *PA4773-4::lux* under limiting Mg<sup>2+</sup> conditions, while the known cation-sensing system PhoPQ is not. As the expression of *PA4773-4::lux* is Mg<sup>2+</sup> regulated, this result suggests that the downstream genes *pmrAB* are expressed and functional, despite the upstream transposon insertion.

To confirm that transposon insertions in the *PA4773*, *PA4773-4::lux*, and *PA4774::lux* mutants do not cause polar effects on the downstream genes *pmrAB*, we examined the expression of each of the genes *PA4773*, *PA4774*, *PA4775*, and *pmrA* in *PA4773*, *PA4773-4::lux*, and *PA4774::lux* mutant backgrounds relative to their expression in PAO1 (Fig. 3B to E). Figure 3B shows that *PA4773* is not expressed in a *PA4773* mutant, indicated by the ~300-fold repression in the mutant relative to the wild-type strain, and *PA4773* expression is unaffected by downstream insertions. The expression of *PA4774* is also repressed in a *PA4773* mutant (120-fold) relative to PAO1, indicating that the upstream insertion has a polar effect on the *PA4774* gene (Fig. 3C). However, there is a greater loss of expression of *PA4773* than of *PA4774* in a *PA4773* mutant (300-fold versus 120-fold), which suggests that although

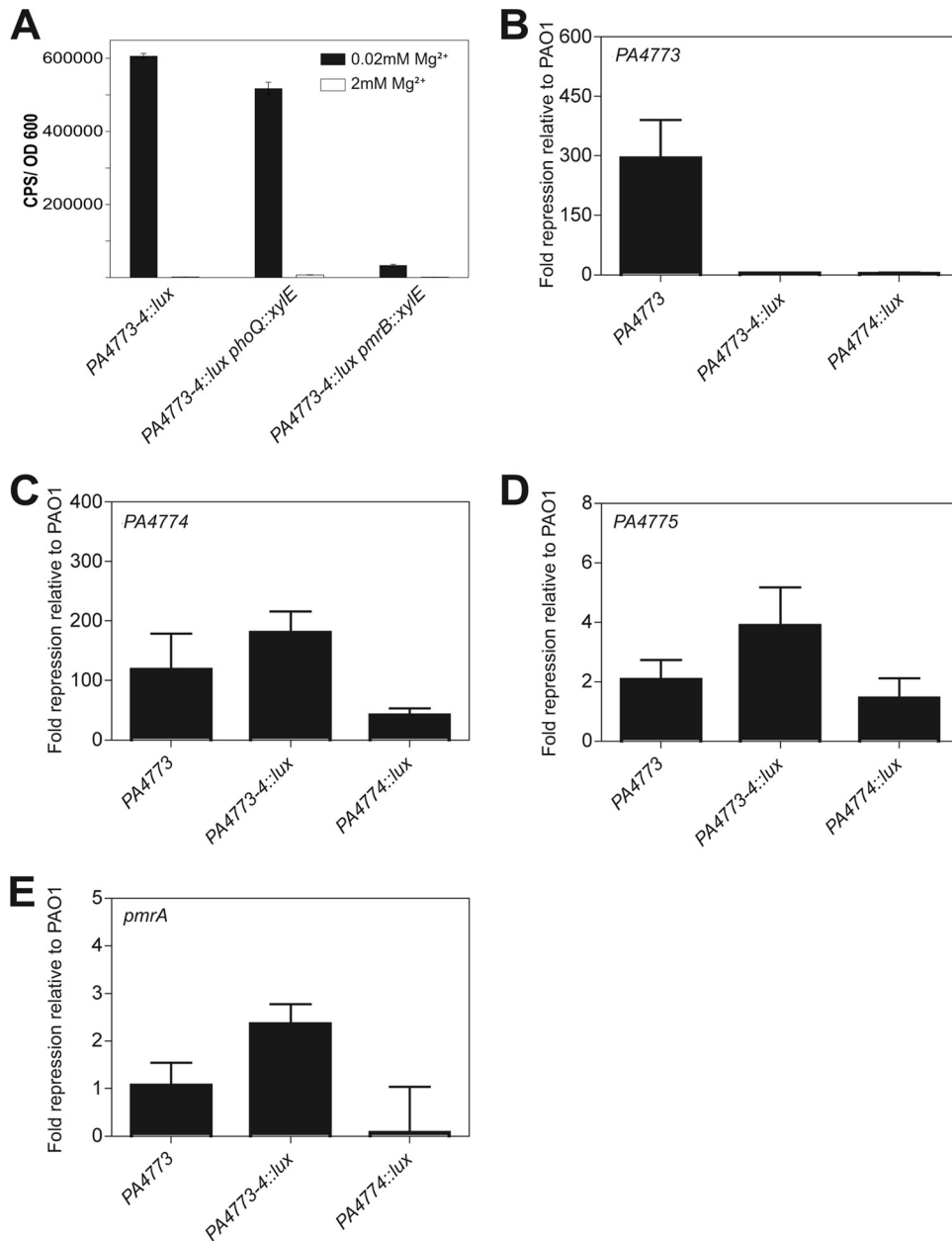


**FIG 2** The Mg<sup>2+</sup>-regulated genes *PA4773* to *PA4775* are induced by membrane-damaging antibiotics and extracellular DNA. (A) Expression of *PA4773-4::lux*, *PA4774::lux*, and *PA4775::lux* in low (0.02 mM) and high (2 mM) Mg<sup>2+</sup> concentrations. (B) The effects of sub-MIC concentrations of colistin (1.25 µg/ml) and polymyxin B (PxB) (1.25 µg/ml) on *PA4774::lux* expression were measured under noninducing conditions (BM2 medium with 2 mM Mg<sup>2+</sup>). Growth was not affected at these peptide concentrations (data not shown). (C) Effects of increasing concentrations of extracellular DNA (0.5% DNA; 5 mg/ml) added to BM2 medium with 2 mM Mg<sup>2+</sup> on *PA4773-4::lux*, *PA4774::lux*, and *PA4775::lux* expression. (D) DNA-mediated induction of *PA4774::lux* with 1% DNA (10 mg/ml) was neutralized with excess Mg<sup>2+</sup> (5 mM), or excess spermidine (Spd) (1.25 mM). Maximum levels of gene expression at 8 h are shown, where gene expression (in counts per second [CPS]) was normalized to growth (OD<sub>600</sub>). Each experiment was performed at least three times, and representative data are shown. The values shown are the averages from at least three technical replicates with standard deviations. (E) Quantitative RT-PCR was used to measure expression of *PA3552*, *PA4773*, and *PA4774* in wild-type PAO1 cultured to mid-log phase (OD, 0.25 to 0.3) in BM2 with 0.02 mM Mg<sup>2+</sup> or 2 mM Mg<sup>2+</sup> with or without 0.75% DNA. The level of gene expression is presented as fold change relative to BM2 with 2 mM Mg<sup>2+</sup>. Values are means and standard errors of the means from triplicate qPCR experiments on RNA isolated from three independent experiments.

there is a polar effect, there is residual expression and not a complete loss of *PA4774* expression. Expression of *PA4774* is also repressed in the *PA4773-4::lux* (180-fold) and *PA4774::lux* (40-fold) mutants, as is to be expected (Fig. 3C). The low level of repression observed for *PA4775* (1.5- to 3.8-fold) (Fig. 3D) and *pmrA* (0.3- to 1.7-fold) (Fig.

3E) in *PA4773*, *PA4773-4::lux*, and *PA4774::lux* mutants indicates that the expression of *PA4775* and *pmrA* is unaffected by upstream transposon insertions.

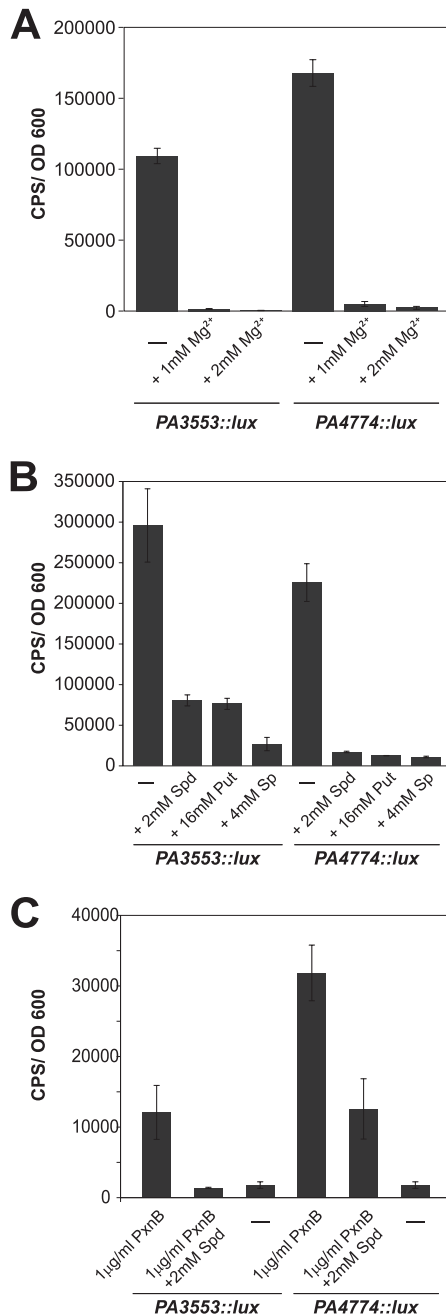
**Polyamines are functionally interchangeable with divalent metal cations.** The divalent metal cation Mg<sup>2+</sup> binds to LPS in the



**FIG 3** Transposon insertion into *PA4773* and *PA4774* does not cause polar effects on the downstream *PA4775* or *pmrAB* genes. (A) Effects of *phoQ* and *pmrB* mutation on *PA4773-4::lux* expression in low (0.02 mM) and high (2 mM) Mg<sup>2+</sup> conditions. (B to E) Quantitative RT-PCR analysis of *PA4773*, *PA4774*, *PA4775*, and *pmrA* gene expression in *PA4773*, *PA4773-4::lux*, and *PA4774::lux* mutants. The level of gene expression is represented as fold change relative to PAO1. All strains were cultured to mid-log phase in BM2 with 0.02 mM Mg<sup>2+</sup>. Values are means and standard errors of the means from triplicate qPCR experiments on RNA isolated from three independent experiments.

outer membrane and acts to stabilize LPS (19). The induction of the putative polyamine synthesis genes *PA4773* and *PA4774* by Mg<sup>2+</sup> limitation or by DNA suggests that *P. aeruginosa* produces its own replacement cation to adapt to the Mg<sup>2+</sup> limitation. DNA-mediated induction can be prevented by excess Mg<sup>2+</sup> but also by addition of excess spermidine (Fig. 2D). The MIC of spermidine is 32 mM, and there were no effects on growth when spermidine was exogenously supplied to cultures at the concentrations used (data not shown). This result suggests that both divalent metal cations and polyamines can bind and neutralize extracellular DNA.

In growth medium with limiting Mg<sup>2+</sup>, the addition of excess Mg<sup>2+</sup> cations (Fig. 4A) or excess polyamines (spermidine, putrescine, and spermine) (Fig. 4B) represses expression of *PA4774::lux*. Similarly, the polymyxin B-mediated induction of *PA4774::lux* can also be reduced by the addition of excess 2 mM spermidine (Fig. 4C). These two observations suggest that polyamines are functionally interchangeable and can compete with antimicrobial peptides for cation binding sites in the outer membrane. As an additional control, we showed that expression of the *arnBCADTEF-ugd* operon (*PA3553::lux*), a second example of a Mg<sup>2+</sup>-regulated



**FIG 4** Polyamines are functionally interchangeable with divalent metal cations. Expression of *PA3553::lux* and *PA4774::lux* was measured under limiting Mg<sup>2+</sup> conditions (0.02 mM) and with the addition of excess 1 mM and 2 mM Mg<sup>2+</sup> (A) or the exogenous addition of 2 mM spermidine (Spd), 16 mM putrescine (Put), or 4 mM spermine (Sp) (B). (C) Expression of *PA3553::lux* and *PA4774::lux* in BM2 with 2 mM Mg<sup>2+</sup> containing polymyxin B (1 µg/ml) with or without 2 mM spermidine or BM2 with 2 mM Mg<sup>2+</sup> alone. Maximum gene expression levels at 8 h are shown, where gene expression (in counts per second [CPS]) was normalized to growth (OD<sub>600</sub>). Each experiment was performed at least three times, and representative data are shown. The values shown are the averages from at least three technical replicates with standard deviations.

operon, can also be prevented by the addition of exogenous divalent metal cations (Fig. 4A) or exogenous polyamines (spermidine, putrescine, and spermine) (Fig. 4B). The polymyxin B-mediated induction of *PA3553::lux* is also repressed by the ad-

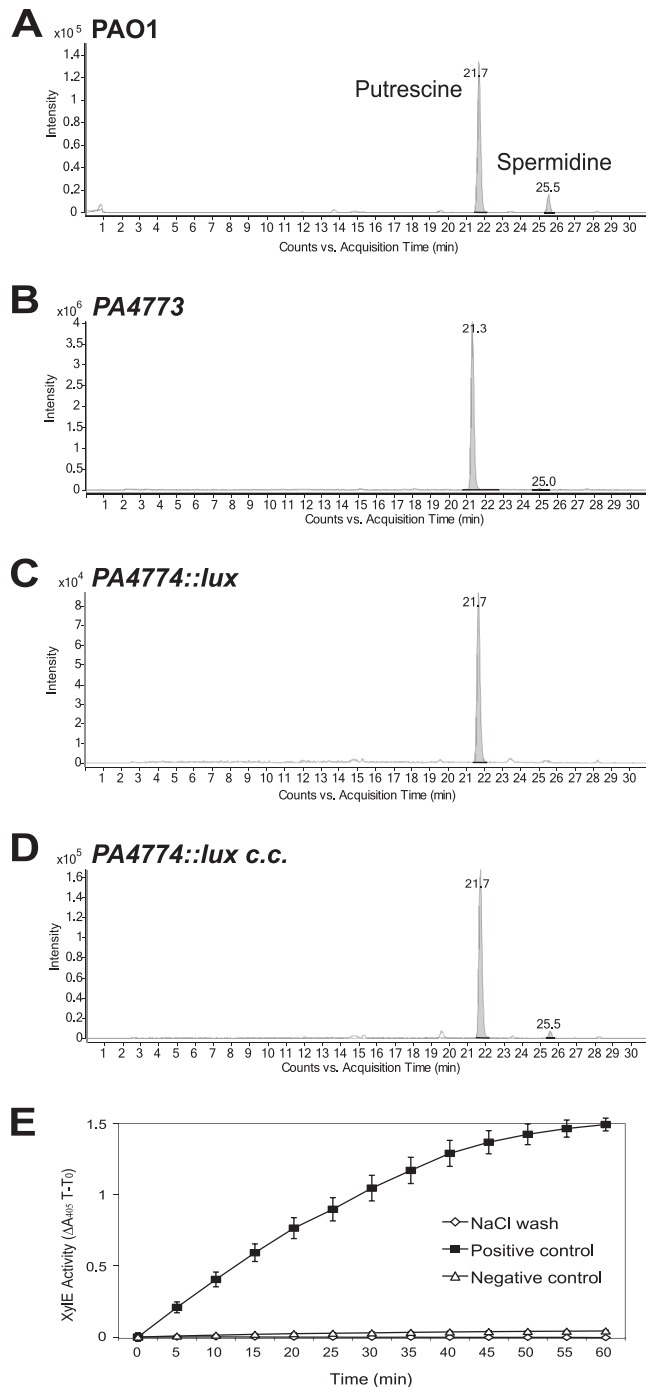
dition of exogenous spermidine (Fig. 4C). These data support the hypothesis that polyamines can function as organic cations and perform a role similar to that of the divalent metal cations.

**Spermidine and putrescine are both localized to the outer membrane of *Pseudomonas aeruginosa*.** Although polyamines are generally found in the cytoplasm, they have also been identified in the outer membrane and LPS of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (31). While the functions of polyamines in the cytoplasm are well studied, their function in the outer bacterial membrane is poorly understood. We employed the method described by Koski et al. (32) to isolate surface-associated polyamines and labeled them by derivatization with dansyl chloride. *P. aeruginosa* was grown under limiting Mg<sup>2+</sup> conditions to induce expression of *PA4773* to *PA4775* for surface polyamine isolation and characterization. Cells were washed with 1 M NaCl to remove any noncovalently attached, surface-associated polyamines. Dansyl chloride-labeled polyamines from the NaCl-washed cells were analyzed by HPLC-UV-MS in order to detect all the dansylated compounds.

Figure 5 shows the extracted ion chromatograms (EIC) for the dilabeled putrescine and trilabeled spermidine. Dilabeled putrescine was observed in the mass spectrometer at a retention time of ~21.7 min as the single-charged species at *m/z* 555.2085 ± 0.0010 (95% confidence level) (theoretical *m/z* 555.2094) and the double-charged species at *m/z* 278.1087 ± 0.0002 (theoretical *m/z* 278.1083). Trilabeled spermidine was observed in the mass spectrometer at a retention time of ~25.5 min as the single-charged species at *m/z* 845.3168 ± 0.0045 (theoretical *m/z* 845.3183), the double-charged species at *m/z* 423.1629 ± 0.0014 (theoretical *m/z* 423.1628), and the triple-charged species at *m/z* 282.4447 ± 0.0003 (theoretical *m/z* 282.4443).

MS characterization of surface polyamines was performed with wild-type PAO1, the *PA4773* and *PA4774::lux* mutants, and a strain in which *PA4774::lux* was chromosomally complemented, referred to here as the *PA4774::lux*<sub>CC</sub> strain. Unlike the surface fractions from the wild type and the *PA4773* mutant, the surface fractions from the *PA4774::lux* mutant did not produce a UV<sub>340</sub> absorbance peak at the 25.5-min retention time of spermidine (Fig. 5C), and only trace amounts of the compound were detected by the mass spectrometer at the limits of detection when a targeted analysis was performed. The production of spermidine was partially restored in the *PA4774::lux*<sub>CC</sub> strain (Fig. 5D). For complementation analysis, the genes *PA4773* to *PA4775* and the 500-bp upstream promoter region of *PA4773* were integrated into the neutral *attTn7* chromosome site using the mini-Tn7 integration system (9).

We performed the following experiment to ensure the 1 M NaCl wash did not cause cell lysis and the release of cytoplasmic polyamines, which may have subsequently contaminated our surface polyamine preparation. XylE is an enzyme expressed in the cytoplasm. We measured XylE activity in the wash fraction from cells treated with 1 M NaCl and found no release of cytoplasmic XylE (Fig. 5E). A buffer wash served as a negative control, and solvent-treated, sonicated cells served as a positive control (Fig. 5E). This experiment confirms that there was no cell lysis with NaCl treatment, and therefore no contamination of surface polyamines with cytoplasmic polyamines. A similar control experiment was also performed in previous polyamine studies with *E. coli* and *Salmonella* and also confirmed that no cytoplasmic contents contaminated the surface polyamines (31).



**FIG 5** Spermidine and putrescine were detected by HPLC-UV-MS on the outer surfaces of *Pseudomonas aeruginosa* cells. Extracted ion chromatograms (EIC) are shown for surface-associated polyamines of *P. aeruginosa*. (A) Di-dansyl chloride putrescine and tri-dansyl chloride spermidine were detected at retention times of  $\sim 21.7$  and  $\sim 25.5$  min, respectively, from the wild-type *P. aeruginosa* strain PAO1. (B) The PA4773 mutant contains both putrescine and spermidine on its surface. (C) The PA4774::lux mutant contains only putrescine, with a retention time of 21.7 min. (D) Spermidine production (retention time, 25.5 min) was restored in the surface-associated polyamines of the PA4774::lux<sub>CC</sub> strain. The EIC shows the *m/z* signal intensity of putrescine or spermidine (*y* axis) relative to the HPLC-UV-MS retention time (*x* axis). (E) XylE activity was measured in the wash fraction of cells treated with buffer (negative control) or washed with 1 M NaCl (NaCl wash), or in the lysate of solvent-treated and sonicated cells (positive control). XylE activity was defined as the change in absorbance over time, after the addition of the substrate catechol.

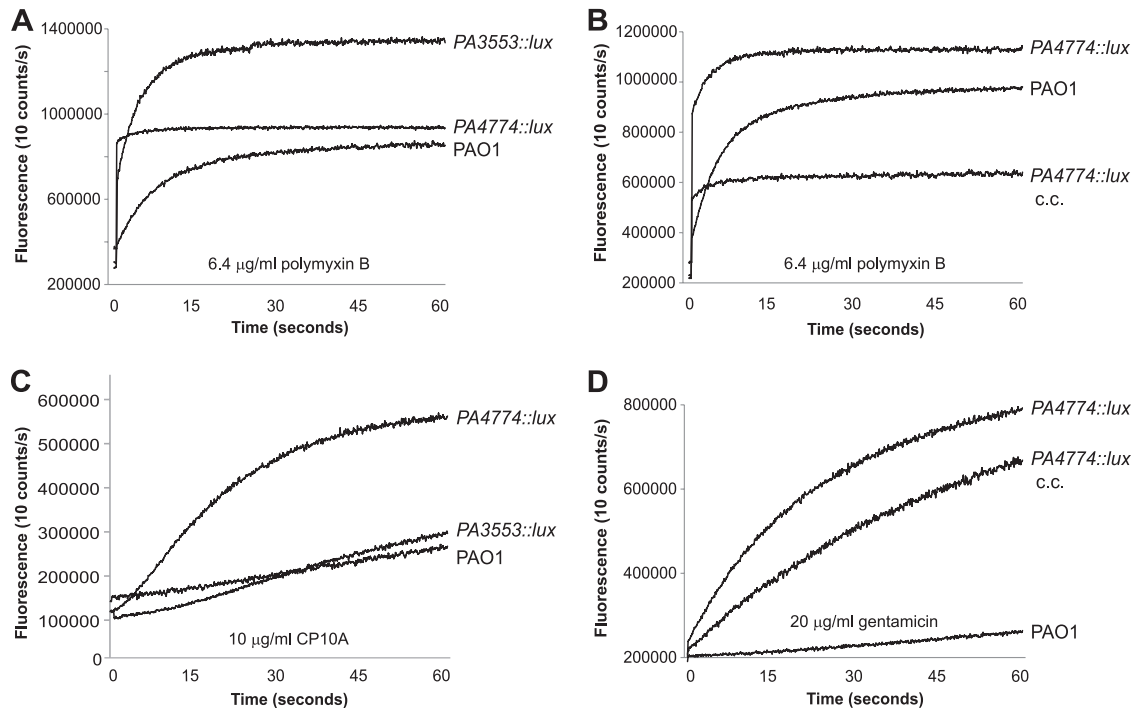
**Spermidine synthesis from the SpeE homolog PA4774 contributes to reduced outer membrane permeability and antimicrobial peptide resistance.** Mechanisms of resistance to antimicrobial peptides involve modifications of teichoic acids and phospholipids in the Gram-positive membrane (33, 55) and modifications to the LPS (47) and phospholipids (22, 30) in Gram-negative bacteria. These membrane modifications reduce the negative surface charge, and we hypothesized that the cationic nature of polyamines would also neutralize the negative surface charge and contribute to antimicrobial peptide resistance. Mutations in PA4773 to PA4775 were previously shown to confer sensitivity to a range of different antimicrobial peptides, implicating this gene cluster in antimicrobial peptide resistance (37, 43). In the MIC assay, the *arn* mutants were most susceptible to polymyxin B (cyclic peptide), while the polyamine synthesis mutants were most sensitive to the indolicidin-derived peptides (37), suggesting that each modification provides resistance to unique peptides and that together the modifications provide broad-spectrum peptide resistance.

To confirm the protective role of surface polyamines, we utilized the NPN (1-*N*-phenyl naphthylamine) uptake assay to measure OM permeability in response to antimicrobial peptides (39). The fluorescent probe NPN is hydrophobic and fluoresces weakly within aqueous environments, but it is strongly fluorescent once integrated into the hydrophobic environment of lipid membranes (21, 23, 39, 64). The active transport processes of the inner membrane were inactivated by treatment with a sub-MIC concentration of the proton motive force (PMF) inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (50). CCCP has previously been shown to inhibit polyamine uptake by intact cells, which is energized by the proton motive force (27).

NPN uptake to both inner and outer membranes after destabilization of the outer membrane by polymyxin B results in a sharp increase in NPN fluorescence (Fig. 6A and B). To determine the relative contribution of the aminoarabinose modification and the effects of polyamines, we compared the NPN uptake profiles of both classes of mutants when they were treated with various peptides. The aminoarabinose modification PA3553::lux mutant was most susceptible to polymyxin B and showed a high degree of NPN integration (Fig. 6A). The polyamine synthesis PA4774::lux mutant had an intermediate susceptibility phenotype compared to PAO1 and PA3553::lux (Fig. 6A). Mutations in PA4773 and PA4775 did not result in outer membrane permeability susceptibility to polymyxin B (data not shown). In addition to measuring the NPN uptake profile, we also calculated the rate of NPN uptake during the first 1,000 ms after polymyxin B treatment. When 6.4  $\mu\text{g/ml}$  of polymyxin B was used, the rate of NPN uptake was 12 times greater in the PA4774::lux (626,660 arbitrary units [AU]/1,000 ms) mutant than in wild-type PAO1 (52,129 AU/1,000 ms). When using 3.2  $\mu\text{g/ml}$  of polymyxin B, the rate of uptake is 6 times greater in the PA4774::lux mutant (138,846 AU/1,000 ms) than in the wild type (23,995 AU/1,000 ms). The chromosomally complemented strain demonstrated a restored polymyxin B resistance phenotype (Fig. 6B).

In addition to polymyxin B treatment, the PA4774::lux mutant also demonstrated a strong susceptibility to CP10A and gentamicin (Fig. 6C and D). The PA4774::lux mutant is more susceptible than the PA3553::lux mutant to the cationic antimicrobial peptide CP10A, an improved variant of indolicidin, which is consistent with the MIC phenotypes of these mutants (37). Gentamicin is an





**FIG 6** Polyamines contribute to antibiotic resistance by protecting the outer membrane from antimicrobial peptide and gentamicin treatment. (A) NPN uptake profiles were measured in the PAO1, PA3553::lux, and PA4774::lux strains from the moment of polymyxin B addition (6.4 μg/ml). (B) NPN uptake profiles of PAO1, the PA4774::lux mutant, and the PA4774::lux<sub>CC</sub> strain challenged with 6.4 μg/ml polymyxin B. (C) NPN uptake profiles of the PAO1, PA3553::lux, and PA4774::lux strains following 10 μg/ml CP10A treatment. (D) NPN uptake profiles following 20 μg/ml gentamicin treatment for PAO1, the PA4774::lux mutant, and the PA4774::lux<sub>CC</sub> strain. The time frame includes the point of addition of antibiotic (0 s) and the following 60 s. Ten readings/s were recorded. Each experiment was performed three times, and results of a representative experiment are shown in each panel.

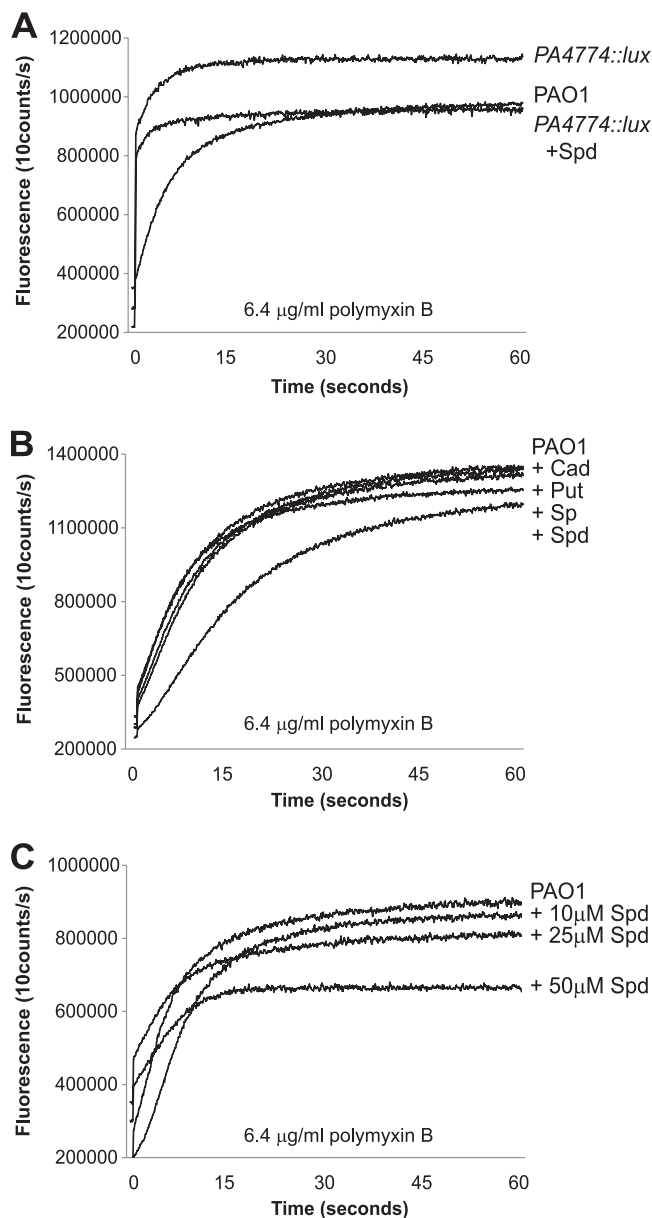
aminoglycoside that is also cationic and must cross the OM through the process of self-promoted uptake, similar to antimicrobial peptides, in order for it to reach the cytoplasm, where it acts to inhibit protein synthesis (18, 19, 39). The PA4774::lux<sub>CC</sub> strain demonstrated a strongly decreased membrane permeability to polymyxin B treatment (Fig. 6B) and a partially restored aminoglycoside resistance phenotype (Fig. 6D). PA4773 and PA4774 are annotated as SpeD and SpeE homologs, catalyzing the first and second steps in spermidine synthesis, respectively. Surprisingly, the PA4773 mutant did not have a peptide sensitivity phenotype as determined by the NPN assay. These data, together with the HPLC-UV-MS surface polyamine analysis (Fig. 5), suggest that only PA4774 and the final step in spermidine synthesis are critical for polymyxin B resistance.

**Exogenous polyamines protect the outer membrane from polymyxin B treatment.** To confirm that polyamines act on the surface of the cell and protect the membrane from antimicrobial peptide treatment, we performed NPN uptake assays in the presence of exogenous polyamines. In Fig. 7A, it is shown that adding exogenous spermidine to the PA4774::lux mutant prior to polymyxin B treatment (within 10 min) reduced the initial rate and final level of NPN uptake following polymyxin B treatment. We next compared the abilities of various polyamines added exogenously to protect the OM from polymyxin B treatment. Figure 7B demonstrates that when low concentrations (10 μM) of cadaverine, putrescine, spermine, and spermidine are added, only spermine and spermidine reduce the OM permeability in the wild-type strain. The addition of increasing concentrations of exogenous

spermidine caused a concentration-dependent reduction of the OM permeability of wild-type PAO1 (Fig. 7C). The addition of exogenous 25 to 50 μM cadaverine and putrescine also protected the OM from polymyxin B treatment, to a level similar to that seen with 10 μM spermidine (data not shown). It should be noted that this assay is a specific measure of the outer membrane properties, and thus surface polyamines can have an effect only if they bind the surface and alter the ability of polymyxin B to act on the membrane. Although polyamines can be imported into the cell, as they are also known to act as carbon sources, the addition of CCCP inhibits polyamine uptake systems (27).

**Spermidine protects membrane lipids from oxidative damage.** Reactive oxygen species (ROS) are produced during the normal course of bacterial respiration and are produced by the host immune response during infection. There is some evidence in the literature that bacterial polyamines can act as antioxidants and protect cells from oxidative stress, but the mechanism is poorly understood (8). Polyamines are found in the cytoplasm and are known to protect DNA from oxidative stress (60, 66). ROS cause damage to DNA, protein, and lipids, and given the membrane localization of spermidine and putrescine in *P. aeruginosa* (Fig. 5), we wanted to determine if polyamines protect membrane lipids from oxidative damage.

The fluorescent probe C<sub>11</sub>-BODIPY<sup>581/591</sup> has been used as an indicator of lipid peroxidation in eukaryotic cells (13, 52), but to our knowledge this is the first time it has been used in live bacteria. The probe integrates into membranes and undergoes a shift from red to green fluorescence emission upon peroxidation (13). The



**FIG 7** Exogenous polyamines protect the outer membrane from polymyxin B treatment. (A) NPN uptake profiles were measured in wild-type PAO1 and the *PA4774::lux* mutant with and without the exogenous addition of 25  $\mu$ M spermidine (Spd), prior to challenge with 6.4  $\mu$ g/ml polymyxin B. (B) NPN uptake profiles of wild-type PAO1 without and with the addition of 10  $\mu$ M cadaverine (Cad), putrescine (Put), spermine (Sp), and spermidine (Spd), prior to challenge with 6.4  $\mu$ g/ml polymyxin B. (C) NPN uptake profiles of wild-type PAO1 in the presence of increasing concentrations of spermidine (Spd), prior to challenge with 6.4  $\mu$ g/ml polymyxin B. The time frame includes the point of addition of antibiotic (1 s) and the following 60 s. Ten readings/s were recorded. Each experiment was performed three times, and results of a representative experiment are shown in each panel.

$C_{11}$  lipid tail likely causes BODIPY<sup>581/591</sup> insertion into the outer membrane, similar to what we have shown for other lipophilic dyes, i.e., FM 4-64 and FM 1-43 (36). We labeled mid-log-phase *P. aeruginosa* wild-type PAO1 and *PA4774::lux* cells with  $C_{11}$ -BODIPY<sup>581/591</sup> and showed that  $C_{11}$ -BODIPY<sup>581/591</sup> uniformly labels the *P. aeruginosa* membrane with red fluorescence

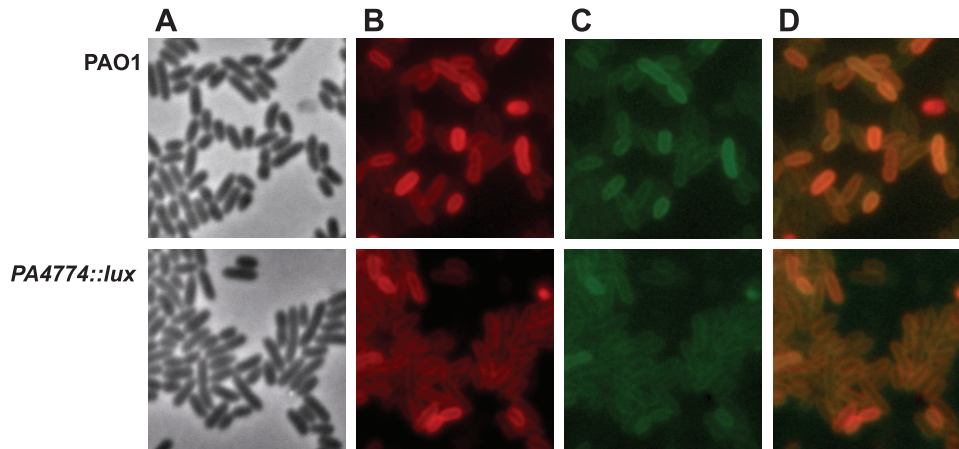
(Fig. 8). To subject the cells to oxidative stress, *P. aeruginosa* was first labeled with  $C_{11}$ -BODIPY<sup>581/591</sup> and then treated with 5 mM  $H_2O_2$ . The conversion of red to green fluorescence in the bacterial membrane upon oxidative stress from  $H_2O_2$  exposure was observed using fluorescence microscopy (Fig. 8).

To measure the degree of oxidative damage, we performed a quantitative analysis of lipid peroxidation using a fixed amount of  $C_{11}$ -BODIPY<sup>581/591</sup> bound to membranes and measured the green fluorescence as an indicator of oxidative damage to lipids. As shown in Fig. 9A, PAO1 cells grown under noninducing conditions for *PA4774* expression (2 mM  $Mg^{2+}$ ) displayed a concentration-dependent increase in peroxidation of membrane lipids upon exposure to increasing amounts of  $H_2O_2$ . Figure 9B illustrates that PAO1 cells grown under inducing conditions for *PA4774* expression (0.02 mM  $Mg^{2+}$ ) showed resistance to oxidative damage by  $H_2O_2$ . To determine if the spermidine synthesis genes *PA4773* and *PA4774* contribute to this increased oxidative stress resistance, the phenotypes of these mutants were compared to that of wild-type PAO1. The *PA4774::lux* mutant showed a statistically significant increase in susceptibility to peroxidation membrane damage (Fig. 9C and D), while the *PA4773* and *PA4775::lux* mutants were unaffected (data not shown). The *PA4774::lux<sub>CC</sub>* strain demonstrated parental levels of resistance to 25 mM  $H_2O_2$  treatment (Fig. 9C). The exogenous addition of 1 mM spermidine was also able to restore near-parental levels of resistance to 10 mM  $H_2O_2$  treatment in the *PA4774::lux* mutant (Fig. 9D). These data indicate that spermidine production protects membrane lipids from oxidative damage.

## DISCUSSION

Polyamines are essential molecules for both prokaryotic and eukaryotic cells. Although the cytoplasmic functions of polyamines are well studied, the role of polyamines in the membrane is poorly understood. Polyamines have been previously identified in the outer membrane and LPS of Gram-negative bacteria (31) and linked to surface-associated functions that include spheroplast stabilization and attachment during biofilm formation (26, 54, 62). Here we present evidence for novel membrane functions of polyamines, obtained using approaches that specifically examined the role of polyamines in protecting the bacterial outer membrane from antibiotic treatment and oxidative stress.

In addition to regulation by membrane-damaging antibiotics and  $Mg^{2+}$  levels, the *PA4773-PA4774* spermidine synthesis genes are also induced by the cation-chelating activity of extracellular DNA (Fig. 2). Collectively, the expression of these genes in the presence of multiple agents that cause membrane stress suggests a role for polyamines in membrane repair and stabilization. We confirmed the protective role of polyamines against antibiotic treatment by using the NPN uptake assay. The outer membrane of the *PA4774::lux* mutant had increased susceptibility to polymyxin B, CP10A, and gentamicin treatment (Fig. 6). The NPN assay also showed that exogenous polyamine addition protected the OM of both the wild-type PAO1 and the *PA4774::lux* strain from polymyxin B damage (Fig. 7). We showed a protective role for exogenous polyamines at low polyamine concentrations (10 to 50  $\mu$ M), which is less than the intracellular concentration of spermidine (6 mM) and putrescine (20 mM) in *E. coli* (62). We also provided evidence that polyamines can act as a substitute for inorganic cations ( $Mg^{2+}$  and  $Ca^{2+}$ ), which are normally found in the outer membrane bound to LPS and function to cross-bridge and stabi-



**FIG 8** The fluorescent probe  $C_{11}$ -BODIPY<sup>581/591</sup> localized to the *P. aeruginosa* membrane as an indicator of lipid peroxidation. Mid-log-phase cultures of PAO1 and the *PA4774::lux* mutant were stained with  $2 \mu\text{M}$   $C_{11}$ -BODIPY<sup>581/591</sup> prior to treatment with  $5 \text{ mM}$   $\text{H}_2\text{O}_2$ . (A) Phase-contrast images; (B) red fluorescence channel; (C) green fluorescence channel; (D) merged images. Images were taken with a  $100\times$  objective.

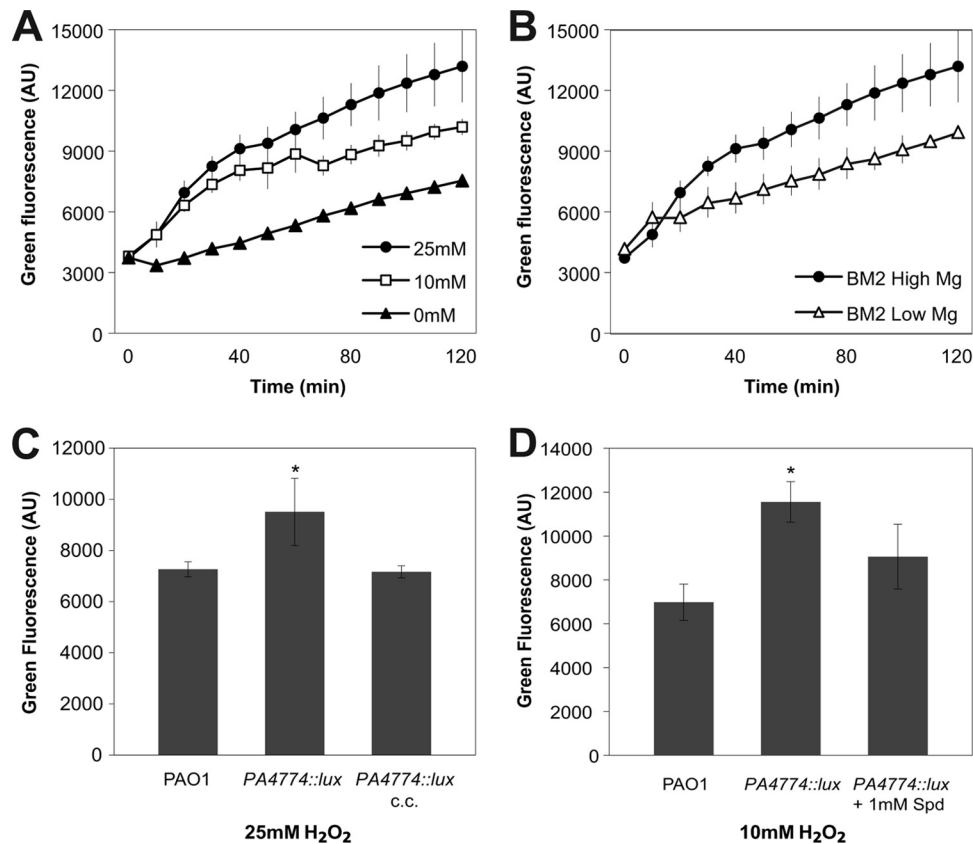
lize LPS in the outer membrane (19, 21) (Fig. 2D). Polyamines were interchangeable with  $\text{Mg}^{2+}$  cations in preventing expression of the spermidine synthesis genes and the *arn* operon, which is required for aminoarabinose modification of LPS (Fig. 4A and B). Exogenous polyamines also reduced peptide-induced expression of both *PA3553::lux* and *PA4774::lux* (Fig. 4C), suggesting that antimicrobial peptides and polyamines can compete for binding sites in the OM, further supporting the idea that polyamines act to stabilize the outer membrane to protect against antimicrobial attack. Spermidine (which has a  $3+$  charge) is more protective than the other bacterial polyamines cadaverine and putrescine ( $2+$  charge) (Fig. 7), which may be due to the extra positive charge. As such, spermine ( $4+$  charge) would be predicted to be more protective than spermidine. However, this was not the case at low concentrations (Fig. 7B), and at concentrations higher than  $10 \mu\text{M}$ , spermine disrupts the OM permeability and has the lowest MIC of all polyamines (data not shown). The higher toxicity of spermine may help to explain why it is not frequently found in bacteria.

Polyamines were isolated from the *P. aeruginosa* cell surface and their identity confirmed using HPLC-UV-MS analysis (Fig. 5). This method of isolating surface polyamines has shown that the majority of polyamines isolated are specifically from the LPS in the outer membrane, with no contamination of cytoplasmic polyamines (Fig. 5E) (31). The lack of spermidine in the outer membrane of the *PA4774::lux* (*speE* homolog) mutant was the cause of increased susceptibility to polymyxin B, CP10A, and gentamicin treatment. The polyamine protection of the outer membrane likely occurs by binding to the negative surface charges, thus preventing the self-promoted uptake of antimicrobial peptides and aminoglycosides, which compete with cations for binding to LPS in the outer membrane (19). Thus, under limiting  $\text{Mg}^{2+}$  conditions or in the presence of extracellular DNA, spermidine production by *P. aeruginosa* is a novel mechanism that contributes to modification of the outer membrane surface. The noncovalent interactions of polyamines with the bacterial surface may work in addition to the aminoarabinose modification of the phosphates of LPS, both functioning to ensure that the negative surface charges are masked and limit membrane destabilization by cationic antibiotics.

*PA4773* is annotated as a SAM decarboxylase, whose end product, dSAM, is a substrate with putrescine for spermidine synthesis by *PA4774*. Within the *P. aeruginosa* genome, there are the *speE* (*PA0654*) and *speD* (*PA1687*) genes, in addition to the homologs studied here. We propose that the genes *PA4773* and *PA4774* represent an inducible pathway for spermidine production. The *PA4773-PA4775* gene cluster is coordinately expressed under limiting  $\text{Mg}^{2+}$  conditions (37, 43, 44) and in the presence of extracellular DNA (Fig. 2). However, mutation in the first gene in the cluster (*PA4773*) did not affect the NPN uptake, lipid peroxidation (data not shown), or spermidine production phenotypes (Fig. 5). One possible explanation is that the production of dSAM by *PA4773* was performed by the true SpeD enzyme, implying a possible redundancy in gene function. And although the transposon insertion in *PA4773* had a polar effect on downstream *PA4774* expression, *PA4774* expression was not completely lost (Fig. 3). A *PA4774::lux* mutant showed increased susceptibility to OM damage by antibiotics and oxidative stress, indicating that the final step in spermidine synthesis is essential for the production of spermidine and resistance to polymyxin B and oxidative stress.

Polyamines have been implicated in antibiotic resistance in *P. aeruginosa*. Paradoxically, high concentrations of exogenous spermidine ( $20 \text{ mM}$ ) can both increase and decrease the susceptibility of *P. aeruginosa* to various classes of antibiotics (34, 35). Similarly, a recent study showed that under conditions of increased intracellular spermidine production, *E. coli*, *P. aeruginosa*, and other bacteria are more resistant to tetracycline but more sensitive to kanamycin (4). The mechanism that accounts for these contrasting effects on antibiotic resistance is not understood. We show here that spermidine can stabilize the outer membrane and contribute to resistance, but the increased susceptibility may be due to the membrane-destabilizing effects of high polycation concentrations on the outer membrane, thus facilitating the entry of certain antibiotics (21).

We also showed that polyamines protect membrane lipids from oxidative damage using a new approach with a fluorescent indicator of lipid peroxidation. Microscopy was used to confirm the membrane localization of  $C_{11}$ -BODIPY<sup>581/591</sup>, which is therefore a targeted indicator of damage to outer membrane lipids. A previous report implicated cadaverine in protecting *E. coli* from



**FIG 9** The *PA4774::lux* mutant is more susceptible to lipid peroxidation than wild-type PAO1. Mid-log-phase cultures were stained with 2  $\mu$ M C<sub>11</sub>-BODIPY<sup>581/591</sup> and exposed to various concentrations of H<sub>2</sub>O<sub>2</sub>, and lipid peroxidation (green fluorescence) was measured every 10 min for 2 h. (A) Lipid peroxidation in PAO1 cultures grown under noninducing conditions with 2 mM Mg<sup>2+</sup> and treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub>. (B) Effect of growth under low-Mg<sup>2+</sup> (0.02 mM) or high-Mg<sup>2+</sup> (2 mM) conditions on resistance to oxidative membrane damage in PAO1 following 25 mM H<sub>2</sub>O<sub>2</sub> treatment. (C) To complement the *PA4774::lux* mutant lipid peroxidation phenotype, the *PA4774::lux*<sub>CC</sub> strain was challenged with 25 mM H<sub>2</sub>O<sub>2</sub> treatment ( $P < 0.01$ ). (D) The addition of 1 mM exogenous spermidine (Spd) was tested for the ability to restore parental levels of lipid peroxidation to the *PA4774::lux* mutant following 10 mM H<sub>2</sub>O<sub>2</sub> treatment ( $P < 0.05$ ). (C and D) Green C<sub>11</sub>-BODIPY<sup>581/591</sup> fluorescence was measured after 100 min of H<sub>2</sub>O<sub>2</sub> exposure. Each value shown is the average of three technical replicates with standard deviations, and each experiment was performed three times. The unpaired Student's *t* test was used to compare the levels of lipid peroxidation among strains.

nitrosative stress, although the mechanism of protection was unclear (6). The presence of polyamines on the cell surface may act to scavenge ROS (16), thereby limiting the damage to the lipids buried in the membrane. It appears that polyamines can protect different macromolecules (lipids and DNA) in different cellular compartments (membrane and cytoplasm) from oxidative damage, dependent on their localization.

The identification of polyamines on the outer surface of *P. aeruginosa* (Fig. 5) indicates that polyamines are transported to the surface, and possibly secreted. There is evidence of putrescine and cadaverine secretion in *E. coli* using antiporter transport proteins (28, 45). A spermidine excretion protein complex, MdtJI, which belongs to the small multidrug resistance family of drug exporters, has been identified in *E. coli* by Higashi et al. (24). The function of secreted polyamines is unclear, but it is tempting to speculate secreted polyamines may play a role in cross-linking the anionic biofilm matrix polymers or may provide an attractive conditioning layer. Polyamines have been implicated in biofilm formation (26, 54), and there are examples of secreted proteins that cross-link and reinforce the exopolysaccharide matrix, thus promoting biofilm integrity (5). Extracellular polyamines have also

been proposed to play a signaling role in biofilm formation (26). Our future work will attempt to identify the polyamine secretion system by testing the role of the known spermidine transport pathways in *P. aeruginosa* (40) and putative polyamine antiporters in the transport of polyamines to the cell surface, and possibly the extracellular environment.

We recently described the cation-chelating activity of DNA, which activates the PhoPQ two-component system and expression of the *arn* antimicrobial peptide resistance operon in *P. aeruginosa* (49). The spermidine synthesis genes *PA4773* and *PA4774* are a second example of DNA-induced genes that contribute to antimicrobial peptide resistance. These genes are likely expressed in *P. aeruginosa* growing in DNA-rich environments, including growth in biofilms, where extracellular DNA accumulates as a matrix polymer (2, 65), or during growth in the CF lung, where neutrophil DNA accumulates to high concentrations (57, 61, 63), possibly due to the formation of neutrophil extracellular traps (42). Herein we describe a dual, protective role for spermidine in resistance to aminoglycoside antibiotics, antimicrobial peptides, and oxidative stress, which can protect *P. aeruginosa* from antibiotic treatment and possibly from the host immune response.

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## REFERENCES

- Aaron SD, et al. 2002. Single and combination antibiotic susceptibilities of planktonic, adherent, and biofilm-grown *Pseudomonas aeruginosa* isolates cultured from sputa of adults with cystic fibrosis. *J. Clin. Microbiol.* 40:4172–4179.
- Allesen-Holm M, et al. 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.* 59:1114–1128.
- Anderson GG, O'Toole GA. 2008. Innate and induced resistance mechanisms of bacterial biofilms. *Curr. Top. Microbiol. Immunol.* 322:85–105.
- Bernier SP, Letoffe S, Delepiere M, Ghigo JM. 2011. Biogenic ammonia modifies antibiotic resistance at a distance in physically separated bacteria. *Mol. Microbiol.* 81:705–716.
- Borlee BR, et al. 2010. *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Mol. Microbiol.* 75:827–842.
- Bower JM, Mulvey MA. 2006. Polyamine-mediated resistance of uropathogenic *Escherichia coli* to nitrosative stress. *J. Bacteriol.* 188:928–933.
- Ceri H, et al. 1999. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* 37:1771–1776.
- Chattopadhyay MK, Tabor CW, Tabor H. 2003. Polyamines protect *Escherichia coli* cells from the toxic effect of oxygen. *Proc. Natl. Acad. Sci. U. S. A.* 100:2261–2265.
- Choi KH, Schweizer HP. 2006. Mini-Tn7 insertion in bacteria with single *attTn7* sites: example *Pseudomonas aeruginosa*. *Nat. Protoc.* 1:153–161.
- Choi KH, et al. 2005. A Tn7-based broad-range bacterial cloning and expression system. *Nat. Methods* 2:443–448.
- Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318–1322.
- Davies D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.* 2:114–122.
- Drummen GP, van Liebergen LC, Op den Kamp JA, Post JA. 2002. C11-BODIPY(581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: (micro)spectroscopic characterization and validation of methodology. *Free Radic. Biol. Med.* 33:473–490.
- Gaynes R, Edwards JR. 2005. Overview of nosocomial infections caused by gram-negative bacilli. *Clin. Infect. Dis.* 41:848–854.
- Guo K, Li L. 2009. Differential 12C-/13C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome. *Anal. Chem.* 81:3919–3932.
- Ha HC, et al. 1998. The natural polyamine spermine functions directly as a free radical scavenger. *Proc. Natl. Acad. Sci. U. S. A.* 95:11140–11145.
- Hancock RE. 1998. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. *Clin. Infect. Dis.* 27(Suppl. 1):S93–S99.
- Hancock RE. 1997. The bacterial outer membrane as a drug barrier. *Trends Microbiol.* 5:37–42.
- Hancock RE. 1984. Alterations in outer membrane permeability. *Annu. Rev. Microbiol.* 38:237–264.
- Hancock RE, Speert DP. 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resist Updat.* 3:247–255.
- Hancock RE, Wong PG. 1984. Compounds which increase the permeability of the *Pseudomonas aeruginosa* outer membrane. *Antimicrob. Agents Chemother.* 26:48–52.
- Hebecker S, et al. 2011. Alanyl-phosphatidylglycerol synthase: mechanism of substrate recognition during tRNA-dependent lipid modification in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 80:935–950.
- Helander IM, Mattila-Sandholm T. 2000. Fluorometric assessment of gram-negative bacterial permeabilization. *J. Appl. Microbiol.* 88:213–219.
- Higashi K, et al. 2008. Identification of a spermidine excretion protein complex (MdtJ) in *Escherichia coli*. *J. Bacteriol.* 190:872–878.
- Jensen PO, Givskov M, Bjarnsholt T, Moser C. 2010. The immune system vs. *Pseudomonas aeruginosa* biofilms. *FEMS Immunol. Med. Microbiol.* 59:292–305.
- Karatan E, Duncan TR, Watnick PI. 2005. NspS, a predicted polyamine sensor, mediates activation of *Vibrio cholerae* biofilm formation by norspermidine. *J. Bacteriol.* 187:7434–7443.
- Kashiwagi K, Kobayashi H, Igarashi K. 1986. Apparently unidirectional polyamine transport by proton motive force in polyamine-deficient *Escherichia coli*. *J. Bacteriol.* 165:972–977.
- Kashiwagi K, Miyamoto S, Suzuki F, Kobayashi H, Igarashi K. 1992. Excretion of putrescine by the putrescine-ornithine antiporter encoded by the *potE* gene of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 89:4529–4533.
- Kirketerp-Moller K, et al. 2008. Distribution, organization, and ecology of bacteria in chronic wounds. *J. Clin. Microbiol.* 46:2717–2722.
- Klein S, et al. 2009. Adaptation of *Pseudomonas aeruginosa* to various conditions includes tRNA-dependent formation of alanyl-phosphatidylglycerol. *Mol. Microbiol.* 71:551–565.
- Koski P, Vaara M. 1991. Polyamines as constituents of the outer membranes of *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* 173:3695–3699.
- Koski P, Helander IM, Sarvas M, Vaara M. 1987. Analysis of polyamines as their dabsyl derivatives by reversed-phase high-performance liquid chromatography. *Anal. Biochem.* 164:261–266.
- Kristian SA, Durr M, Van Strijp JA, Neumeister B, Peschel A. 2003. MprF-mediated lysinylation of phospholipids in *Staphylococcus aureus* leads to protection against oxygen-independent neutrophil killing. *Infect. Immun.* 71:546–549.
- Kwon DH, Lu CD. 2006. Polyamines increase antibiotic susceptibility in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 50:1623–1627.
- Kwon DH, Lu CD. 2006. Polyamines induce resistance to cationic peptide, aminoglycoside, and quinolone antibiotics in *Pseudomonas aeruginosa* PAO1. *Antimicrob. Agents Chemother.* 50:1615–1622.
- Lewenza S, Vidal-Ingigliardi D, Pugsley AP. 2006. Direct visualization of red fluorescent lipoproteins indicates conservation of the membrane sorting rules in the family *Enterobacteriaceae*. *J. Bacteriol.* 188:3516–3524.
- Lewenza S, et al. 2005. Construction of a mini-Tn5-*luxCDABE* mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes. *Genome Res.* 15:583–589.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* 25:402–408.
- Loh B, Grant C, Hancock RE. 1984. Use of the fluorescent probe 1-N-phenylanthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 26:546–551.
- Lu CD, Itoh Y, Nakada Y, Jiang Y. 2002. Functional analysis and regulation of the divergent *spuABCDEFHG-spul* operons for polyamine uptake and utilization in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 184:3765–3773.
- Mah TF, et al. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426:306–310.
- Marcos V, et al. 2010. CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. *Nat. Med.* 16:1018–1023.
- McPhee JB, Lewenza S, Hancock RE. 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 50:205–217.
- McPhee JB, et al. 2006. Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg<sup>2+</sup>-induced gene regulation in *Pseudomonas aeruginosa*. *J. Bacteriol.* 188:3995–4006.
- Meng SY, Bennett GN. 1992. Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH. *J. Bacteriol.* 174:2659–2669.
- Moreau-Marquis S, Stanton BA, O'Toole GA. 2008. *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. *Pulm. Pharmacol. Ther.* 21:595–599.
- Moskowitz SM, Ernst RK, Miller SI. 2004. PmrAB, a two-component

- regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *J. Bacteriol.* **186**:575–579.
48. Mulcahy H, Charron-Mazenod L, Lewenza S. 2010. *Pseudomonas aeruginosa* produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source. *Environ. Microbiol.* **12**:1621–1629.
  49. Mulcahy H, Charron-Mazenod L, Lewenza S. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog.* **4**:e1000213.
  50. Murata T, Tseng W, Guina T, Miller SI, Nikaido H. 2007. PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **189**:7213–7222.
  51. Nickel JC, Ruseska I, Wright JB, Costerton JW. 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrob. Agents Chemother.* **27**:619–624.
  52. Pap EH, et al. 1999. Ratio-fluorescence microscopy of lipid oxidation in living cells using C11-BODIPY(581/591). *FEBS Lett.* **453**:278–282.
  53. Parsek MR, Singh PK. 2003. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu. Rev. Microbiol.* **57**:677–701.
  54. Patel CN, et al. 2006. Polyamines are essential for the formation of plague biofilm. *J. Bacteriol.* **188**:2355–2363.
  55. Peschel A, et al. 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J. Biol. Chem.* **274**:8405–8410.
  56. Ramadan HH. 2006. Chronic rhinosinusitis and bacterial biofilms. *Curr. Opin. Otolaryngol. Head Neck Surg.* **14**:183–186.
  57. Ranasinha C, et al. 1993. Efficacy and safety of short-term administration of aerosolised recombinant human DNase I in adults with stable stage cystic fibrosis. *Lancet* **342**:199–202.
  58. Savli H, et al. 2003. Expression stability of six housekeeping genes: A proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J. Med. Microbiol.* **52**:403–408.
  59. Schurek KN, et al. 2009. Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **53**:4345–4351.
  60. Shah P, Swiatlo E. 2008. A multifaceted role for polyamines in bacterial pathogens. *Mol. Microbiol.* **68**:4–16.
  61. Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL. 1990. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc. Natl. Acad. Sci. U. S. A.* **87**:9188–9192.
  62. Tabor CW, Tabor H. 1985. Polyamines in microorganisms. *Microbiol. Rev.* **49**:81–99.
  63. Ulmer JS, et al. 1996. Engineering actin-resistant human DNase I for treatment of cystic fibrosis. *Proc. Natl. Acad. Sci. U. S. A.* **93**:8225–8229.
  64. Vaara M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **56**:395–411.
  - 64a. West SE, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**:81–86.
  65. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* **295**:1487.
  66. Wortham BW, Patel CN, Oliveira MA. 2007. Polyamines in bacteria: pleiotropic effects yet specific mechanisms. *Adv. Exp. Med. Biol.* **603**:106–115.