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Bis-(3′-5′)-Cyclic Dimeric GMP Regulates Antimicrobial Peptide Resistance in *Pseudomonas aeruginosa*

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Bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) is an intracellular second messenger that controls the lifestyles of many bacteria. A high intracellular level of c-di-GMP induces a biofilm lifestyle, whereas a low intracellular level of c-di-GMP stimulates dispersal of biofilms and promotes a planktonic lifestyle. Here, we used the expression of different reporters to show that planktonic cells, biofilm cells, and cells dispersed from biofilms (DCells) had distinct intracellular c-di-GMP levels. Proteomics analysis showed that the low intracellular c-di-GMP level of DCells induced the expression of proteins required for the virulence and development of antimicrobial peptide resistance in *Pseudomonas aeruginosa*. In accordance with this, *P. aeruginosa* cells with low c-di-GMP levels were found to be more resistant to colistin than *P. aeruginosa* cells with high c-di-GMP levels. This finding contradicts the current dogma stating that dispersed cells are inevitably more susceptible to antibiotics than their sessile counterparts.

It is now widely accepted that microbes are able to form surface-attached biofilm communities in the environment and during infection as an alternative to the planktonic or free-living style. Biofilm formation proceeds through several distinct steps, including initial attachment, with subsequent development of dense microcolonies embedded in self-generated extracellular matrix materials (1) and finally dispersal to seed new areas of biofilm formation (2).

Bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) is a global, intracellular second messenger that controls the lifestyles of many bacteria (3). The intracellular c-di-GMP concentration is controlled by diguanylate cyclases (DGCs) which catalyze the formation of c-di-GMP and phosphodiesterases (PDEs) which degrade c-di-GMP (4). Many bacteria contain multiple copies of DGCs and PDEs, which allow bacterial cells to sense and respond to diverse sets of environmental signals by adjusting the intracellular c-di-GMP content accordingly.

As a secondary messenger that binds to specific domains of regulatory proteins, high level of c-di-GMP stimulates bacteria to form biofilm by enhancing the synthesis of adhesive structures and biofilm matrix components and by reducing motility and chemotaxis (5, 6). In the aggregated biofilm mode, quorum sensing contributes to the production of matrix components that facilitate protection of the biofilm cells against cellular immunity attack and antimicrobial treatments (7–10). Recently, however, a low intracellular level of c-di-GMP has been shown to be necessary for the pathogenesis of bacteria (11, 12). The CheY-EAL-HTH domain protein VieA of *Vibrio cholerae* is required for the activation of certain virulence factors (13). Another EAL domain-containing protein, CdgR, has been shown to be required by *Salmonella* to resist phagocytosis and virulence during infection of mice (14).

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that can cause a wide range of infections, including those in cystic fibrosis, wounds, and the urinary tract (15). The success of *P. aeruginosa* as a human pathogen is largely dependent on its ability to form biofilms, produce virulence factors, and launch immune protective measures in an organized fashion, as well as its notorious resistance to antimicrobial agents (16, 17), all of which may allow infections to develop into chronic conditions (16, 17). Here, we studied the effects of modulating the intracellular content of c-di-GMP in *P. aeruginosa* in relation to biofilm dispersal and antimicrobial peptide resistance.

MATERIALS AND METHODS

Bacteria and growth conditions. The bacterial strains, plasmids, and primers used in the present study are listed in Table 1. *Escherichia coli* DH5a strain was used for standard DNA manipulations. Luria-Bertani medium (18) was used to cultivate *E. coli* strains. Batch cultivation of *P. aeruginosa* was carried out at 37°C in ABT minimal medium (19) supplemented with 5 g of glucose liter^{-1} (ABTG) or 2 g of glucose liter^{-1} plus 2 g of Casamino Acids liter^{-1} (ABTGC). For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg of ampicillin ml^{-1}, 15 μg of gentamicin (Gm) ml^{-1}, 15 μg of tetracycline (Tc) ml^{-1}, or 8 μg of chloramphenicol ml^{-1}. For marker selection in *P. aeruginosa*, 30 μg of Gm ml^{-1}, 50 μg of Tc ml^{-1}, and 200 μg of carbenicillin ml^{-1} were used, as appropriate.

Construction of p_{BAD}-*yhjH* vector. Plasmid pJN105 contains an *araC*-P_{BAD} promoter, which has been well studied and induced in the presence of L-arabinose (23). The *yhjH* gene of *E. coli* MG1655 was amplified by PCR using primers yhjH-rev and yhjH-fwd. The PCR product was cloned into the vector pJN105 by restriction with PstI and XbaI. DNA

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TABLE 1 Strains,plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s) or sequence (5′–3′)[a]	Source or reference
Strains		
P. aeruginosa		
PAO1	Prototypic nonmucoid wild-type strain	20
PAO1Δ*wspF*	*wspF* derivative of PAO1 constructed by allelic exchange	21
PAO1/p_{lac}-*yhjH*	Tc^r; PAO1 containing the p_{lac}-*yhjH* vector	This study
PAO1/p_{BAD}-*yhjH*	Gm^r; PAO1 containing the p_{BAD}-*yhjH* vector	This study
PAO1Δ*wspF*/p_{lac}-*yhjH*	Tc^r; PAO1Δ*wspF* containing the p_{lac}-*yhjH* vector	This study
PAO1Δ*wspF*/p_{BAD}-*yhjH*	Gm^r; PAO1Δ*wspF* containing the p_{BAD}-*yhjH* vector	This study
PAO1Δ*pelA*Δ*pslBCD*/p_{cdrA}-*gfp*	Gm^r Cb^r; low intracellular c-di-GMP content derivative of PAO1	21
PAO1/p_{cdrA}-*gfp*	Gm^r; PAO1 containing the p_{cdrA}-*gfp* vector	This study
PAO1Δ*wspF*/p_{cdrA}-*gfp*	Gm^r Cb^r; PAO1Δ*wspF* containing the p_{cdrA}-*gfp* vector	This study
PAO1/p_{lac}-*yhjH*/p_{cdrA}-*gfp*	Tc^r Cb^r; PAO1/p_{lac}-*yhjH* containing the p_{cdrA}-*gfp* vector	This study
PAO1/p_{BAD}-*yhjH*/p_{cdrA}-*gfp*	Gm^r Cb^r; PAO1/p_{BAD}-*yhjH* containing the p_{cdrA}-*gfp* vector	This study
PAO1-p_{pmr}-*gfp*	Gm^r; PAO1 tagged by miniTn*7*-p_{pmr}-*gfp*	This study
PAO1Δ*wspF*/p_{pmr}-*gfp*	Gm^r; PAO1Δ*wspF* tagged by miniTn*7*-p_{pmr}-*gfp*	This study
PAO1/p_{lac}-*yhjH*/p_{pmr}-*gfp*	Tc^r Gm^r; PAO1/p_{lac}-*yhjH* tagged by miniTn*7*-p_{pmr}-*gfp*	This study
PAO1/p_{pelA}-*lacZ*	Tc^r; PAO1 tagged by miniCTX-p_{pelA}-*lacZ*	This study
PAO1Δ*wspF*/p_{pelA}-*lacZ*	Tc^r; PAO1Δ*wspF* tagged by miniCTX-p_{pelA}-*lacZ*	This study
PAO1/p_{BAD}-*yhjH*/p_{pelA}-*lacZ*	Tc^r Gm^r; PAO1/p_{BAD}-*yhjH* tagged by miniCTX-p_{pelA}-*lacZ*	This study
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Laboratory collection
Plasmids		
pUCP22	Ap^r Gm^r; broad-host-range cloning vector	22
pJN105	Gm^r; broad-host-range vector carrying the *araBAD* promoter	23
pBBR1MCS3	Tc^r; broad-host-range *ori* from *Bordetella bronchiseptica* S87	24
miniTn*7*-p_{pmr}-*gfp*	Ap^r Gm^r; miniTn*7* vector carrying the p_{pmr}-*gfp* fusion	25
miniCTX-p_{pelA}-*lacZ*	Tc^r; miniCTX vector carrying the p_{pelA}-*lacZ* fusion	26
p_{lac}-*yhjH*	Tc^r; pBBR1MCS3 carrying the *yhjH* gene	27
p_{BAD}-*yhjH*	Gm^r; pJN105 carrying the *yhjH* gene	This study
p_{cdrA}-*gfp*	Ap^r Gm^r; pUCP22 carrying the p_{cdrA}-*gfp* fusion	21
pRK600	Cm^r; *ori* ColE1 RK2-Mob^+ RK2-Tra^+; helper vector for conjugation	28
Primers		
yhjH-fwd	AAACTGCAGTAGTGGAGGAATTTGATGATAAGGCAGGTTATCCAGC	This study
yhjH-rev	AAATCTAGAGAAAATGAGGCAGCTTATAGCGC	This study

[a] Cm^r, chloramphenicol resistance; Tc^r,tetracycline resistance; Ap^r, ampicillin resistance; Gm^r,gentamicin resistance; Cb^r, carbenicillin resistance.

restriction enzyme digestions and modifications were performed according to the manufacturer's instructions (Fermentas and Invitrogen). The resulting plasmid p_{BAD}-*yhjH* was transferred into *E. coli* DH5α by electroporation. Correct insertion of the *yhjH* gene into the vector pJN105 was verified by sequencing. The p_{BAD}-*yhjH* plasmid was transformed into *E. coli* S17-1 by electroporation and thereafter conjugated into *P. aeruginosa*.

CdrA-*gfp* assay. *P. aeruginosa* strains containing p_{cdrA}-*gfp* reporter were cultivated in ABTGC medium at 37°C with shaking. Portions (200 μl) of overnight cultures were transferred into each of the wells of a 96-well microplate. The expression of p_{cdrA}-*gfp* in *P. aeruginosa* was measured using a Tecan Infinite Pro2000 microplate reader. The optical density at 600 nm (OD_{600}) and green fluorescent protein (GFP) fluorescence (in relative fluorescence units) were recorded for each well of the 96-well microplate.

For measuring p_{cdrA}-*gfp* expression in biofilm cells of the PAO1 strain, the *P. aeruginosa* PAO1/p_{cdrA}-*gfp* strain were cultivated in 50-ml BD Falcon tubes containing 15 ml of ABTGC medium. A sterile glass cover slide (24 by 60 mm) was inserted into each Falcon tube to support biofilm growth. After overnight incubation, PAO1 biofilms on the slides were washed twice with 1 ml of 0.9% NaCl and imaged using fluorescence microscopy (Carl Zeiss). The planktonically growing PAO1/p_{cdrA}-*gfp* strain and strain PAO1Δ*wspF*/p_{cdrA}-*gfp* were also imaged using fluorescence microscopy for comparison.

Pel-*lacZ* assay. The mini-CTX-p_{pel}-*lacZ* reporter fusion (26) was inserted into the chromosomes of *P. aeruginosa* PAO1, PAO1Δ*wspF*, and PAO1/p_{BAD}-*yhjH* strains by triparental mating with the help of pRK600 vectors as previously described (29). PAO1/p_{BAD}-*yhjH* biofilms were cultivated in ABTGC medium in a 24-well plate (Nunc) overnight at 37°C. The biofilms were washed twice with 1 ml of 0.9% NaCl and supplemented with ABTGC medium containing 0.25, 0.5, or 1% arabinose for 5 h to induce dispersal. Biofilms formed by the PAO1 and PAO1Δ*wspF* strains were dispersed by 5 μM NO donor sodium nitroprusside (SNP; Sigma). As controls, PAO1 and PAO1/p_{BAD}-*yhjH* planktonic cultures were diluted 10 times to fresh ABTGC medium and incubated for 5 h at 37°C with shaking. The OD_{600} values of planktonic cells were measured and normalized to the same OD_{600} values of dispersed cells. A classical β-galactosidase assay was used to measure expression of the p_{pel}-*lacZ* fusion in *P. aeruginosa* cells (30).

Intracellular c-di-GMP concentration in biofilm cells. To assay c-di-GMP concentrations of biofilm cells, the glass slide biofilm assay was performed as previously reported (31). The p_{cdrA}-*gfp* containing *P. aeruginosa* PAO1 and PAO1/p_{BAD}-*yhjH* strain were cultivated in 50-ml BD Falcon tubes containing 15 ml of ABTGC medium. A sterile glass

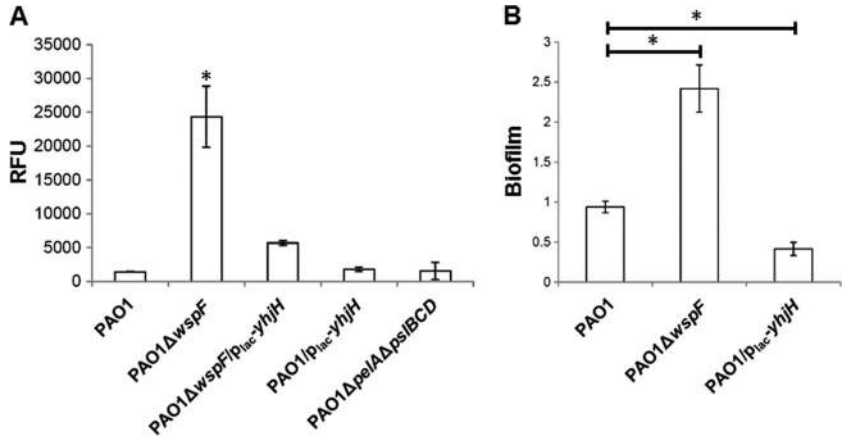

FIG 1 (A) Expression of p_{cdrA}-*gfp* fusion in *P. aeruginosa* PAO1 (PCells), PAO1Δ*wspF* (BCells), PAO1Δ*wspF*/p_{lac}-*yhjH*, and PAO1/p_{lac}-*yhjH* (DCells) strains. Means and standard deviations (SD) in relative fluorescence units (RFU) from triplicate experiments are shown. *, $P < 0.01$. (B) Biofilm formation of *P. aeruginosa* PAO1 (PCells), PAO1Δ*wspF* (BCells), and PAO1/p_{lac}-*yhjH* (DCells) strains in microplates. Means and SD from triplicate experiments are shown. *, $P < 0.01$.

cover slide (24 by 60 mm) was inserted into each Falcon tube to support biofilm growth. After overnight incubation, slide biofilms were washed twice with 0.9% NaCl and imaged by using fluorescence microscopy (Carl Zeiss).

Microplate biofilm formation assay. A microplate biofilm formation assay was carried out in ABTGC medium as previously described (32).

iTRAQ-based proteomics analyses. *P. aeruginosa* cells were harvested after 48 h of cultivation in AB minimal medium supplemented with 5 g of glucose liter^{-1} and subjected to iTRAQ-based proteomics analyses (additional details for these analyses are provided in the supplemental material).

Pyoverdine quantification. PAO1, PAO1Δ*wspF*, and PAO1/p_{lac}-*yhjH* strains were grown in ABTGC medium overnight. The pyoverdine fluorescence (excitation wavelength, 400 nm; emission wavelength, 450 nm) of each supernatant of *P. aeruginosa* overnight cultures was recorded by using the Tecan Infinite Pro2000 microplate reader as previously reported (33).

Pmr-*gfp* assay. The miniTn*7*-Gm-p_{pmr}-*gfp* fusion was inserted into the chromosomes of PAO1, PAO1Δ*wspF*, and PAO1/p_{lac}-*yhjH* strains by four-parental mating with the help of pBF13 and pRK600 vectors as previously described (29). *P. aeruginosa* PAO1, PAO1Δ*wspF*, and PAO1/p_{lac}-*yhjH* strains were grown in ABTGC medium overnight. The cultures were then diluted 10-fold into fresh ABTGC medium with or without 1 μg of colistin ml^{-1}. Cultures, 3 μl for each condition, were spotted onto cover slides after 7 h growth for fluorescence microscopy imaging (Carl Zeiss). The level of fluorescence of 30 individual p_{pmr}-*gfp*-tagged bacterial cells was measured for each sample by using ImageJ (http://rsbweb.nih.gov/ij/). The corrected total cell fluorescence of each cell was calculated as the sum of the fluorescence intensity within the region of interest minus the background intensity.

Antimicrobial peptide resistance assay of planktonic cells. For comparison of the resistance of strains PAO1, PAO1Δ*wspF*, and PAO1/p_{lac}-*yhjH* to colistin, the growth curves of the three strains in the presence of 0, 0.125, and 2 μg of colistin ml^{-1} were produced in triplicate, as previously described (34). Colistin (0.25 μg ml^{-1}) was selected to represent a concentration lower than the MIC of PAO1 (which is 1 μg ml^{-1}), and 2 μg of colistin ml^{-1} was chosen for a concentration higher than the MIC. Overnight cultures were diluted to an OD_{600} of 0.15 with ABTGC minimal medium containing the appropriate concentrations of colistin. The OD_{600} was recorded every hour for 9 h using the Tecan Infinite Pro2000 microplate reader.

A time-kill kinetic assay was also performed to compare the resistance of PAO1, PAO1Δ*wspF*, and PAO1/p_{lac}-*yhjH* to colistin to concentrations of 2, 4, and 8 μg ml^{-1}, respectively. Overnight cultures of PAO1, PAO1Δ*wspF*, and PAO1/p_{lac}-*yhjH* strains were diluted to an OD_{600} of ~0.2 in fresh ABTGC medium containing 2, 4, and 8 μg of colistin ml^{-1}, respectively. The absorbance of the surviving bacterial cells was monitored by using the Tecan Infinite Pro2000 microplate reader and Live/Dead BacLight bacterial viability kits (Invitrogen).

Colistin resistance assay of dispersed cells. In order to compare the tolerance of cells that dispersed from biofilms with tube-cultivated planktonic cells to colistin, biofilms of PAO1 and PAO1/p_{BAD}-*yhjH* were cultivated in ABTGC medium in a 24-well plate (Nunc) overnight at 37°C. The biofilms were washed twice with 1 ml of 0.9% NaCl and supplemented with ABTGC medium containing 5 μM SNP (PAO1 biofilms) or 0, 0.5, or 1% arabinose (PAO1/p_{BAD}-*yhj*H biofilms) for 5 h to induce dispersal. Biofilms were stained with 0.01% crystal violet as previously described (32). As controls, biofilms of PAO1 and PAO1/p_{BAD}-*yhjH* were washed twice with 1 ml of 0.9% NaCl and supplemented with ABTGC medium for 5 h. Planktonic cells were derived from both biofilms. The OD_{600} of dispersed cells and planktonic cells was measured and adjusted to an OD_{600}

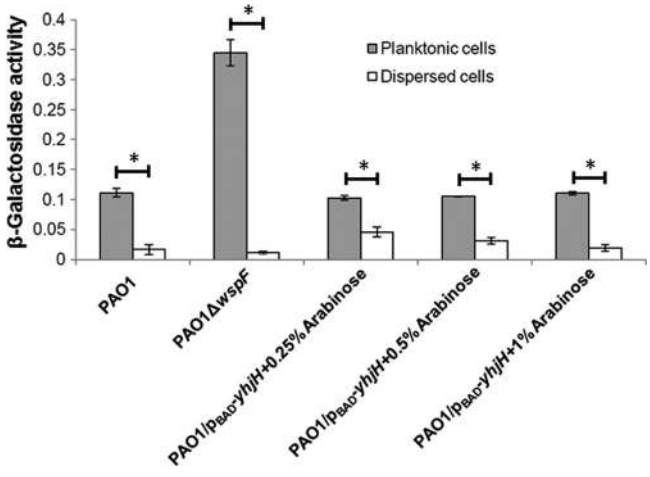

FIG 2 β-Galactosidase activity of *P. aeruginosa* strains grown as planktonic cells or biofilm cells containing the *pel-lacZ* biosensors. SNP was added to both PAO1 and the PAO1Δ*wspF* (BCells) at final concentration of 5 μM, whereas 0.25, 0.5, and 1% arabinose was added to the PAO1/p_{BAD}-*yhjH* strain. For the biofilm cells, the β-galactosidase activity was measured in the dispersed cells. Means and SD in β-galactosidase activity from triplicate experiments are shown. *, $P < 0.01$.

TABLE 2 Proteins whose abundance in *P. aeruginosa* PAO1 increased significantly in conditions of low intracellular levels of c-di-GMP

PA no.	Gene	Description of product	Peptides (95%)	Coverage (95%)	Ratio (115:114)	P^a (115:114)
PA2452		Enterochelin esterase	43	53.42	99.08	5.22E–03
PA1092	*fliC*	Flagellin type B	146	54.3	99.08	3.19E–02
PA3531	*bfrB*	Bacterioferritin	33	34.18	22.28	2.82E–04
PA4777	*pmrB*	PmrB: two-component regulator system signal sensor kinase PmrB	2	5.451	11.91	3.28E–02
PA1596	*htpG*	Heat shock protein 90	30	22.4	10.57	1.58E–02
PA2821	*gstA*	Glutathione *S*-transferase	5	20.91	10.28	3.68E–02
PA4386	*groES*	Cochaperonin GroES	2	45.36	9.20	3.15E–02
PA3126	*ibpA*	Heat shock protein Hsp20	15	24.16	8.95	1.20E–02
PA4228	*pchD*	Pyochelin biosynthesis protein PchD	14	13.71	8.24	3.35E–03
PA1039	*ychJ*	Hypothetical protein O1Q_07577	2	15.29	7.94	3.79E–02
PA3552	*arnB*	UDP-4-amino-4-deoxy-L-arabinose–oxoglutarate aminotransferase	4	13.09	7.38	2.85E–02
PA4942	*hflK*	Protease subunit HflK	6	17	7.31	2.24E–03
PA1534	*recR*	Recombination protein RecR	2	21.21	7.31	3.72E–02
PA4670	*prs*	Ribose-phosphate pyrophosphokinase	43	56.55	7.11	8.95E–05
PA4710	*phuR*	Heme/hemoglobin uptake outer membrane receptor PhuR	20	25.52	6.55	1.57E–04
PA4227	*pchR*	Transcriptional regulator PchR	3	15.54	6.31	2.34E–02
PA4224	*pchG*	Pyochelin biosynthetic protein PchG	16	30.37	6.25	9.10E–03
PA3158	*wbpB*	UDP-2-acetamido-2-deoxy-D-glucuronic acid 3-dehydrogenase, WbpB	10	22.78	6.08	3.03E–02
PA0427	*oprM*	Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM	13	32.16	5.40	1.56E–03
PA0018	*fmt*	Bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase	3	8.006	5.15	2.80E–02
PA1803	*lon*	Putative ATP-dependent protease	23	25.28	4.79	1.21E–04
PA3135		Putative transcriptional regulator	2	11.11	4.61	3.86E–02
PA4231	*pchA*	Salicylate biosynthesis isochorismate synthase	4	12.39	4.57	4.12E–02
PA3114	*truA*	tRNA pseudouridine synthase A	3	17.54	3.98	3.39E–02
PA3831	*pepA*	Multifunctional aminopeptidase A	42	38.59	3.80	2.66E–02
PA3159	*wbpA*	UDP-glucose/GDP-mannose dehydrogenase	17	31.42	3.66	5.69E–03
PA5054	*hslU*	ATP-dependent protease ATP-binding subunit HslU	6	13.87	3.44	4.85E–02
PA4225	*pchF*	*pchF* gene product	24	16.8	3.25	5.80E–06
PA0426	*mexB*	RND multidrug efflux transporter MexB	6	5.067	3.25	3.05E–03
PA1288	*ompP1*	Putative outer membrane protein	8	24.76	3.19	2.64E–02
PA4749	*glmM*	Phosphoglucosamine mutase	5	8.09	3.16	3.36E–02
PA5213	*gcvP1*	Glycine dehydrogenase	3	3.967	2.91	8.00E–03
PA3478	*rhlB*	Rhamnosyltransferase chain B	2	7.512	2.86	3.40E–03
PA2086	*ephx*	Epoxide hydrolase	3	19.33	2.75	3.59E–02
PA4336		Hypothetical protein O1Q_03368	9	23.32	2.73	3.78E–03
PA4595	*yjjK*	Putative ABC transporter ATP-binding protein	15	24.37	2.68	9.43E–04
PA4476		Hypothetical protein O1Q_04078	6	5.956	2.51	9.67E–03
PA2290	*gcd*	Glucose dehydrogenase	9	9.34	2.42	3.72E–02
PA5237	*yigC*	3-Octaprenyl-4-hydroxybenzoate carboxy-lyase	3	6.967	2.42	4.15E–02
PA2302	*ambE*	Protein AmbE	20	10.34	2.27	1.66E–02
PA4307	*pctC*	*pctC* gene product	20	31.65	2.11	2.50E–03
PA3707	*wspB*	Hypothetical protein O1Q_02948	2	14.79	2.07	3.07E–02
PA4588	*gdhA*	Glutamate dehydrogenase	30	41.57	2.05	3.19E–02
PA4226	*pchE*	*pchE* gene product	78	30.6	2.03	1.85E–05

[a] Significance was defined as having a 115:114 abundance score of >2.0 and a *P* value for 115:114 of <0.05. "115:114" refers to the ratio of the protein's abundance in the low c-di-GMP PAO1/p_{lac}-*yhjH* strain (strain 115) versus the high c-di-GMP PAO1Δ*wspF* strain (strain 114).

of 0.15. The growth curves of planktonic PAO1/p_{BAD}-*yhjH* cells and dispersed biofilm cells from PAO1/p_{BAD}-*yhjH* and PAO1 biofilms were measured in ABTGC medium containing 0, 0.125, and 2 μg of colistin ml^{-1}. The OD_{600} was recorded every 15 min for 5 h using the Tecan Infinite Pro2000 microplate reader.

Biofilm colistin treatment assay. The *P. aeruginosa* PAO1 strain was cultivated in 50-ml BD Falcon tubes containing 15 ml of ABTGC medium. A sterile glass cover slide (24 by 60 mm) was inserted into each Falcon tube to support biofilm growth. After overnight incubation, slide PAO1 biofilms were washed twice with 1 ml of 0.9% NaCl and supplemented with ABTGC medium containing 4 μg of colistin ml^{-1}. PAO1 biofilms incubated in ABTGC medium without colistin were used as a control. After 2 h of treatment, the biofilms were washed twice with 0.9% NaCl, stained by using a Live/Dead bacterial viability kit (Invitrogen), and imaged using fluorescence microscopy (Carl Zeiss).

RESULTS

Construction of *P. aeruginosa* cells with different intracellular c-di-GMP levels. We constructed *P. aeruginosa* strains with

TABLE 3 Proteins whose abundance in *P. aeruginosa* PAO1 decreased significantly in conditions of low intracellular levels of c-di-GMP

PA no.	Gene	Description of product	Peptides (95%)	Coverage (95%)	Ratio (115:114)	P^a (115:114)
PA1245	*aprX*	Hypothetical protein O1Q_25902	21	33.82	0.03	2.74E–02
PA3064	*pelA*	PelA protein	2	2.743	0.04	1.71E–02
PA2395	*pvdO*	Protein PvdO	3	16.2	0.05	8.64E–03
PA4554	*pilY1*	Type 4 fimbrial biogenesis protein PilY1	6	7.666	0.05	3.52E–02
PA3613		Hypothetical protein O1Q_02478	12	20.85	0.05	6.41E–03
PA2398	*fpvA*	*fpvA* gene product	40	25.64	0.06	8.02E–06
PA2394	*pvdN*	Protein PvdN	57	50.82	0.06	1.24E–02
PA0059	*osmC*	Osmotically inducible protein OsmC	2	26.24	0.07	3.22E–02
PA5192	*pckA*	Phosphoenolpyruvate carboxykinase	8	18.13	0.07	7.83E–03
PA0423	*pasP*	Hypothetical protein O1Q_22583	17	59.16	0.08	1.49E–03
PA0781		Hypothetical protein O1Q_01027	4	6.259	0.08	1.13E–02
PA5171	*arcA*	Arginine deiminase	68	50.72	0.09	2.68E–05
PA5427	*adhA*	Alcohol dehydrogenase	18	47.08	0.10	2.33E–02
PA2397	*pvdE*	Pyoverdine biosynthesis protein PvdE	3	9.107	0.10	3.47E–02
PA2392	*pvdP*	*pvdP* gene product	15	28.31	0.11	4.16E–04
PA3117	*asd*	Aspartate-semialdehyde dehydrogenase	11	24.32	0.11	8.29E–03
PA0764	*mucB*	Sigma E regulatory protein, MucB/RseB	4	21.52	0.13	2.89E–02
PA3313		Hypothetical protein	17	44.48	0.13	6.00E–03
PA3190	*gltB*	Putative binding protein component of ABC sugar transporter	45	50	0.14	3.53E–03
PA4624	*cdrB*	Hypothetical protein O1Q_15965	5	12.16	0.14	3.22E–02
PA5046	*maeB*	Malic enzyme	22	41.23	0.15	1.34E–03
PA5312	*pauC*	Putative aldehyde dehydrogenase	15	16.1	0.15	4.37E–02
PA3330		Putative short-chain dehydrogenase	21	36.18	0.15	1.05E–02
PA0895	*aruC*	Bifunctional *N*-succinyldiaminopimelate-aminotransferase/acetylornithine transaminase protein	23	49.26	0.15	1.40E–02
PA4450	*murA*	Bifunctional cyclohexadienyl dehydrogenase/3-phosphoshikimate 1-carboxyvinyltransferase	32	35.12	0.15	9.69E–03
PA3327		Nonribosomal peptide synthetase	31	15.48	0.15	2.35E–05
PA0482	*glcB*	Malate synthase G	33	24.69	0.16	3.22E–02
PA3769	*guaA*	GMP synthase	31	35.62	0.17	1.49E–02
PA3977	*hemL*	Glutamate-1-semialdehyde aminotransferase	27	37	0.18	6.48E–03
PA2119	*adh*	Alcohol dehydrogenase	6	24.59	0.18	3.22E–02
PA0552	*pgk*	Phosphoglycerate kinase	12	30.23	0.19	2.51E–02
PA4687	*hitA*	Ferric iron-binding periplasmic protein HitA	10	29.55	0.19	2.03E–02
PA2413	*pvdH*	Diaminobutyrate–2-oxoglutarate aminotransferase	22	41.36	0.19	7.34E–04
PA4448	*hisD*	Bifunctional histidinal dehydrogenase/histidinol dehydrogenase	11	29.55	0.20	3.40E–02
PA5172	*arcB*	Ornithine carbamoyltransferase	67	44.94	0.20	3.04E–02
PA3686	*adk*	Adenylate kinase	4	25.58	0.20	3.45E–02
PA3922		Hypothetical protein O1Q_15760	7	21.54	0.21	4.15E–02
PA2385	*pvdQ*	3-Oxo-C_{12}-homoserine lactone acylase PvdQ	19	22.31	0.21	4.23E–04
PA3729		Hypothetical protein O1Q_03058	3	3.634	0.22	2.48E–02
PA0314	*fliY*	L-Cysteine transporter of ABC system FliY	7	30.86	0.24	1.02E–02
PA2445	*gcvP2*	Glycine dehydrogenase	21	24.19	0.24	2.85E–04
PA0084	*tssC1*	Hypothetical protein O1Q_08024	8	19.48	0.24	2.89E–02
PA3452	*mqoA*	Malate:quinone oxidoreductase	4	10.52	0.26	7.25E–03
PA0400	*metB*	Putative cystathionine gamma-lyase	26	48.48	0.26	7.74E–03
PA4138	*tyrS*	Tyrosyl-tRNA synthetase	7	19.8	0.26	4.93E–02
PA4236	*katA*	Catalase	14	28.42	0.27	8.08E–03
PA0139	*ahpC*	Alkyl hydroperoxide reductase subunit C	16	40.64	0.27	2.34E–02
PA5322	*algC*	Phosphomannomutase	12	16.94	0.27	1.63E–02
PA2944	*cobN*	Cobaltochelatase subunit CobN	8	9.936	0.29	1.96E–02
PA3186	*oprB*	Glucose-sensitive porin, partial	7	7.432	0.29	3.49E–02
PA0036	*trpB*	Tryptophan synthase subunit beta	12	25.87	0.30	2.98E–02
PA4560	*ileS*	Isoleucyl-tRNA synthetase	20	16.65	0.32	4.14E–03
PA4938	*purA*	Adenylosuccinate synthetase	17	41.86	0.34	5.56E–03
PA0077	*tssM1*	Hypothetical protein O1Q_28197	5	6.378	0.34	4.85E–02
PA4266	*fusA1*	Elongation factor G	75	44.62	0.34	8.93E–04
PA4829	*lpd3*	Dihydrolipoamide dehydrogenase	20	45.91	0.35	6.32E–03
PA3328		Putative FAD-dependent monooxygenase	12	19.85	0.35	1.96E–02
PA3666	*dapD*	2,3,4,5-Tetrahydropyridine-2-carboxylate *N*-succinyltransferase	8	28.49	0.36	1.20E–02
PA2391	*opmQ*	Hypothetical protein O1Q_22777	7	23.94	0.37	7.47E–03
PA0956	*proS*	Prolyl-tRNA synthetase	14	26.8	0.37	8.13E–03
PA5131	*pgm*	Phosphoglyceromutase	5	11.46	0.37	3.54E–02
PA3213		Hypothetical protein O1Q_21511	7	46.4	0.41	2.14E–02
PA1833	*yhfP*	Putative oxidoreductase	13	34.24	0.42	4.81E–03
PA0548	*tktA*	Transketolase	10	17.74	0.42	4.74E–03
PA5497	*nrdJa*	*nrdJa* gene product	9	13.08	0.43	1.94E–02
PA1342		Putative binding protein component of ABC transporter	10	31.79	0.44	1.39E–02
PA0067	*prlC*	Oligopeptidase A	13	19.82	0.44	4.51E–02
PA1588	*sucC*	Succinyl coenzyme A synthetase subunit beta	52	42.53	0.45	6.66E–03
PA1010	*dapA*	Dihydrodipicolinate synthase	21	45.89	0.47	6.41E–03
PA3790	*oprC*	Outer membrane copper receptor OprC	15	24.76	0.48	7.21E–03
PA3194	*edd*	Phosphogluconate dehydratase	36	36.84	0.49	3.74E–03
PA1583	*sdhA*	Succinate dehydrogenase flavoprotein subunit	25	22.88	0.50	3.35E–02

[a] Significance was defined as having a 115:114 abundance score of <0.5 and a P value for 115:114 of <0.05. "115:114" refers to the ratio of the protein's abundance in the low c-di-GMP PAO1/p_{lac}-*yhjH* strain (strain 115) versus the high c-di-GMP PAO1Δ*wspF* strain (train 114).

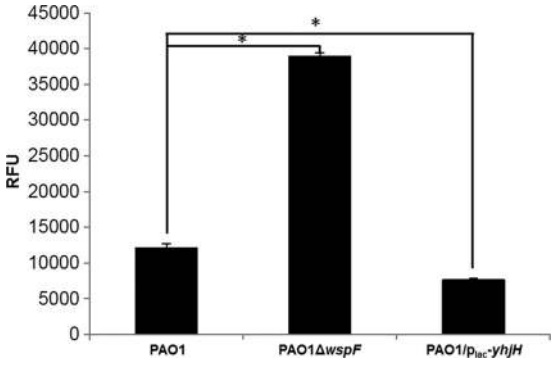

FIG 3 Pyoverdine production by *P. aeruginosa* PAO1 (PCells), PAO1Δ*wspF* (BCells), and PAO1/p_{lac}-*yhjH* (DCells). The pyoverdine fluorescence levels (excitation wavelength, 400 nm; emission wavelength, 450 nm) of supernatants of *P. aeruginosa* overnight cultures were recorded using the Tecan Infinite Pro2000 microplate reader.

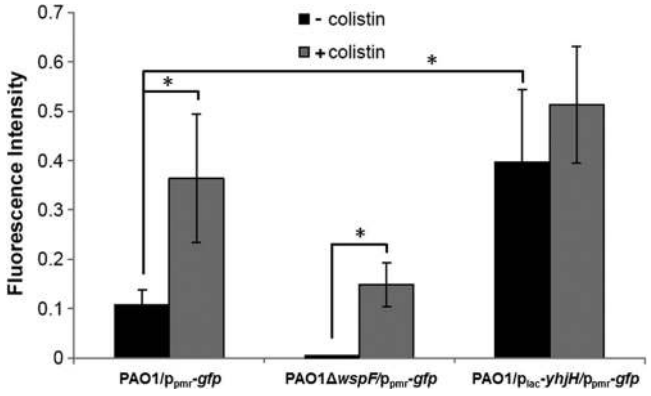

FIG 4 P_{pmr}-*gfp* expression in *P. aeruginosa* PAO1 (PCells), PAO1Δ*wspF* (BCells), and PAO1/p_{lac}-*yhjH* (DCells) strains. Overnight cultures were diluted 10-fold into fresh ABTGC medium with or without 1 μg of colistin ml^{-1}. Portions (3 μl) of cultures representing each condition were spotted onto cover slides after 7 h of growth for imaging by fluorescence microscopy. The level of fluorescence of 30 individual p_{pmr}-*gfp* tagged bacterial cells was measured for each sample by using ImageJ. Means and SD in relative fluorescence intensity units (RFU) from 30 individual cells are shown. *, $P < 0.01$.

controllable intracellular levels of c-di-GMP so that we can mimic the three phases of the biofilm life cycle: planktonic cells (PCells), biofilm cells (BCells), and dispersed cells (DCells). The PAO1Δ*wspF* strain, which overexpresses the diguanylatecyclase WspR (21), is known to contain a high intracellular level of c-di-GMP (11) and can be used to mimic BCells. The PAO1/p_{lac}-*yhjH* strain contains a PBBRMCS-2 plasmid carrying the phosphodiesterase gene *yhjH* fused to a *lac* promoter, which is constitutively expressed in *Pseudomonas* (27), and can be used to mimic DCells. The PAO1/p_{BAD}-*yhjH* strain contains a pJN105 plasmid carrying *yhjH* fused to an arabinose-inducible promoter. The intracellular content of c-di-GMP of the PAO1/p_{BAD}-*yhjH* strain can be adjusted by arabinose and can therefore be used to mimic all of the three phases dependent on the conditions.

The *cdrA* and *pel* genes are both positively regulated by c-di-GMP in *P. aeruginosa* (35), and the fusions p_{cdrA}-*gfp* and p_{pel}-*lacZ* can therefore be used to monitor intracellular c-di-GMP levels in *P. aeruginosa* (21, 26). We measured the expression of the c-di-GMP biosensor p_{cdrA}-*gfp* (21) in the PAO1 (PCells), PAO1Δ*wspF* strain (BCells), PAO1/p_{lac}-*yhjH* strain (DCells) and found that the intracellular level of c-di-GMP in the PAO1Δ*wspF* strain (BCells) was significantly higher than in the PAO1 strain (PCells) and the PAO1/p_{lac}-*yhjH* strain (DCells) (Fig. 1A). The *wspF* mutation was shown before to increase the intracellular content of c-di-GMP of *P. aeruginosa* up to 7-fold in planktonic growth (21). Expression of the p_{lac}-*yhjH* in PAO1Δ*wspF* strain was found to decrease the p_{cdrA}-*gfp* fluorescence intensity (Fig. 1). PAO1 cells from biofilms had a high level of *cdrA-gfp* fluorescence intensity close to that of the PAO1Δ*wspF* cells (see Fig. S1 in the supplemental material). However, the p_{cdrA}-*gfp* expression in PAO1 PCells and PAO1/p_{lac}-*yhjH* DCells was too low to indicate differences in the c-di-GMP level (Fig. 1). Nevertheless, the PAO1/p_{lac}-*yhjH* strain (DCells) was unable to form biofilms similar to the PAO1 (PCells) and PAO1Δ*wspF* strain during static cultivation (Fig. 1B), indicating that it had a low c-di-GMP level.

Due to the detection limit of the p_{cdrA}-*gfp* biosensor, we then used the p_{pel}-*lacZ* reporter gene (26) to compare the intracellular levels of c-di-GMP in SNP-dispersed biofilm cells (DCells*) and *yhjH*-dispersed biofilm cells (DCells). SNP reduces the intracellular c-di-GMP level in *P. aeruginosa* through activation of the DipA PDE (36), as an alternative to direct induction of the ectopically expressed YhjH PDE (27). The PAO1Δ*wspF* BCells showed a higher β-galactosidase activity than that of the PAO1 PCells (Fig. 2). SNP-dispersed PAO1 biofilm cells (DCells*) showed a level of β-galactosidase activity similar to that of PAO1/p_{BAD}-*yhjH* cells dispersed by the addition of 0.5% arabinose (DCells) (Fig. 2). The dispersed cells (DCells* and DCells) expressed lower levels of β-galactosidase activity than did the planktonic PAO1 cells (PCells) (Fig. 2). This finding suggests that freshly dispersed *P. aeruginosa* cells (DCells) from the biofilms had lower levels of c-di-GMP than the planktonic cells (PCells).

Proteomics analysis of *P. aeruginosa* cells with different intracellular c-di-GMP levels. Proteomics analysis of *P. aeruginosa* cells with different intracellular c-di-GMP levels was performed. Using a *P* value cutoff of 0.05, the abundances of 116 proteins were found to be significantly affected by low intracellular levels of c-di-GMP; the abundance of 44 proteins was upregulated, while the abundance of 72 proteins was downregulated (shown in Tables 2 and 3, respectively). As expected (3), extracellular matrix proteins were expressed more abundantly in PAO1Δ*wspF* strain (BCells) (Table 3), while motility and chemotaxis proteins were more abundant in PAO1/p_{lac}-*yhjH* (DCells) (Table 2).

High intracellular levels of c-di-GMP were correlated with the increased expression of proteins for synthesis of the major iron siderophore, pyoverdine (Table 3). The data were corroborated by pyoverdine fluorescence measurements showing that the production of pyoverdine in the *P. aeruginosa* PAO1, PAO1Δ*wspF*, and PAO1/p_{lac}-*yhjH* strains was in accordance with the proteomics analysis (Fig. 3).

Low intracellular levels of c-di-GMP were found to favor the expression of a set of virulence-associated proteins (Table 2). Surprisingly, we found that dispersal correlated with the expression of proteins that contributes to the antimicrobial peptide resistance of *P. aeruginosa*. Antimicrobial peptides (AMPs; e.g., defensins) are secreted by a wide-range of host cells as a response to microbial infections and act by disrupting the bacterial cell (37). Bacteria have evolved a set of inducible AMP-sensing systems (38, 39). In *P. aeruginosa*, the PhoP/PhoQ system and the PmrA/PmrB two-

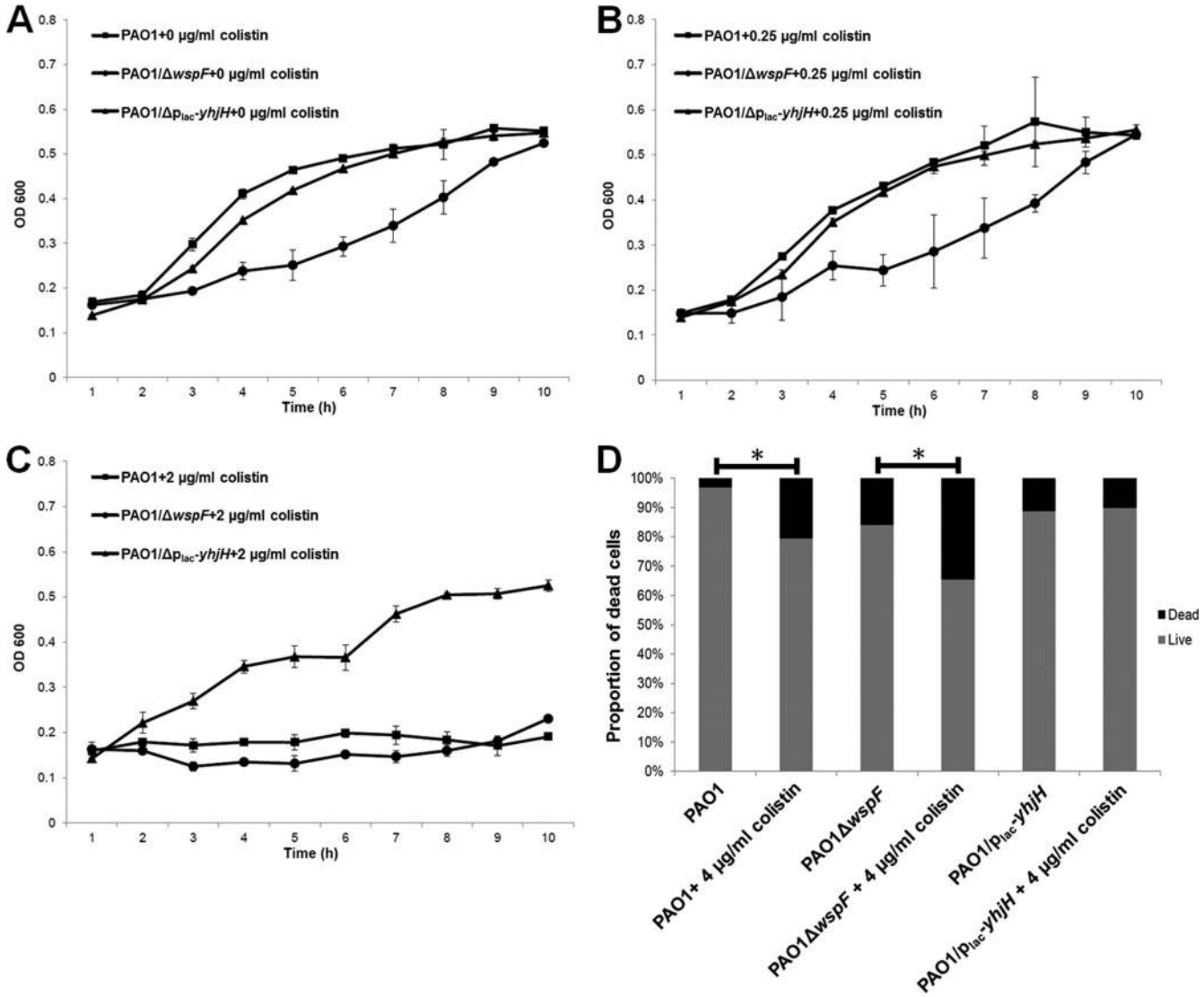

FIG 5 Colistin resistance assay. *P. aeruginosa* PAO1 (PCells) (A), PAO1Δ*wspF* (BCells) (B), and PAO1/p_{lac}-*yhjH* (DCells) (C) were cultivated at 37°C in ABTGC medium with 0, 0.25, or 2 μg of colistin ml^{-1}. The OD_{600} was monitored for 10 h. Means and SD from triplicate experiments are shown. (D) Fast-kill assay of *P. aeruginosa* PAO1 (PCells), PAO1Δ*wspF* (BCells), and PAO1/p_{lac}-*yhjH* (DCells) by 4 μg of colistin ml^{-1}. The proportion of dead bacterial cells was monitored by using the Live/Dead BacLight bacterial viability kits (Invitrogen) after 10 min of treatment. *, $P < 0.01$.

component systems can sense the presence of AMPs and upregulate genes involved in AMP resistance, including lipopolysaccharide modification (40). The *arn* operon (PA3552-PA3559) can also be induced by AMPs, and its expression is partially regulated by the PmrA/PmrB two-component system (40). PmrB and ArnB, typically induced by antimicrobial peptides (41, 42), were observed to be induced here by low intracellular levels of c-di-GMP (Table 2).

To examine whether the c-di-GMP effect found by proteomic analysis is on the level of transcription, we analyzed the expression of a p_{pmrA}-*gfp* transcriptional fusion (25) in PAO1 (PCells), PAO1Δ*wspF* (BCells), and PAO1/p_{lac}-*yhjH* (DCells). The p_{pmrA}-*gfp* fusion was expressed in all three strains in the presence of sublethal concentrations of colistin, but in the absence of any colistin, the fusion was only expressed in the PAO1/p_{lac}-*yhjH* (DCells) (Fig. 4 and see Fig. S2 in the supplemental material).

Antimicrobial peptide resistance of *P. aeruginosa* cells with different intracellular c-di-GMP levels. Growth monitored in the presence of different concentrations of colistin revealed that the PAO1/p_{lac}-*yhjH* DCells were more resistant to colistin than PAO1 PCells and PAO1Δ*wspF* BCells during planktonic growth (Fig. 5A, B, and C). Colistin is a fast-killing bactericidal agent, and we thus measured the killing kinetics of 4 μg of colistin ml^{-1} in PAO1 PCells and PAO1/p_{lac}-*yhjH* DCells. PAO1 PCells and PAO1Δ*wspF* BCells were killed rapidly by 4 μg of colistin ml^{-1}, whereas PAO1/p_{lac}-*yhjH* DCells were able to survive in the presence of 4 μg of colistin ml^{-1} (Fig. 5D and see Fig. S3 in the supplemental material).

To examine whether cells that dispersed from biofilms were also more resistant to colistin than planktonic cells, the dispersal cells from biofilms of PAO1 and PAO1/p_{BAD}-*yhjH* were tested for colistin resistance, and it was observed that PAO1/p_{BAD}-*yhjH* cells

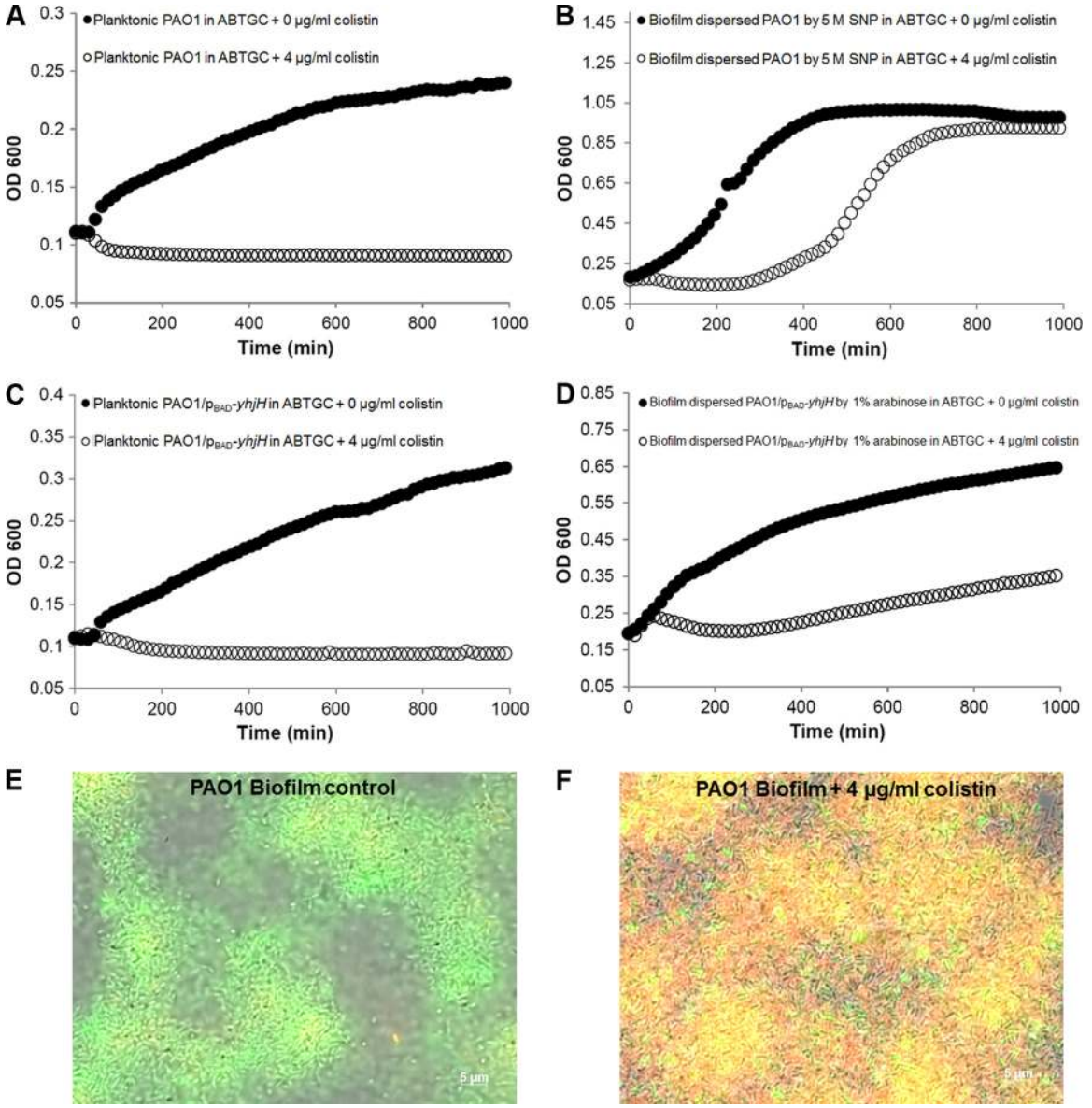

FIG 6 Colistin resistance of planktonic cells (PCells), biofilm cells (BCells), and dispersed cells (DCells). Planktonic cells (PCells) of PAO1 (A), biofilm-dispersed cells (BCells) from PAO1 biofilm by 5 μM SNP (B), planktonic cells of (PCells) PAO1/p_{BAD}-*yhjH* (C), and biofilm-dispersed cells (DCells) from PAO1/p_{BAD}-*yhjH* biofilms by 1% arabinose (D) were cultivated at 37°C in ABTGC medium with 0 or 4 μg of colistin/ml. The OD_{600} was monitored for 300 min. Means of three replicates are shown. (E and F) Biofilms formed by PAO1 strain on glass slides were submerged into ABTGC medium with 0 (E) and 4 (F) μg of colistin ml^{-1} for 2 h. Live and dead cells in treated biofilms were stained by using Live/Dead BacLight bacterial viability kits, followed by confocal laser scanning microscopy imaging.

dispersed from biofilms by the expression of *yhjH* were more resistant to colistin based on differences in growth rates (Fig. 6A and B). It was also observed that *P. aeruginosa* biofilms treated with dispersing agents (either arabinose or SNP) were more resistant to colistin than planktonic cells (Fig. 6C and D). In contrast, exposure of biofilms to colistin resulted in the killing of most biofilm cells and showed that a large fraction of the biofilm cells remained sensitive to colistin (Fig. 6E and F).

DISCUSSION

In this work, *P. aeruginosa* strains were constructed with a controllable intracellular c-di-GMP content to mimic the natural biofilm cells (BCells) and dispersed cells (DCells) from biofilms. Unlike the natural biofilm cells with a high level of physiological heterogeneity (43), our cells are cultivated as homogeneous planktonic cultures and are easy to manipulate. These *P. aeruginosa* strains thus enable us to study the overall impact of c-di-GMP on *P. aeruginosa* cells. Of course, the *P. aeruginosa* PAO1Δ*wspF* cells in planktonic cultures cannot functionally mimic the late stage biofilm cells since cells from mature biofilms have a slow growth rate, oxygen limitation, and a large amount of extracellular matrix material around them. Nevertheless, we showed here that the PAO1/p_{lac}-*yhjH* cells (DCells) have an intracellular c-di-GMP content similar to that of chemically dispersed

cells (DCells*), which have a distinct physiology compared to planktonic cells (PCells). In fact, the PAO1/p_{lac}-*yhjH* cells were unable to form normal amounts of biofilms compared to the PAO1 cells (Fig. 1B).

It was also observed that the intracellular c-di-GMP level plays an important role in production of pyoverdine by *P. aeruginosa*. Pyoverdine is the major siderophore of *P. aeruginosa* and is required for subpopulation interactions and biofilm maturation (44). Previous work showed that pyoverdine is mainly produced by the nonmotile subpopulation at the bottom part of mature *P. aeruginosa* biofilms (44). The present result suggests that the nonmotile subpopulation might have a higher intracellular level of c-di-GMP compared to the motile subpopulation at the top part of mature *P. aeruginosa* biofilms. Further studies will be carried out to study the detailed regulation mechanism exerted by c-di-GMP on pyoverdine production.

Induction of expression of *arnB* and PA4773 (from the *pmr* operon) with 2 μg of polymyxin B ml^{-1} but not with 0.125 μg of polymyxin B ml^{-1} was reported to increase the polymyxin B resistance of *P. aeruginosa* clinical isolates from cystic fibrosis patients (34). A number of unique mutations in the *pmrAB* and *phoPQ* operons enable these clinical isolates to show an adaptive growth in medium containing 2 μg of polymyxin B ml^{-1} after a long lag phase (34). Our study has shown for the first time that c-di-GMP signaling plays a role in AMP resistance in *P. aeruginosa*. Reduced c-di-GMP levels were found to induce the expression of PmrB and AnrB even without the presence of AMPs. PhoP was recently found to be able to bind c-di-GMP (45); thus, it might be an effector of c-di-GMP in regulation of AMP resistance. However, further studies are needed to elucidate the mechanistic basis of induction of the *pmr* and *arn* genes by low levels of c-di-GMP. The reported induced resistance thus confers a "protection in advance" mechanism to protect dispersed cells from the otherwise detrimental action of antibiotics on planktonic cells and may be the first finding to contradict the current dogma stating that dispersed cells are inevitably more susceptible than their sessile counterparts.

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