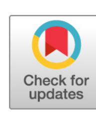
RESEARCH ARTICLE



Putrescine and Its Metabolic Precursor Arginine Promote Biofilm and c-di-GMP Synthesis in *Pseudomonas aeruginosa*

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ABSTRACT *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen, can synthesize and catabolize several small cationic molecules known as polyamines. In several clades of bacteria, polyamines regulate biofilm formation, a lifestyle-switching process that confers resistance to environmental stress. The polyamine putrescine and its biosynthetic precursors, L-arginine and agmatine, promote biofilm formation in *Pseudomonas* spp. However, it remains unclear whether the effect is a direct effect of polyamines or occurs through a metabolic derivative. Here, we used a genetic approach to demonstrate that putrescine accumulation, either through disruption of the spermidine biosynthesis pathway or the catabolic putrescine aminotransferase pathway, promoted biofilm formation in *P. aeruginosa*. Consistent with this observation, exogenous putrescine robustly induced biofilm formation in *P. aeruginosa* that was dependent on putrescine uptake and biosynthesis pathways. Additionally, we show that L-arginine, the biosynthetic precursor of putrescine, also promoted biofilm formation but did so by a mechanism independent of putrescine or agmatine conversion. We found that both putrescine and L-arginine induced a significant increase in the intracellular level of bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) (c-di-GMP), a bacterial second messenger widely found in *Proteobacteria* that upregulates biofilm formation. Collectively these data show that putrescine and its metabolic precursor, arginine, promote biofilm and c-di-GMP synthesis in *P. aeruginosa*.

IMPORTANCE Biofilm formation allows bacteria to physically attach to a surface, confer tolerance to antimicrobial agents, and promote resistance to host immune responses. As a result, the regulation of biofilm formation is often crucial for bacterial pathogens to establish chronic infections. A primary mechanism of biofilm promotion in bacteria is the molecule c-di-GMP, which promotes biofilm formation. The level of c-di-GMP is tightly regulated by bacterial enzymes. In this study, we found that putrescine, a small molecule ubiquitously found in eukaryotic cells, robustly enhances *P. aeruginosa* biofilm and c-di-GMP. We propose that *P. aeruginosa* may sense putrescine as a host-associated signal that triggers a lifestyle switch that favors chronic infection.

KEYWORDS *Pseudomonas aeruginosa*, arginine, biofilm, c-di-GMP, polyamines, putrescine

Pseudomonas aeruginosa is a Gram-negative gammaproteobacterium that opportunistically causes disease in both animals and plants (1–3). *P. aeruginosa* frequently forms biofilm-associated infections in cystic fibrosis (CF) airways, contributing to its adaptive resistance to antimicrobials and long-term colonization of the CF lung (4). While attachment to biotic surfaces is required for successful host colonization in both animal and plant roots (5, 6), an increasing body of evidence suggests that increased biofilm formation and attachment to host cells may trigger a more robust host immune response, potentially leading to clearance of the biofilm-associated

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microbes in plants and human epithelial cells (7, 8). We previously demonstrated that *Pseudomonas* must modulate its biofilm in the rhizosphere to evade triggering a plant immune response (8).

One mechanism through which *P. aeruginosa* regulates biofilm formation (9) and virulence (10) is the modulation of bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP), a ubiquitous second messenger in *Proteobacteria* (11). The turnover of c-di-GMP is mediated by diguanylate cyclase (DGC) enzymes and c-di-GMP phosphodiesterase (PDE) enzymes. *P. aeruginosa* encodes 41 putative c-di-GMP-modulating enzymes (CMEs) (11), suggesting that intricate spatiotemporal regulation of CME activities is in place to maintain c-di-GMP homeostasis. Many of the CMEs contain ligand-binding domains, such as Per-Arnt-Sim (PAS) or cyclases/histidine kinase associated sensory extracellular (CHASE) domains (12), suggesting that these CMEs may regulate their enzymatic activities in response to specific ligands. *Vibrio cholerae*, a gastrointestinal pathogen, downregulates its c-di-GMP levels in response to gastrointestinal tract-related molecules, such as bile salt and bicarbonate (13). However, it is not known whether *Pseudomonas* sense similar host-associated signals to trigger changes in c-di-GMP and bacterial physiology.

We previously identified the putrescine aminotransferase gene *spuC* as a negative regulator of *Pseudomonas fluorescens* biofilm formation and a plant rhizosphere fitness determinant (8). Furthermore, similar paradigms have been observed in divergent bacterial taxa, where polyamines either positively or negatively regulate biofilm formation. However, the responses to polyamines differ between taxa. For example, spermine is a biofilm inhibitor in *Vibrio cholerae* while norspermidine is a robust inducer of pellicle formation in *Bacillus subtilis* and spermidine and putrescine negatively regulate biofilm formation in *Agrobacterium tumefaciens* (14–16). These observations suggest that putrescine and related molecules involved in polyamine metabolism may act as a host-associated signal that triggers *Pseudomonas* lifestyle switching. Importantly, *P. aeruginosa* has an intricate network of enzymes involved in polyamine metabolism (17), and previous studies identified L-arginine and agmatine, which are known precursors of putrescine biosynthesis (17), as robust signals that upregulate biofilm formation in *Pseudomonas* (12, 18, 19). In this study, we used systematic mutagenesis of genes involved in polyamine metabolism coupled with exogenous polyamine application and c-di-GMP quantification to disentangle the roles of putrescine derivatives and biosynthetic precursors in biofilm regulation in *P. aeruginosa*.

RESULTS

Genes predicted to encode proteins required for polyamine uptake, biosynthesis, and catabolism are conserved across the genus *Pseudomonas*. We previously demonstrated that putrescine catabolism inhibits *P. fluorescens* biofilm formation (8). *P. aeruginosa* has an extensive polyamine metabolism network that includes catabolic and biosynthetic pathways of putrescine, spermidine, and spermine (17). Furthermore, *P. aeruginosa* is a well-studied model organism for biofilm formation with available genetic tools that allow for the investigation of biofilm and c-di-GMP regulation (20–22). As a result, we chose to study *P. aeruginosa* to elucidate the mechanisms underpinning the polyamine-mediated biofilm enhancement that is observed in *P. fluorescens*.

To determine how similar putrescine metabolism is across *Pseudomonas* strains, we identified the orthologous genes involved in putrescine uptake, biosynthesis, and catabolism pathways in both *P. fluorescens* WCS365 and *P. aeruginosa* PAO1. Based on what is known in *P. aeruginosa* (17), we identified orthologs in *P. fluorescens* WCS365 (Fig. 1A). The *P. aeruginosa* genome encodes genes required for putrescine biosynthesis (*speA* and *speC*), uptake (*spuD*), catabolism (*spuC*), and conversion to spermidine (*speD* and *speE*). Interestingly, *speD* and *speE* are absent in *P. fluorescens* WCS365, so we queried whether carboxyspermidine dehydrogenase (CASDH) and decarboxylase (CASDC), which catalyzes an alternate spermidine biosynthesis pathway from putrescine (23), were present. We identified CASDH/C homologs in *P. fluorescens* WCS365 but not in PAO1 (Fig. 1A). We used a previously described comparative genomics platform (24) to query the presence and absence of these polyamine biosynthesis genes in diverse *Pseudomonas* spp. We found that the majority of polyamine metabolism genes

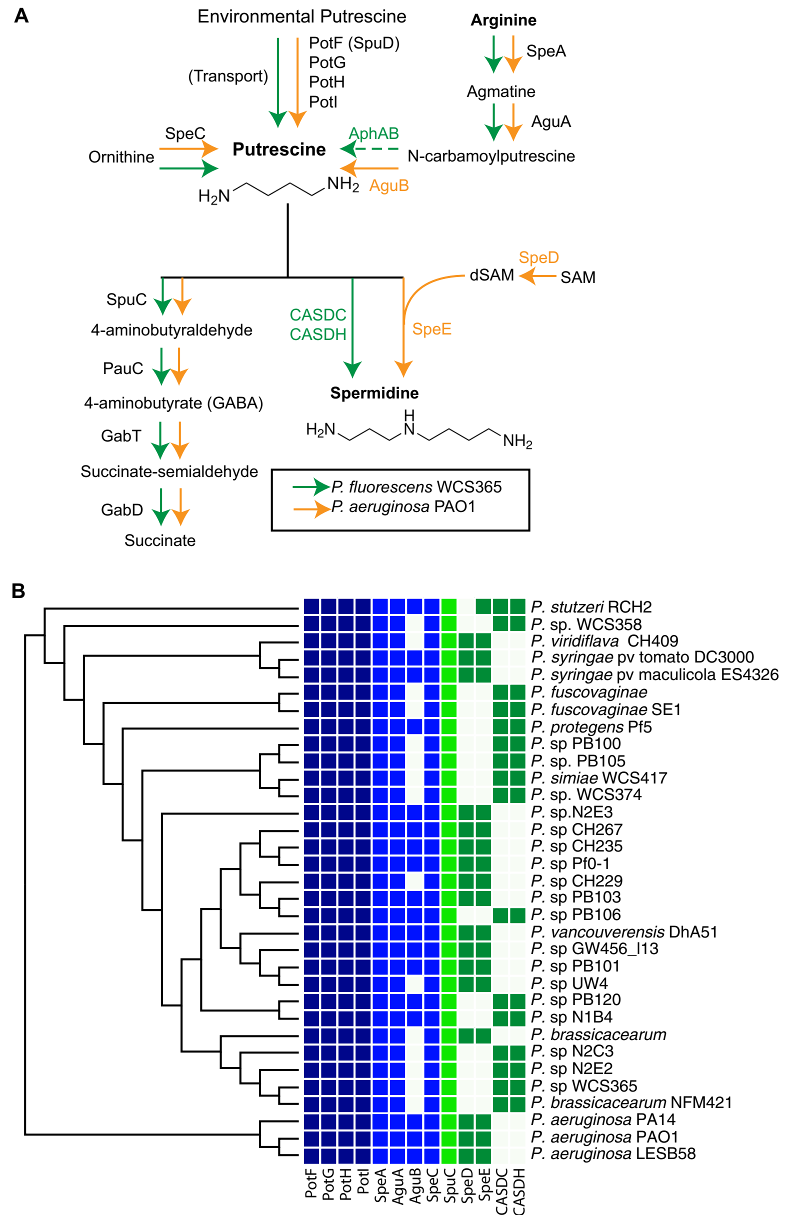

FIG 1 Polyamine metabolism pathway in *Pseudomonas* spp. (A) Predicted polyamine metabolism pathways in *P. aeruginosa* PAO1 and *P. fluorescens* WCS365. The pathway was reconstructed based on the work of Lu et al. (17). *P. fluorescens* WCS365 genes were identified by BLASTp using *P. aeruginosa* homologs as query sequences. (B) Phylogenetic tree of *Pseudomonas* spp. including the presence and absence of genes involved in polyamine metabolism. Genes predicted to encode proteins involved in putrescine uptake (PotFGHI), putrescine biosynthesis (SpeA, AguA, AguB, SpeC), putrescine catabolism (SpuC), or spermidine biosynthesis (SpeDE and CADSC/H) are shown.

are conserved across diverse *Pseudomonas*. Interestingly, all strains contained predicted enzymes to convert putrescine to spermidine either through *speD/E* or CASDH/C but never both (Fig. 1B). This suggests that *Pseudomonas* strains have either a CASDH/C or *speD/E* pathway to generate spermidine from putrescine, but they do not maintain both in their genomes. These findings show that the potential for putrescine uptake, biosynthesis and catabolism, and spermidine biosynthesis is broadly conserved across the *Pseudomonas* genus (Fig. 1B).

Exogenous putrescine promotes biofilm formation in *P. aeruginosa*. To test whether the accumulation of putrescine promotes biofilm formation, we performed

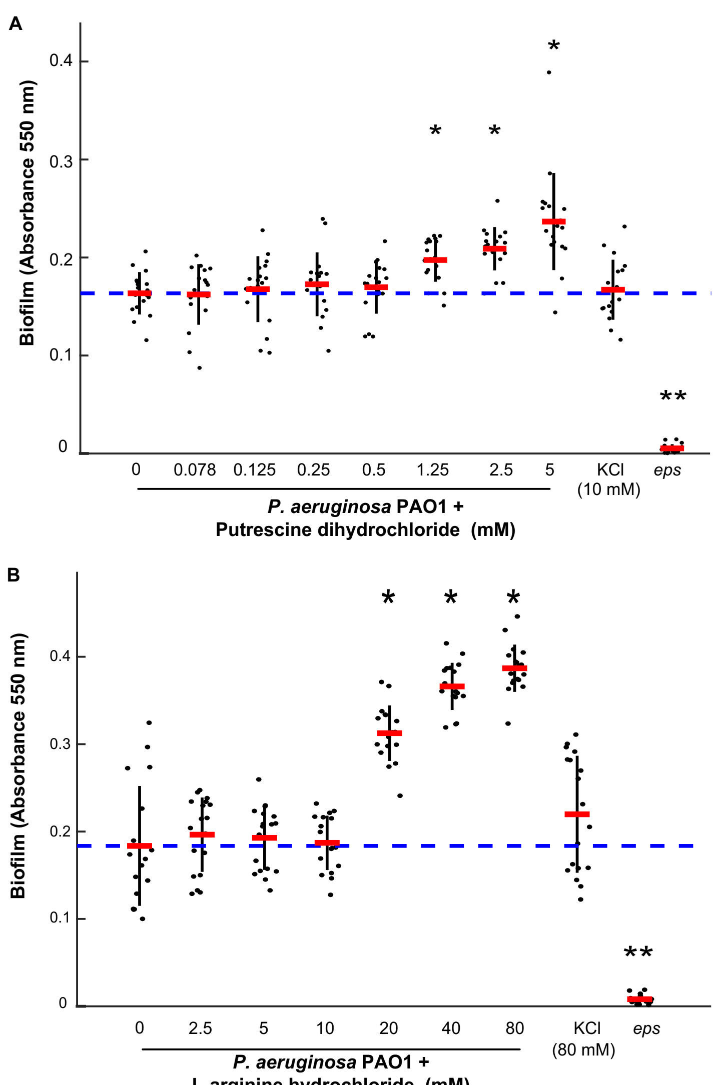

FIG 2 The biofilm-promoting effect of L-arginine and putrescine in *P. aeruginosa* PAO1 is dosage dependent. Putrescine can promote biofilm at 1.25 mM, and L-arginine enhances biofilm formation at 20 mM. The *eps* mutant has decreased biofilm and was used as a control. * indicates a *P* value of $<$0.0001 by Student's *t* test. Error bars represent standard deviation. Data points show all technical replicates from 3 biological replicates.

crystal violet biofilm assays in *P. aeruginosa* subline H103 (*P. aeruginosa* PAO1) with and without the presence of exogenous putrescine. We titrated the concentration of exogenous L-arginine hydrochloride or putrescine dihydrochloride and observed that exogenous putrescine promotes biofilm at 1.25 mM (Fig. 2A) and L-arginine promotes biofilm at 20 mM (Fig. 2B). We found that KCl does not affect biofilm in *P. aeruginosa* PAO1 at concentrations up to 80 mM, ruling out an effect of chlorine ions in L-arginine hydrochloride or putrescine dihydrochloride in modulating biofilm (Fig. 2). While previous reports show that the concentration of putrescine in bacterial cultures is around 50 μM (25), bacteria routinely colonize environments with much higher putrescine concentrations. Putrescine is estimated at 0.5 mM in cystic fibrosis sputum (26), 1 mM in the human gut (27), and up to 11 mM in tomato root exudate (28). These data show that the addition of putrescine or arginine is sufficient to promote biofilm formation in wild-type *P. aeruginosa* PAO1 at concentrations they may encounter during associations with hosts (Fig. 2).

To determine whether putrescine accumulation promotes biofilm formation in *P. aeruginosa* PAO1, we generated clean deletions of genes encoding key enzymes involved in putrescine biosynthesis (*speA* and *speC*), catabolism (*spuC*), and spermidine biosynthesis (*speD*) in *P. aeruginosa* PAO1 (Fig. 1A). We first validated that these mutations affected polyamine biosynthesis through targeted metabolomics and found that the PAO1 Δ*spuC*, Δ*speAC,* and Δ*speD* mutants all had significantly reduced intracellular putrescine when grown in the absence of exogenous putrescine and arginine (see Fig. S1A in the supplemental material) and spermidine (Fig. S1B) levels compared to wild-type PAO1. This indicates that, in the absence of precursors, disruption of putrescine biosynthesis (Δ*speAC*), catabolism (Δ*spuC*), and spermidine biosynthesis (Δ*speD*) affects putrescine and spermidine accumulation in *P. aeruginosa* PAO1.

To determine if putrescine accumulation promotes biofilm, we tested whether mutants predicted to accumulate putrescine had enhanced biofilm formation capacity using crystal violet staining with and without exogenous putrescine. Consistent with the hypothesis that the loss of putrescine catabolism results in putrescine accumulation and increased biofilm and the previous observation that deletion of *spuC* in *P. fluorescens* enhances biofilm formation, we found that deletion of PAO1 *spuC* leads to a significant increase in biofilm formation (Fig. 3A). This response was further enhanced by the addition of exogenous putrescine (Fig. 3A), suggesting that putrescine accumulation robustly enhances biofilm in *P. aeruginosa* PAO1 through the loss of putrescine catabolism.

P. aeruginosa can synthesize spermidine from putrescine via an aminopropylation reaction using decarboxylated *S*-adenosyl methionine (dSAM) as an aminopropyl donor. To test whether putrescine conversion to spermidine is necessary for enhanced biofilm formation, we generated a deletion mutant in *speD*, which is required for spermidine biosynthesis from putrescine. SpeD decarboxylates *S*-adenosyl methionine to generate dSAM. Similar to the deletion of *spuC*, deletions of *speD* enhanced biofilm formation in *P. aeruginosa* (Fig. 3A), which was further enhanced by exogenous putrescine, suggesting that the putrescine-induced enhancement of biofilm formation is not mediated by the conversion of putrescine to spermidine via *speD/E*. Collectively, these data showed that the accumulation of putrescine promotes biofilm formation or that putrescine catabolism inhibits biofilm formation in *P. aeruginosa*.

P. aeruginosa can synthesize putrescine via arginine decarboxylation or ornithine decarboxylation pathways or take up environmental putrescine through the putative putrescine transporter system encoded by *spuABCDEFGH-spuI* (17, 29). To test whether putrescine biosynthesis and uptake promote biofilm formation in *P. aeruginosa*, we tested a deletion mutant in *spuD* (encoding a putative periplasmic putrescine substrate-binding protein) and a double deletion mutant in the putrescine biosynthesis genes *speAC* (*speA* encodes an arginine decarboxylase [17] and *speC* encodes an ornithine decarboxylase [17]) for biofilm formation. We found that abolishing the biosynthesis of putrescine from L-arginine and L-ornithine decarboxylation did not impair biofilm formation (Fig. 3B) nor did disrupting the putrescine uptake pathway with *spuD* deletion (Fig. 3A). This suggests that disrupting a single intrinsic putrescine biosynthesis or uptake system is not sufficient to impair biofilm formation and that either redundancy or additional pathways are required.

To test whether abolishing both putrescine uptake via SpuD and putrescine biosynthesis through SpeAC would result in a loss of biofilm in the presence of exogenous putrescine, we tested a *P. aeruginosa* PAO1 Δ*spuD* Δ*speAC* triple mutant (predicted to be impaired in both putrescine uptake and biosynthesis). We performed a biofilm assay and found that the triple mutant had a reduced response to putrescine, suggesting that the reduction of intracellular putrescine in this mutant results in lower enhancement of biofilm (Fig. 3B). To quantify intracellular levels of putrescine in the triple mutant Δ*spuD* Δ*speAC*, we grew PAO1 WT and the triple mutant in the presence of 2.5 mM putrescine then spun down and washed the cells before performing targeted metabolomics. We found that the triple mutant has a significant reduction in intracellular putrescine (Fig. 3C), suggesting

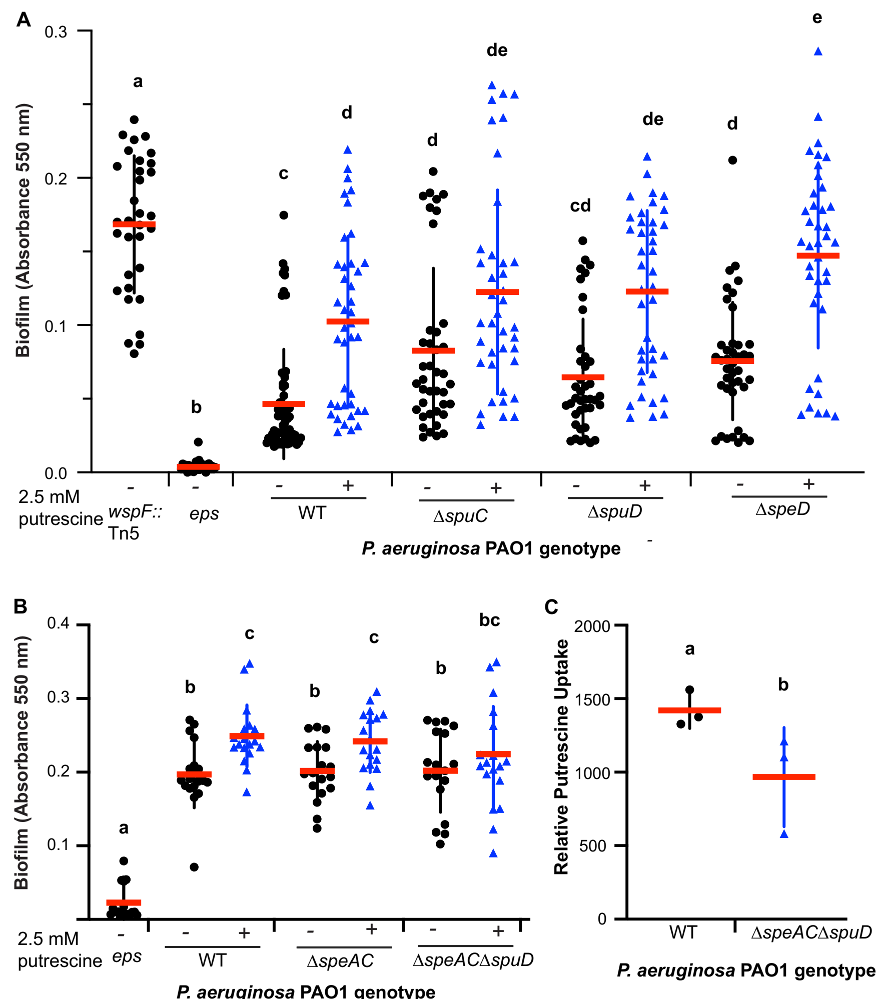

FIG 3 Uptake of exogenous putrescine and L-arginine is sufficient to promote biofilm formation in *P. aeruginosa*. (A) Wild-type *P. aeruginosa* PAO1 and Δ*spuC*, Δ*spuD*, and Δ*speD* mutants were treated with 2.5 mM putrescine dihydrochloride. Mutants predicted to accumulate putrescine (Δ*spuC* and Δ*speD*) had enhanced biofilm that was further enhanced by exogenous putrescine. Putrescine induction of biofilm formation is independent of SpuD, a periplasmic substrate-binding protein involved in putrescine uptake, and SpeD, an enzyme required for the production of spermidine through putrescine aminopropylation. (B) Putrescine-mediated biofilm induction is reduced in a Δ*spuD* Δ*speAC* triple mutant. (C) The triple Δ*spuD* Δ*speAC* mutant had reduced levels of intracellular putrescine. Cells were grown with exogenous putrescine and washed followed by targeted metabolomics. (A to C) Error bars represent standard deviation. Data represent at least 3 biological replicates. Letters indicate significant differences by one-way ANOVA followed by Tukey's honest significant difference (HSD).

that these three pathways provide a significant contribution to putrescine uptake and biosynthesis.

Exogenous arginine promotes biofilm formation in *P. aeruginosa* independent of conversion to putrescine. Previous studies reported that L-arginine is a strong inducer of biofilm formation in *P. aeruginosa* PA14 and in *P. fluorescens* WCS365 within a modified M63 medium that uses L-arginine as the sole carbon and nitrogen source (8, 12, 18). The addition of exogenous putrescine has been shown to inhibit transcription of *aguAB* (30), an operon encoding enzymes that convert agmatine to putrescine in the L-arginine decarboxylation pathway (31). As a result, exogenous supplementation of putrescine could lead to the accumulation of agmatine, L-arginine, and L-ornithine, given their roles as putrescine biosynthetic precursors (Fig. 1A). Importantly, agmatine and L-arginine are known to promote biofilm formation in *Pseudomonas* (12, 18, 19). To investigate whether putrescine causes biofilm induction by feedback inhibition of

L-arginine conversion or by L-arginine triggering biofilm formation through putrescine biosynthesis, we tested the *P. aeruginosa* PAO1 Δ*speAC* double mutant, which cannot convert L-arginine (in Δ*speA* strain) to agmatine (and subsequently, putrescine) or L-ornithine (in Δ*speC* strain) to putrescine via decarboxylation pathways (Fig. 1A). Notably, the Δ*speAC* double mutant does not have a biofilm formation defect (Fig. 3B), suggesting that neither the depletion of putrescine nor the accumulation of L-arginine, achieved by abolishing the putrescine biosynthesis pathway, interferes with biofilm formation.

Previous studies that reported L-arginine as a robust biofilm-promoting molecule used a defined, modified M63 medium in which L-arginine was used at 0.4% (wt/vol) as the sole carbon and nitrogen source in M63 salts (dM63-Arg [12, 18]). However, because dM63-Arg replaces Casamino Acids and D-glucose with an excess of L-arginine, this medium may have limited the intracellular availability of amino acids other than L-arginine because *P. aeruginosa* would need to synthesize these amino acids *de novo*. Amino acid unavailability has been shown to trigger RelA-mediated production of (p)ppGpp via ribosome stalling (32, 33). (p)ppGpp is a stringent response second messenger that mediates physiological changes, including biofilm formation, in *Proteobacteria* (33, 34). To avoid the confounding effect of nutrient starvation on biofilm formation, we used a modified medium that contains the standard nutrients in M63 medium with additional L-arginine supplementation to test the effect of L-arginine as a biofilm-promoting agent. We found that while 2.5 mM putrescine robustly induced biofilm formation (Fig. 2A and 3A), equimolar L-arginine HCl did not affect the biofilm formation in the wild-type or an L-arginine-accumulating Δ*speAC* background (Fig. S2). As previous studies that examined the effect of L-arginine and agmatine used concentrations consistant with our titration results (22.69 mM for L-arginine [12, 18] or 10 mM for agmatine [19]), we confirmed the effect of L-arginine as a biofilm-promoting agent at 20 mM. At 20 mM, L-arginine robustly increased biofilm formation in wild-type *P. aeruginosa* PAO1 (Fig. S2). Importantly, we also noticed that L-arginine-induced biofilm enhancement is independent of *speA* and *speC* (Fig. S2). This suggested that L-arginine rather than its downstream decarboxylation pathway metabolites, such as agmatine or putrescine, is responsible for the enhanced biofilm formation.

Because L-arginine can be used as a carbon and nitrogen source by *P. aeruginosa*, we examined whether the increased biofilm formation with the addition of 20 mM L-arginine was the result of increased growth. Because previous reports suggested that *P. aeruginosa* exopolysaccharide (EPS) production can confound the measured optical density at 600 nm (OD_{600}) in growth assays (22), we measured the growth of *P. aeruginosa* PAO1 with L-arginine supplementation in both wild-type and an EPS-defective background (Δ*eps::FRT*). The addition of L-arginine in M63 medium at 20 mM did not alter the maximum growth rate achieved by either the wild-type or Δ*eps::FRT* strains (Fig. S3). Furthermore, while growth curves showed that L-arginine supplementation increased both the maximum OD_{600} reached by *P. aeruginosa* PAO1 during the growth assay (Fig. S3) and the OD_{600} at 8 h (Fig. S3), a time point consistent with our biofilm assay measurement, we noted that these increases in OD_{600} are observed only in the wild-type background and are dependent on exopolysaccharide (EPS) biosynthesis genes (Fig. S3). This is consistent with our hypothesis that L-arginine supplementation led to increased OD_{600} in an EPS-dependent manner via biofilm enhancement rather than bacterial growth promotion. Collectively these data suggest that putrescine and L-arginine promote biofilm in *P. aeruginosa* independently of their role as carbon and nitrogen sources.

Putrescine and arginine promote the intracellular accumulation of c-di-GMP. c-di-GMP is a second messenger ubiquitous in *Proteobacteria* that governs lifestyle switching between biofilm and planktonic cells (16). Exogenous L-arginine can lead to increased c-di-GMP levels in *Pseudomonas putida* (35). Therefore, we hypothesized that putrescine and L-arginine modulate *P. aeruginosa* biofilm formation by regulating intracellular c-di-GMP levels. We tested this hypothesis using a c-di-GMP-dependent GFP reporter (22). We found that the addition of exogenous putrescine or L-arginine in M63 medium resulted in a significant increase in fluorescence signal, suggesting that

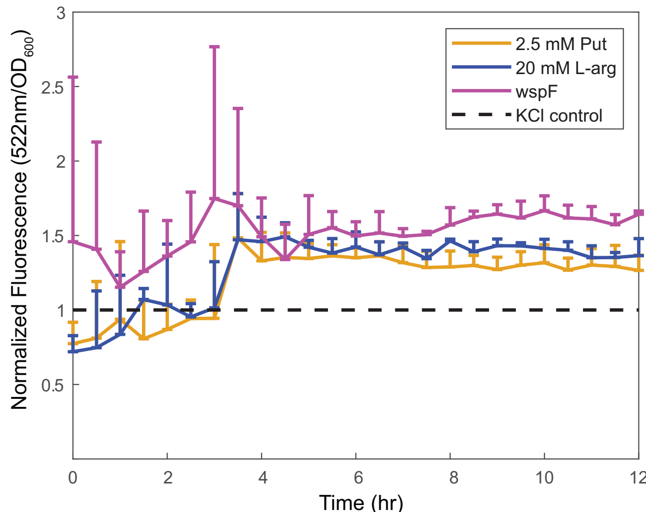

FIG 4 Addition of exogenous L-arginine and putrescine induces c-di-GMP accumulation. *P. aeruginosa* carrying a reporter pCdrA-*gfp*^{S} was grown in M63 medium supplemented with 20 mM L-arginine hydrochloride or 2.5 mM putrescine dihydrochloride. *wspF*::Tn*5*, a genetic background with constitutive high levels of c-di-GMP, was used as a control for the pCdrA-*gfp*^{S} reporter. Putrescine dihydrochloride or L-arginine HCl supplementation promotes GFP expression relative to equimolar KCl, suggesting that polyamines upregulate intracellular c-di-GMP levels. Error bars represent standard deviation. Each time point represents at least 3 biological replicates with 8 technical replicates in each biological replicate.

exogenous polyamines and the polyamine biosynthetic precursor L-arginine induce c-di-GMP biosynthesis in a timescale similar to that of the biofilm assays starting at 4 h (Fig. 4). These data indicated that putrescine and L-arginine may promote biofilm formation by regulating intracellular levels of c-di-GMP.

DISCUSSION

We previously demonstrated that biofilm downregulation and putrescine catabolism via SpuC are required for successful plant rhizosphere colonization by *Pseudomonas fluorescens* (8). Additionally, putrescine and spermidine are components of tomato rhizosphere exudate (28, 36), suggesting a role of putrescine in biofilm regulation in a host-associated context. However, no mechanistic link exists between putrescine metabolism and biofilm attenuation. We found that putrescine-mediated enhancement of biofilm formation coincided with increased intracellular level of c-di-GMP in *P. aeruginosa* PAO1 as reflected by a c-di-GMP-induced GFP expression reporter. This is consistent with the previous observation that polyamine regulation of *Agrobacterium tumefaciens* biofilm formation is dependent on the intracellular c-di-GMP pool (16) and that L-arginine-induced c-di-GMP increase correlates with biofilm enhancement in *P. putida* (35). It is unclear how putrescine supplementation is linked to the increase of c-di-GMP. Our data suggest that SpuD-mediated uptake of putrescine is not necessary for biofilm promotion. One possibility is that the *P. aeruginosa* PAO1 genome encodes an unknown, redundant periplasm substrate-binding protein for putrescine uptake. However, SpuE, the periplasm polyamine substrate-binding protein with the highest homology to SpuD in *P. aeruginosa* PAO1, has been shown to have no detectable putrescine-binding ability (29).

An alternative hypothesis is that there may be a periplasmic sensor for putrescine that triggers the downstream signaling that leads to biofilm enhancement. A recent study in *Vibrio cholerae* proposes a model in which norspermidine and spermidine can competitively bind a periplasmic protein, NspS, allowing NspS to regulate the antagonistic activities of a dual activity diguanylate cyclase/c-di-GMP phosphodiesterase, MbaA (37). Additionally, previous reports suggested that c-di-GMP-modulating enzymes (CMEs) containing ligand-binding domains may be regulated by the direct binding of exogenous signals (38). As a result, our current hypothesis is that one or more *P. aeruginosa*

TABLE 1 Strains and plasmids

Strains or plasmids	Genotype or description	Source or reference
E. coli strains		
DH5α λpir	*supE44* Δ*lacU169* (Φ*lacZ*Δ*M15*), *recA1 endA1 hsdR17 thi-1 gyrA96 relA1* λ*pir*	
SM10 λpir	*thi thr leu tonA lacY supE recA*::RP4-2-*Tc*::Mu Km λ*pir*; conjugating strain	
P. aeruginosa strains		
PAO1-H103	H103 subline of PAO1 strain; parental strains of all mutants	41, 42
PAO1 Δ*speA*	*speA* (PA4839) in frame deletion; L-arginine decarboxylase mutant	This study
PAO1 Δ*speC*	*speC* (PA4519) in frame deletion; L-ornithine decarboxylase mutant	This study
PAO1 Δ*speD*	*speD* (PA0654) in frame deletion; SAM decarboxylase mutant	This study
PAO1 Δ*spuC*	*spuC* (PA0299) in frame deletion; putrescine aminotransferase mutant	This study
PAO1 Δ*spuD*	*spuD* (PA0300) in frame deletion; putrescine periplasmic SBP mutant	This study
PAO1 Δ*speAC*	*speAC* in frame deletions	This study
PAO1 Δ*speAC* Δ*spuD*	Δ*speAC* Δ*spuD* in frame deletions	This study
PAO1 *pel*	Δ*pelA*::*FRT*; markerless FLP-mediated excision of *pelA*	This study
PAO1 *psl*	Δ*pslBCDE*::*FRT*; markerless FLP-mediated excision of *pslBCDE*	This study
PAO1 *eps*	Δ*pelA*::*FRT* Δ*pslBCDE*::*FRT*; exopolysaccharide (EPS) mutant	This study
PAO1 *wspF*::Tn*5*	*wspF*(PA3703)::mini-Tn*5*-*luxCDABE*; ID PAO1_lux_75_D8; 91/1,008 nt	42
PAO1 *wspF pel*	*wspF*::mini-Tn*5*-*luxCDABE* Δ*pelA*::*FRT*	This study
PAO1 *wspF psl*	*wspF*::mini-Tn*5*-*luxCDABE* Δ*pslBCDE*::*FRT*	This study
PAO1 *wspF eps*	*wspF*::mini-Tn*5*-*luxCDABE* Δ*pelA*::*FRT* Δ*pslBCDE*::*FRT*	This study
PAO1 ZXL001	Δ*pelA*::*FRT* Δ*pslBCDE*::*FRT* pCdrA-*gfp*^{S}	This study
PAO1 ZXL002	*wspF*::mini-Tn*5*-*luxCDABE* Δ*pelA*::*FRT* Δ*pslBCDE*::*FRT* pCdrA-*gfp*^{S}	This study
Plasmids		
pEXG2	Vector for *Pseudomonas* spp. allelic exchange; *aacC1 sacB oriT;* pBR322 *oriC*	43
pΔspeA	pEXG2::speA-flanking; allelic exchange construct	This study
pΔ*speC*	pEXG2::speC-flanking; allelic exchange construct	This study
pΔ*speD*	pEXG2::speD-flanking; allelic exchange construct	This study
pΔspeE	pEXG2::speE-flanking; allelic exchange construct	This study
pΔ*spuC*	pEXG2::spuC-flanking; allelic exchange construct	This study
pΔ*spuD*	pEXG2::spuD-flanking; allelic exchange construct	This study
pMPELA	pEX18.Ap::*pelA::FRT-aacC1-gfp-FRT*	45
pMPSL-KO1	pEX18.Ap::*pslBCDE::FRT-aacC1-gfp-FRT*	46
pFLP3	Source of FLP recombinase; Ap^r Tc^r	47
pCdrA-*gfp*^{S}	pUCP22Not-P*cdrA*-RBS-CDS-RNaseIII-*gfp*(Mut3)-T0-T1 Ap^r Gm^r	22

CMEs respond to polyamine compounds either through their ligand-binding domains or through another periplasmic sensor protein. Interestingly, a prior study demonstrated that, while a diguanylate cyclase may only contribute to a subtle increase in the intracellular global concentration of c-di-GMP, its effect on biofilm formation is much more pronounced (39, 40). This is due to the localization of diguanylate cyclase, which allows direct "loading" of c-di-GMP into a receptor protein that mediates adhesion protein secretion (39, 40). Hence, it should be noted that, while the change in c-di-GMP level induced by exogenous polyamines is modest as reflected by the relative GFP signal, polyamines can still lead to a robust physiological response in *Pseudomonas*.

While we demonstrated that L-arginine and putrescine serve as environmental signals that promote biofilm in *Pseudomonas*, the identities of potential polyamine sensors are unknown. Our current hypothesis is that CMEs can directly bind polyamines via ligand-binding domains. In addition to PAS domain- and CHASE domain-containing CMEs, *Pseudomonas fluorescens* also encodes Ca^{2+} channel and chemotaxis (CACHE) domain-containing CMEs that can recognize other ligands that modulate their enzymatic activities (12, 38). Therefore, a comprehensive screening of a *Pseudomonas aeruginosa* CME deletion library (12) could identify genes required for sensing and responding putrescine. This work lays the foundation for future mechanistic studies that can identify such polyamine metabolite sensors.

MATERIALS AND METHODS

Strains, media, and culture conditions. All strains and plasmids used in this study are in Table 1. For routine culture, *P. aeruginosa* PAO1 H103 subline (*P. aeruginosa* PAO1 [41, 42]), *Escherichia coli* DH5α

λpir, and *E. coli* SM10 λpir strains were grown on lysogeny broth (LB) agar or in LB medium at 37°C with shaking at 200 rpm. When appropriate, antibiotics and counterselection agents were supplemented at the following concentrations: 10% (wt/vol) sucrose, 5 μg/mL (*E. coli*) or 50 μg/mL (*P. aeruginosa*) gentamicin (Gm), 100 μg/mL (*E. coli*) or 250 μg/mL (*P. aeruginosa*) carbenicillin (Cb), and 10 μg/mL Irgasan.

Strain construction. All primers used in this study are described in Table S1 in the supplemental material. Construction of Δ*speA*, Δ*spuC*, Δ*spuD*, Δ*speC*, Δ*speD*, and Δ*speE* mutants in *P. aeruginosa* was performed as previously described (8, 43). Briefly, 450 to 600 bp of the upstream and downstream flanking regions of the target genes were amplified and joined via overlap extension PCR (44) then ligated into the pEXG2 vector (43). Correct insertions were verified by PCR and Sanger sequencing. The deletion constructs were transformed into calcium competent *E. coli* SM10 λpir, and subsequently mobilized into *P. aeruginosa* PAO1 by conjugation (8). To allow for conjugation, *E. coli* and *P. aeruginosa* cultures were mixed, plated on solid LB medium, and incubated at 37°C for 4 h. The mating spots were then scraped off, resuspended in 1 mL of 10 mM $MgSO_4$, and plated on LB agar containing 50 μg/mL Gm and 10 μg/mL Irgasan. Second homologous recombination events were selected for using sucrose counterselection (8). Briefly, single recombinant colonies are grown in lysogeny broth with no selection overnight before plating on LB-sucrose to select for the double recombinant colonies that excised the *sacB*-containing plasmid backbone. Successful deletions were verified by testing for Gm sensitivity and by colony PCR.

To construct Δ*pelA*::*FRT* Δ*pslBCDE*::*FRT* (Δ*eps*) double mutants in *P. aeruginosa* PAO1 H103 subline, the gene disruption constructs pMPELA (45) and pMPSL-KO1 (46) that were originally developed based on the Flp-*FRT* gene disruption system (47) were introduced into *P. aeruginosa* by *E. coli* SM10 λpir conjugation. To select for the first homologous recombination events, the mating spots were resuspended and plated on LB agar containing 50 μg/mL Gm and 10 μg/mL Irgasan. To confirm plasmid integration, *P. aeruginosa* single recombinant colonies were either streaked on LB-Cb or LB-Gm plates. Additionally, single recombinant colonies contain *FRT-aacC1-gfp-FRT* gene disruption cassettes and, therefore, could be distinguished from the wild type by measuring the green fluorescent protein (GFP) signal from cultures grown overnight (diluted to OD_{600} of 0.5) using a 96-well plate reader. To remove the pMPELA or pMPSL-KO1 plasmid backbones by second homologous recombination events, single recombinant *P. aeruginosa* were cultured overnight in LB broth with no selection before plating on LB-sucrose. Double recombinant colonies (Δ*pelA*::*FRT-aacC1-gfp-FRT* or Δ*pslBCDE*::*FRT-aacC1-gfp-FRT*) were confirmed by selecting for Gm resistance and Cb sensitivity. The *FRT-aacC1-gfp-FRT* cassettes were excised via Flp-mediated recombination as previously described (48). Briefly, double recombinant colonies were transformed with pFLP3. Cb^r colonies were grown overnight on LB agar before patching onto LB-Gm and LB-Cb. Gm^s Cb^r colonies were picked and grown in LB broth with no selection before plating on sucrose to select for colonies cured of the *sacB*-harboring pFLP3 plasmid. The final colonies carrying Δ*pelA*::*FRT* or Δ*pslBCDE*::*FRT* scars were further confirmed by the lack of GFP signal in the cultures grown overnight. Double knockout mutants of *pelA* and *pslBCDE* were created by performing gene disruption sequentially with pMPELA and pMPSL-KO1.

To create the c-di-GMP reporter strains *P. aeruginosa* PAO1 ZXL001 and *P. aeruginosa* PAO1 ZXL002, *P. aeruginosa* PAO1 Δ*eps*::*FRT* and *P. aeruginosa* PAO1 Δ*eps*::*FRT wspF*::mini-Tn*5-luxCDABE* (*P. aeruginosa* PAO1 Δ*eps wspF*::Tn*5* [42]) strains were transformed with pCdrA-*gfp*^{S} (22) by electroporation as previously described (49). Plasmids were confirmed by PCR using GFPmut3-specific primers and maintained in LB-Gm.

Polyamine quantification through targeted metabolomics. To quantify intracellular spermidine and putrescine levels, wild-type PAO1 and the Δ*spuC*, Δ*speD*, and Δ*speAC* mutants were grown overnight in standard M63 medium (comprised of a modified M63 medium with M63 salts, glucose [0.2%] as the carbon source, magnesium sulfate [1 mM], and ammonium chloride [20 mM] as the nitrogen source). The next day, the cultures were spun down at 13,000 $\times$ *g* for 2 min, washed twice, and resuspended in the same medium they were grown in overnight. The bacteria were diluted to a final OD_{600} of 0.1. To quantify putrescine uptake in the Δ*spuD* Δ*speAC* triple mutant, the triple mutant and the wild-type PAO1 strains were grown overnight in standard M63 medium with the addition of putrescine dihydrochloride (2.5 mM). They were spun down the next day at 13,000 $\times$ *g* for 2 min, washed twice, resuspended in standard M63 medium (without putrescine dihydrochloride), and diluted to a final OD_{600} of 0.1. Targeted metabolomics to quantify putrescine and spermidine levels was performed by West Coast Metabolomics.

Crystal violet biofilm assays. Biofilm assays were performed as previously described (18, 50). Overnight cultures of *P. aeruginosa* PAO1 in LB were spun down at 13,000 $\times$ *g* for 2 min, washed twice, resuspended, and diluted to an OD_{600} of 0.1 in 1.1$\times$ M63 medium (1$\times$ M63 salt, 0.2% glucose, 0.5% Casamino Acids, 1 mM $MgSO_4$). Putrescine and L-arginine were supplemented at the indicated concentrations (2.5 mM putrescine dihydrochloride for M63-Put, 2.5 mM L-arginine hydrochloride for M63-Arg, and 20 mM L-arginine for M63-Arg20). When putrescine dihydrochloride or L-arginine hydrochloride were added, the control medium were supplemented with KCl to control for the chloride ion concentration (5 mM KCl for putrescine dihydrochloride control and 20 mM KCl for L-arginine hydrochloride control). 100 μL of the diluted cultures were incubated at 37°C under a static condition in non-tissue culture-treated 96-well plates (Falcon; product no. 351177) for 8 h. After incubation, the plates were rinsed in distilled water twice and stained with 125 μL of 0.1% crystal violet aqueous solution for 10 min. After staining, plates were washed in distilled water 3 times to remove excess stain and dried for at least 6 h before solubilizing the crystal violet with 150 μL of 30% acetic acid for 10 min. One hundred microliters of the resolubilized crystal violet was transferred to a flat bottom 96-well plate for absorbance reading at 550 nm (SpectraMax i3x fluorescence plate reader). Background signals were measured from wells containing 100 μL of 30% unstained acetic acid and subtracted from the absorbance readings.

Growth curves. Growth curves were performed in tissue culture-treated 96-well plates. Cultures of *P. aeruginosa* PAO1 grown overnight in LB were spun down at 13,000 × *g* for 2 min, washed twice, resuspended, and diluted to an OD_{600} of 0.11 in 1.1× M63 medium. 10 μL of 200 mM L-arginine, 200 mM KCl, or H_2O was first added to the appropriate wells before adding 90 μL of the bacterial suspension (OD_{600} 0.11) in 1.1× M63 medium for a final OD_{600} of 0.1 in 1× M63 medium. Wells on the edge of the plates were not used to minimize the effects of evaporation. The OD_{600} was measured every 15 min in a plate reader (Molecular Devices, VersaMax) at 37°C with constant shaking for at least 20 h.

Comparative genomics. The species tree used in Fig. 1B was generated as described using 122 single-copy genes we previously found to be conserved in all *Pseudomonas* strains (24). Determination of the presence/absence of the polyamine uptake, biosynthesis, and catabolism genes was determined using the PyParanoid comparative genomics tool (24). We used the annotated sequences for *potFGHI* (PA0300, PA0302-0304), *speA* (PA4839), *aguA* (PA0292), *aguB* (PA0293), *spuC* (PA0299), *speC* (PA4519), *speD* (PA0654), and *speE* (PA1687) from *P. aeruginosa* PAO1, and *CASDH* (WCS365_04584) and *CASDC* (WCS365_04583) from *P. fluorescens* WCS365 to query the database and then plotted the presence/absence data against the species tree.

c-di-GMP GFP reporter assays. Cultures of *P. aeruginosa* PAO1 ZXL001 and *P. aeruginosa* PAO1 ZXL002 strains were grown overnight in LB-Gm for 20 h. Bacterial cells were pelleted by centrifugation at 13,000 × *g* for 2 min, washed three times with M63 medium, resuspended, and diluted to an OD_{600} of 0.5 in fresh 1.1× M63 medium with no antibiotics. In a black-welled 96-well plate (Corning; cat. no. CLS3603), 90 μL of diluted bacterial cultures was added to each well. When appropriate, each well was supplemented with either 10 μL of 50 mM KCl, 25 mM putrescine dihydrochloride, 200 mM KCl, or 200 mM L-arginine hydrochloride for a final concentration of 2.5 mM putrescine or 20 mM L-arginine with appropriate KCl molarity in the control medium (5 mM KCl for putrescine dihydrochloride and 20 mM KCl for L-arginine hydrochloride). The OD_{600} and GFP signals of each well were read immediately and again every 30 min for 12 h on a 96-well plate reader (SpectraMax i3x fluorescence plate reader) at 497 nm excitation and 522 nm emission. Relative fluorescence intensity (the ratio of A_{522} and OD_{600}) was used as a proxy for c-di-GMP levels in the cells.

Data processing and availability. Growth curves and c-di-GMP GFP reporter assays data were processed and visualized with custom scripts in Python 3.0 and MATLAB. Raw data and scripts used for data analysis and visualization are available at https://github.com/fzxliu/liu_et_al_polyamine_2021.git.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We declare no conflict of interest.

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