

# Pyocyanin Stimulates Quorum Sensing-Mediated Tolerance to Oxidative Stress and Increases Persister Cell Populations in *Acinetobacter baumannii*

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*Acinetobacter baumannii* and *Pseudomonas aeruginosa* are nosocomial pathogens with overlapping sites of infection. This work reports that the two can coexist stably in mixed-culture biofilms. In a study intended to improve our understanding of the mechanism of their coexistence, it was found that pyocyanin, produced by *P. aeruginosa* that generally eliminates competition from other pathogens, led to the generation of reactive oxygen species (ROS) in *A. baumannii* cells, which in response showed a significant ( $P \leq 0.05$ ) increase in production of enzymes, specifically, catalase and superoxide dismutase (SOD). This work shows for the first time that the expression of catalase and SOD is under the control of a quorum-sensing system in *A. baumannii*. In support of this observation, a quorum-sensing mutant of *A. baumannii* (*abaI::Km*) was found to be sensitive to pyocyanin compared to its wild type and showed significantly ( $P \leq 0.001$ ) lower levels of the antioxidant enzymes, which increased on addition of 5  $\mu\text{M}$  *N*-(3-hydroxydodecanoyl)-L-homoserine lactone. Likewise, in wild-type *A. baumannii*, there was a significant ( $P < 0.01$ ) decrease in the level of anti-oxidant enzymes in the presence of salicylic acid, a known quencher of quorum sensing. In the presence of amikacin and carbenicillin, *A. baumannii* formed 0.07 and 0.02% persister cells, which increased 4- and 3-fold, respectively, in the presence of pyocyanin. These findings show that pyocyanin induces a protective mechanism in *A. baumannii* against oxidative stress and also increases its persistence against antibiotics which could be of clinical significance in the case of coinfections with *A. baumannii* and *P. aeruginosa*.

*Acinetobacter baumannii* is an emerging opportunistic human pathogen that causes a plethora of nosocomial infections (1). The ability of this pathogen to sense and react to environmental and host stress signals allows it to persist in medical settings and in human hosts (2). *A. baumannii* is often associated with coinfection by other pathogens (3). Infection by *A. baumannii* and *Pseudomonas aeruginosa* in the upper respiratory tract and in lungs of patients with cystic fibrosis has been reported (4–6). *P. aeruginosa* competes with coinfecting organisms through the secretion of pyocyanin which is a redox-active phenazine compound, known to act through the generation of reactive oxygen species (ROS) (7). Pyocyanin was identified as the active component responsible for inhibition of *Burkholderia cepacia* in mixed-species biofilms with *P. aeruginosa* (8), while it did not have any such inhibitory effect on coexisting *Klebsiella pneumoniae* (9). *A. baumannii* has also been shown to coexist *in vitro* with *P. aeruginosa*, with evidence of interspecies interactions between them (10). Interspecies interactions may affect virulence and antibiotic sensitivities in the interacting pathogens (11).

ROS stress causes macromolecular damage, and bacteria respond to it by producing enzymatic scavengers, such as catalase and superoxide dismutase (SOD), the two conspicuous antioxidant enzymes widely distributed in nature (12). *P. aeruginosa* survives oxidative stress by raising cellular catalase and SOD enzyme activities, which have been demonstrated to be controlled by *lasI-lasR*- and *rhlI-rhlR*-mediated quorum-sensing systems (13). *A. baumannii* has only one quorum-sensing system, involving the transcriptional regulator ABA, which forms a complex with *N*-(3-hydroxydodecanoyl)-L-homoserine lactone synthesized by the autoinducer synthase gene (*abaI*) (14). However, no link between quorum sensing and oxidative stress in *A. baumannii* has been reported.

Reports on multiple drug tolerance in *A. baumannii* are emerging in response to antibiotics (1). Generally, pathogens produce persister cells as phenotypic variants of the wild type that are tolerant to antibiotics. Persister cells can also form in response to starvation or oxidative stress (15). Very little is known about the phenotypic variant subpopulation of persister cells in *A. baumannii*. In view of coinfection by *A. baumannii* and *P. aeruginosa* in respiratory tract infections, especially in cystic fibrosis patients, where pyocyanin has been detected in the sputum and pulmonary secretions (16), it is pertinent to study the mechanism(s) by which *A. baumannii* survives oxidative stress caused by pyocyanin exposure and also its effect with regard to persistence. This work reports that *A. baumannii* and *P. aeruginosa* can coexist in mixed-species biofilms, as the oxidative stress caused by *P. aeruginosa* pyocyanin induced protective antioxidative enzymes in *A. baumannii* and enhanced its tolerance to antibiotics.

## MATERIALS AND METHODS

**Organisms and culture conditions.** Strains used in this study are listed in Table 1. For pyocyanin production, *P. aeruginosa* PAO1 was grown in peptone water (2% [wt/vol] peptone, pH 7.0), as it resulted in higher

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TABLE 1 *A. baumannii* and *P. aeruginosa* strains

Strain	Characteristic(s)	Reference or source
<i>A. baumannii</i>		
M2	Clinical isolate	14
M2 ( <i>abaI::Km</i> )	Autoinducer synthase ( <i>abaI</i> ) mutant of M2 strain; Kan <sup>r</sup>	14
M2, GFP tagged	pMU125 encoding GFP expressed under tet promoter; Amp <sup>r</sup>	This study
ATCC 17978	Standard strain	American Type Culture Collection
ATCC 19606	Standard strain	American Type Culture Collection
C1 to C4	Clinical isolates from endoscopic tracheal secretions	Maintained in lab
<i>P. aeruginosa</i>		
PAO1	Wild type	17
PAO1, YFP tagged	Chromosomally encoded YFP	18

production of pyocyanin. All other strains were grown in Luria-Bertani broth at 37°C under shaking conditions.

**Biofilm formation.** For mixed-culture biofilms, *A. baumannii* C4, a clinical isolate from tracheal secretion, was grown with *P. aeruginosa* PAO1, as C4 is resistant to amikacin and could be differentially separated from sensitive *P. aeruginosa* on LB agar plates containing amikacin. Pure-culture biofilms were formed by inoculating LB containing glycerol (1% vol/vol) with *A. baumannii* C4 or *P. aeruginosa* PAO1 (10<sup>8</sup> CFU/ml) in polystyrene tubes at 37°C under batch conditions for a period of 48 h. A binary-culture biofilm was formed by cocultivation of *A. baumannii* C4 and *P. aeruginosa* PAO1 (10<sup>8</sup> CFU/ml each) under similar conditions. The loosely adherent planktonic cells in the biofilm were removed by three washings with 10 mM phosphate-buffered saline (PBS, pH 7.0) while the tightly adherent cells were harvested by three consecutive cycles of alternate vortexing (15 s) and sonication (35% amplitude, 4 cycles of 30 s on and 30 s off) with the sonicator probe outside the tube in the water bath (19). Viable-cell counting of recovered cells was done to determine the growth of individual species in coculture using LB agar plates containing amikacin (100 µg/ml) for *A. baumannii* C4 and *Pseudomonas* agar with 0.03% (wt/vol) cetrimide for *P. aeruginosa* PAO1.

**Microscopy of biofilms.** Electrocompetent *A. baumannii* M2 cells (20) were electroporated with pMU125 (21), an *Escherichia coli*-*Acinetobacter* shuttle vector expressing green fluorescent protein (GFP), using a Bio-Rad Gene Pulser (1.8 kV, 200 W, 25 µF) in a sterile prechilled electroporation cuvette (1 mm), and the transformants were selected on LB agar containing ampicillin (100 µg/ml). GFP was induced by tetracycline (100 ng/ml), and tagging of *A. baumannii* M2 was confirmed by fluores-

cence microscopy (excitation, 488 nm; emission, 530 nm) of the transformed culture.

The distribution of GFP-tagged *A. baumannii* M2 cocultured with yellow fluorescent protein (YFP)-tagged *P. aeruginosa* PAO1 in the mixed biofilm was studied by confocal laser scanning microscopy (CLSM) (Nikon). A one-day-old static biofilm was developed on the surface of a glass coverslip by immersing it in LB containing *A. baumannii* M2 and *P. aeruginosa* PAO1 (10<sup>8</sup> CFU/ml each) at 37°C. The coverslip was washed three times with 10 mM PBS (pH 7.0) to remove the weakly adherent cells. GFP fluorescence was recorded at 517 nm after excitation at 488 nm, while YFP fluorescence was captured at 527 nm after excitation at 514 nm. The spatial arrangement of both GFP-tagged *A. baumannii* and YFP-tagged *P. aeruginosa* under coculture conditions was observed at a magnification of ×40. Images from three independent biofilms were analyzed using NIS Elements AR 4.12.01 software, and the coefficient of variation (CoV) was calculated as the standard deviation divided by the mean fluorescence intensity (22).

**Extraction of pyocyanin from *P. aeruginosa* PAO1.** *P. aeruginosa* PAO1 was inoculated in peptone water (2% [wt/vol], pH 7.0) and incubated at 37°C and 180 rpm for 24 h. The soluble pyocyanin pigment was extracted with chloroform, followed by re-extraction in 0.5 N HCl, and quantitated by measuring the absorbance at 520 nm (23). The aqueous fraction was lyophilized and dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 500 mM. The presence of pyocyanin in the extract was confirmed by liquid chromatography-mass spectrometry (LC-MS) (*m/z* as 210).

**Effect of pyocyanin on *A. baumannii* growth.** *A. baumannii* was grown in the presence (250 µM) and absence of pyocyanin at 37°C for 24 h at 180 rpm. Viable cells which escaped pyocyanin stress converted triphenyl tetrazolium chloride (TTC) dye (0.005% [wt/vol]) to red formazan, which was quantitated at an optical density at 560 nm (OD<sub>560</sub>) (24). A culture grown without pyocyanin was treated as the control, and the percent survival was calculated as the OD<sub>560</sub> of pyocyanin-treated cells divided by the OD<sub>560</sub> of untreated cells.

**Detection of pyocyanin-stimulated reactive oxygen species (ROS) in *A. baumannii*.** *A. baumannii* M2 cells were grown in the presence of pyocyanin (250 µM) as described earlier. Pyocyanin was removed by centrifugation at 8,000 × *g* for 10 min at 4°C. The cell pellet was suspended in PBS (pH 7.2) containing a 5 µM concentration of 5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCFDA; Invitrogen), and incubated at 37°C for 30 min in the dark (25). Stained cells were then pelleted and resuspended in PBS to obtain a dilute cell suspension (10<sup>5</sup> CFU/ml), a wet mount of which was imaged on an Olympus fluorescence microscope (excitation/emission, 495 nm/510 nm). Quantitation of fluorescence of the stained bacterial suspension was done on a plate reader fluorimeter (Spectramax) by taking the ratio of the

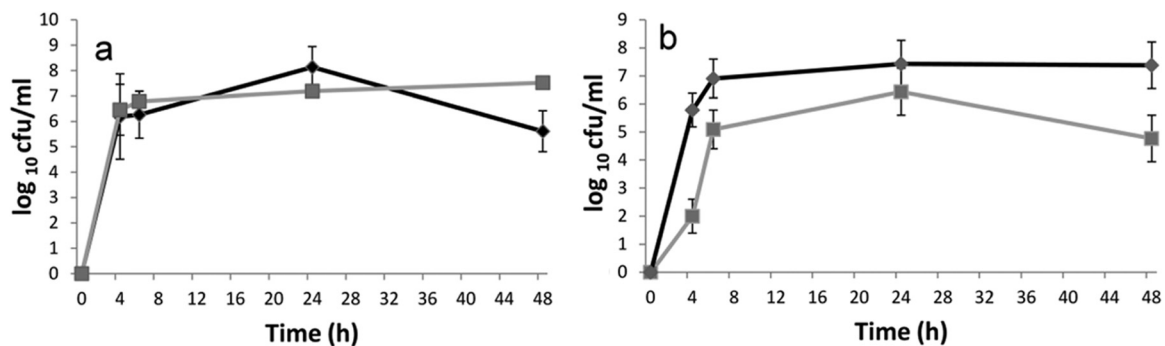


FIG 1 Viable-cell counts of *A. baumannii* C4 (■) and *P. aeruginosa* PAO1 (◆) from (a) pure cultures and (b) mixed-culture biofilms. *A. baumannii* C4 and *P. aeruginosa* PAO1 were cocultivated in LB to form batch biofilms at 37°C. The cells were harvested from the biofilm in a water bath sonicator at the indicated times. Viable-cell counts were obtained by spread plating appropriate dilutions on LB plates containing amikacin (100 µg/ml) and *Pseudomonas* agar plates (containing 0.03% cetrimide [wt/vol]), respectively. Data are means from three experiments.

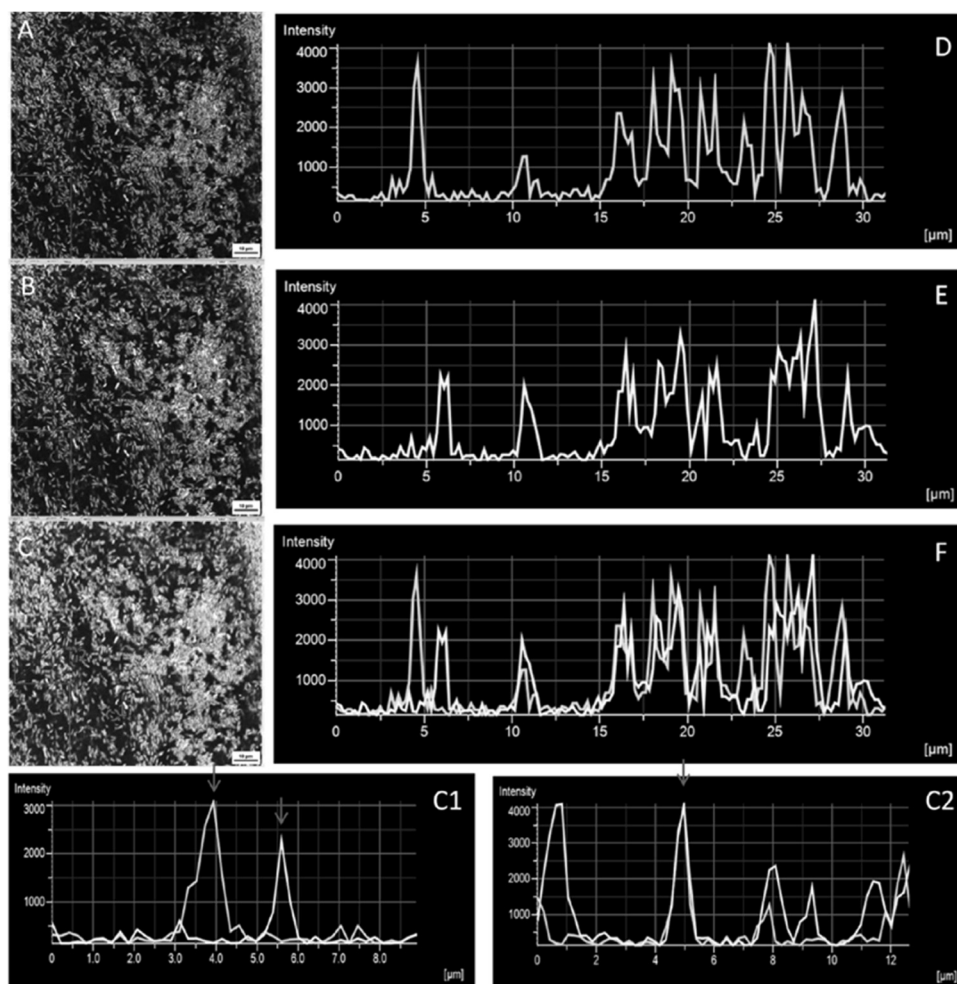


FIG 2 (A to C) CLSM images of biofilms (cultured under batch conditions), formed on glass coverslips by (A) a GFP-tagged culture of *A. baumannii* M2, (B) a YFP-tagged culture of *P. aeruginosa* PAO1, and (C) cocultured *A. baumannii* M2 and *P. aeruginosa* PAO1. (D to F) Intensity graphs of fluorescence quantitated along the line indicated by arrows in the boxes in panels A, B, and C using NIS Elements viewer software. Highlighted area 1 in image C shows the two species in close proximity to each other (corresponding to the intensity graph in panel C1), and highlighted area 2 shows overlapping *A. baumannii* M2 and *P. aeruginosa* PAO1 cells (intensity graph in panel C2). Red arrows in panels C1 and C2 indicate intensity peaks. Magnification,  $\times 63$ .

OD<sub>510</sub> to the OD<sub>600</sub>. *A. baumannii* M2 cells without exposure to pyocyanin were stained in a similar way to be used as controls.

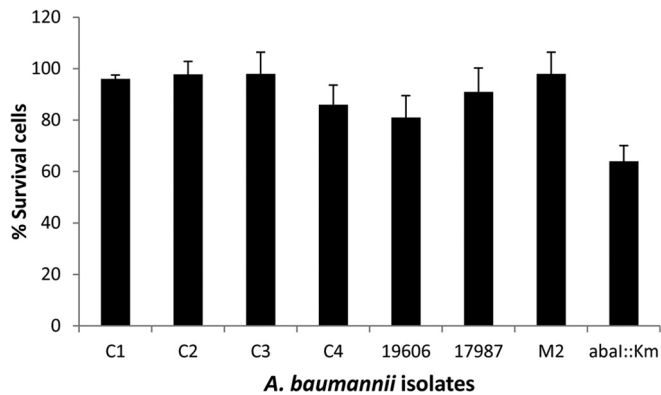
**Effect of pyocyanin on catalase and superoxide dismutase activities of *A. baumannii*.** (i) **Enzyme preparation.** Ten milliliters of LB was inoculated with 1% (vol/vol) of overnight grown culture of *A. baumannii* and grown in the presence (250  $\mu$ M) and absence of pyocyanin at 37°C and 180 rpm for 16 h. The culture was centrifuged at 10,000  $\times g$  for 15 min at 4°C. The cell pellet was washed twice and resuspended in 2 ml of 50 mM phosphate buffer (pH 7.0) before disruption by sonication (five cycles of a pulse, 30 s on and 30 s off, 21% amplitude). The cell lysate was cleared of cell debris by centrifugation at 10,000  $\times g$  and 4°C for 15 min. The protein was quantitated at 280 nm with a Nanodrop spectrophotometer and used as crude enzyme (26).

(ii) **Catalase assay.** H<sub>2</sub>O<sub>2</sub> (500  $\mu$ l; 5 mM) and phosphate buffer (400  $\mu$ l; 50 mM, pH 7.0) were mixed with 100  $\mu$ l of crude enzyme. The mixture was scanned at 240 nm every 10 s for 5 min. The enzyme solution containing H<sub>2</sub>O<sub>2</sub>-free phosphate buffer served as control. One unit of catalase activity was the amount of enzyme that decomposed 1 mM H<sub>2</sub>O<sub>2</sub> per min at room temperature. The catalase activity was expressed as units per mg of protein (13).

(iii) **Superoxide dismutase (SOD) assay.** The assay mixture contained 140  $\mu$ l of 25 mM phosphate buffer (pH 8.3), 10  $\mu$ l of 186  $\mu$ M PMS

(phenazine methosulfate), 30  $\mu$ l of 300  $\mu$ M NBT (nitroblue tetrazolium), and 100  $\mu$ l of the enzyme preparation in a total volume of 280  $\mu$ l. The reaction was initiated by the addition of 20  $\mu$ l of 780  $\mu$ M NADH, the mixture was incubated at 30°C for 90 s, and the reaction was stalled by the addition of 100  $\mu$ l of glacial acetic acid. The reaction mixture was shaken with 400  $\mu$ l of butanol, allowed to stand for 10 min, and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm. One unit of enzyme activity was the amount of enzyme that resulted in 50% inhibition of NBT reduction (27). The SOD activity was expressed as units per mg of protein.

**Quorum sensing and oxidative stress in *A. baumannii*.** A 10-ml culture of *A. baumannii* M2 and its quorum-sensing mutant M2 (*abaI::Km*) were grown in the presence (0.5 and 5  $\mu$ M) and absence of synthetic 3-OH-C<sub>12</sub>-HSL (Cayman Chemicals; 10 mM stock in dimethyl formamide), the quorum-sensing signal molecule of *A. baumannii*, at 37°C and 180 rpm for 16 h. Both the cultures were also grown in the absence and presence of 20 and 40  $\mu$ M salicylic acid (50 mM stock in DMSO), a known quencher of quorum sensing, at 37°C and 180 rpm for 16 h. Enzyme assays for catalase and SOD were performed as described earlier. Overnight-grown cultures of *A. baumannii* M2 and M2 (*abaI::Km*) were inoculated (10% [vol/vol]) into 10 ml fresh LB broth. A 200- $\mu$ l portion of each freshly inoculated culture was exposed to 100 mM H<sub>2</sub>O<sub>2</sub> and incubated at



**FIG 3** Survival profile of *A. baumannii* isolates (C1 to C4, M2 [clinical isolates], ATCC 19606, ATCC 17978, and the *abal::Km* quorum-sensing mutant of M2) in the presence of pyocyanin (250  $\mu$ M). Data are means from three experiments. The level corresponding to 100% survival in the absence of pyocyanin was  $2$  to  $6 \times 10^{12}$  CFU/ml in different isolates.

37°C and 180 rpm for 30 min. The reaction was stopped with 5% (wt/vol) sodium thiosulfate. Viable cells which escaped  $H_2O_2$  stress were quantitated at  $OD_{560}$  with TTC dye (0.005% [wt/vol]) (24). The surviving cells were also enumerated as CFU by spreading appropriate dilutions of  $H_2O_2$ -treated culture on LB agar. Percent survival was calculated as the CFU in the  $H_2O_2$ -treated culture divided by the CFU in the untreated culture.

**Determination of persister cell populations.** The MICs of carbenicillin and amikacin against *A. baumannii* M2 were determined (28). The persister cell population in *A. baumannii* M2 was determined in a time-dependent manner by growing cells to log phase followed by antibiotic exposure (MIC) at 37°C. Samples were withdrawn at various intervals (0, 3, 5, and 24 h), washed once to remove antibiotic completely, serially diluted, and spread on LB plates for a persister cell count (29). To determine dose-dependent persister cell formation in *A. baumannii*, the log-phase culture was exposed to high concentrations of antibiotic ( $3 \times$  MIC and  $5 \times$  MIC) for 3 h followed by a wash with LB to remove antibiotic. The culture was then serially diluted and plated for enumeration of persister cells (30).

The effect of pyocyanin on persister cell formation by *A. baumannii* was determined by growing the cells in the presence (250  $\mu$ M) of pyocyanin followed by challenge with antibiotic under conditions as described above. Following the challenge, the cells were washed, and an appropriate dilution was spread on LB agar for determination of surviving persister cells.

The nonheritable nature of the persister cells was determined by inoculating the persister cells surviving the antibiotic into LB without antibiotic and allowing it to grow for 24 h at 37°C. These cells were further reinoculated into LB with antibiotic (amikacin or carbenicillin at MIC).

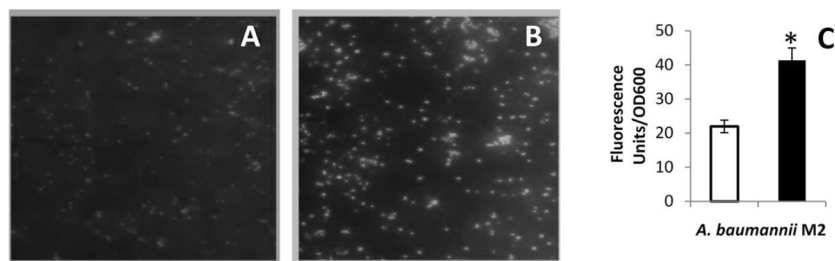
This cycle was repeated three times, and samples from each set were withdrawn from antibiotic medium at various times (0, 3, 5 and 24 h), washed once to remove antibiotic, and then plated on LB agar to determine persister cells.

**Statistical analysis.** Data were expressed as means  $\pm$  standard deviations (SD), and all experiments were repeated at least three times in triplicate. Student's *t* test was used to determine the significance. A *P* value of  $\leq 0.05$  was considered significant.

## RESULTS

**Biofilm formation by *A. baumannii* and *P. aeruginosa*.** Both *A. baumannii* C4 and *P. aeruginosa* PAO1 adhered well to polystyrene tubes used for the formation of biofilm under static conditions. After 4 h of incubation,  $6.2 \pm 1.6$  and  $6.5 \pm 1.0$  log CFU/ml of *A. baumannii* C4 and *P. aeruginosa* PAO1, respectively, were recovered from their pure-culture biofilms (Fig. 1a), which increased to  $8.1 \pm 0.81$  and  $7.2 \pm 0.23$  log CFU/ml, respectively, after 24 h. *A. baumannii* C4 counts decreased to  $5.6 \pm 0.81$  log CFU/ml, compared to  $7.5 \pm 0.34$  log CFU/ml for *P. aeruginosa* PAO1 after 48 h. When the coculture of biofilms composed of *A. baumannii* C4 and *P. aeruginosa* PAO1 was assessed, it was found that both showed growth patterns similar to those of the pure-culture biofilms (Fig. 1b). After 4 h of incubation,  $2 \pm 0.6$  and  $5.7 \pm 1.4$  log CFU/ml of *A. baumannii* C4 and *P. aeruginosa* PAO1 were recovered from mixed-culture biofilm, and the counts steadily increased to  $4.7 \pm 0.8$  log CFU/ml and  $7.5 \pm 0.3$  log CFU/ml, respectively, after 24 h, clearly indicating that the two organisms coexisted in the mixed-species biofilm. From 48-h-old cocultured biofilm,  $4.8 \pm 0.8$  and  $7.3 \pm 0.3$  log CFU/ml *A. baumannii* C4 and *P. aeruginosa* PAO1, respectively, were recovered. The decrease in *A. baumannii* C4 counts in mixed biofilm was similar to the trend seen in its pure-culture biofilm.

To study the spatial distribution of *A. baumannii* M2 and *P. aeruginosa* PAO1 in mixed-culture biofilm, GFP- and YFP-tagged cultures were used. Confocal microscopy confirmed that both *A. baumannii* M2 and *P. aeruginosa* PAO1 were able to coexist in mixed-species biofilms and formed mat-like structures that covered extensive surface areas of the glass coverslip (Fig. 2A, B, and C). *P. aeruginosa* PAO1 appeared to colonize the substratum before *A. baumannii*, as only yellow signal could be captured at 4 h of incubation (data not shown). Both green and yellow fluorescence signals of *A. baumannii* M2 and *P. aeruginosa* PAO1, respectively, were captured from the 6-h-old mixed biofilm specimen, indicating the coexistence of the two species. Intensity graphs of fluorescence generated from a 1-day-old mixed-species biofilm showed close proximity of the two signals, indicating cell-to-cell contact and coaggregation (Fig. 2C1). At certain places in the micrograph,



**FIG 4** (A and B) Representative fluorescence microscope images of CM- $H_2$ DCFDA-stained *A. baumannii* M2 grown in the absence (A) and presence of pyocyanin (B). Magnification,  $\times 40$ . (C) Quantitation of fluorescence in *A. baumannii* M2 grown in the absence (□) and presence (■) of pyocyanin. Data are means from three experiments. \*,  $P \leq 0.001$ .

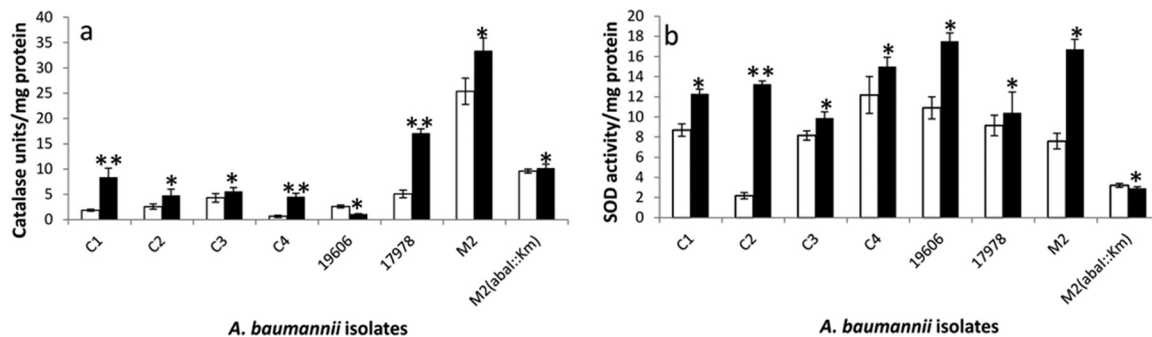


FIG 5 Catalase (a) and SOD (b) activities in *A. baumannii* isolates (C1 to C4, M2 [clinical isolates], ATCC 19606, ATCC 17978, and the *abaI::Km* quorum-sensing mutant of M2) grown in the absence (□) and presence (■) of pyocyanin. Data are means from three experiments. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ .

a complete overlap was also observed (Fig. 2C2). Quantification of the variable distribution of *A. baumannii* M2 and *P. aeruginosa* PAO1 in 1-day-old biofilm was done by averaging the signal intensity of green and yellow fluorescence captured from three randomly selected areas of three different biofilms. The coefficients of variation of the averaged intensities of green and yellow fluorescence in the selected area were 0.22 and 0.32, respectively, indicating the coexistence of *A. baumannii* M2 and *P. aeruginosa* PAO1.

**Response of *A. baumannii* to pyocyanin.** *P. aeruginosa* produces pyocyanin, a phenazine with redox activity, as one of the virulence factors which have been detected in lungs and sputum of cystic fibrosis patients. It has biocidal activity against coinfecting organisms. Since *A. baumannii* M2 and *P. aeruginosa* PAO1 were found to coexist, the effect of pyocyanin on the growth of *A. baumannii* M2 was checked, and the MIC was found to be  $>18$  mM (data not shown). *A. baumannii* isolates grown in the presence of exogenous pyocyanin (250  $\mu$ M) showed growth inhibition ranging from 0.8 to 15% only, indicating that *A. baumannii* isolates could survive pyocyanin-related stress (Fig. 3).

*A. baumannii* M2 cells grown in the presence of pyocyanin showed the generation of ROS (Fig. 4A and B), which led to 1.8-fold-higher ( $P < 0.001$ ) fluorescence in pyocyanin-treated cells compared to the untreated cells stained with CM-H<sub>2</sub>DCFDA dye (Fig. 4C).

In order to study the protective mechanism(s) against ROS in *A. baumannii* generated by pyocyanin, the levels of antioxidant enzymes were evaluated. Significant increases in catalase (1.3- to

6.9-fold) and SOD (1.2- to 6.1-fold) activities in the clinical isolates of *A. baumannii* were observed (Fig. 5a and b). In *A. baumannii* ATCC 17978, catalase and SOD activities increased 3.3 ( $P \leq 0.01$ )- and 1.1-fold, respectively, whereas *A. baumannii* ATCC 19606 showed 2.3-fold ( $P \leq 0.05$ ) less catalase activity 1.6-fold ( $P \leq 0.05$ ) more SOD activity (Fig. 5b), which alone may not have been sufficient to protect it against pyocyanin stress. This was reflected as maximum (15%) growth inhibition of this strain in the presence of pyocyanin (Fig. 3).

**Quorum sensing and oxidative stress management in *A. baumannii*.** *A. baumannii* M2 (*abaI::Km*), the quorum-sensing mutant, showed 30% growth inhibition, compared to only a 4% reduction in growth of the wild type, in response to pyocyanin (Fig. 3). Exposure of *A. baumannii* M2 to H<sub>2</sub>O<sub>2</sub> (100 mM) also resulted in  $>2$ -fold-higher ( $P < 0.001$ ) survival in the wild type than the quorum-sensing mutant of M2, as determined by TTC dye reduction assay. This was also confirmed from viable-cell counts of M2 ( $6.6 \pm 0.6$  log CFU/ml) and M2 (*abaI::Km*) ( $6.3 \pm 0.6$  log CFU/ml) after exposure to H<sub>2</sub>O<sub>2</sub>, which showed 55 and 35% survival compared to the unexposed cells ( $7 \pm 0.3$  log CFU/ml was taken as 100% survival).

The catalase and SOD activities in the mutant were 3-fold ( $P \leq 0.001$ ) and 2.6-fold ( $P \leq 0.001$ ) lower than those in its wild-type strain, which could be the reason for its increased sensitivity to pyocyanin compared to the wild type (Fig. 6a and b). Catalase and SOD activities of M2 (*abaI::Km*) increased 2-fold ( $P \leq 0.001$ ) and 1.7-fold ( $P \leq 0.001$ ) in response to pyocyanin in the presence of

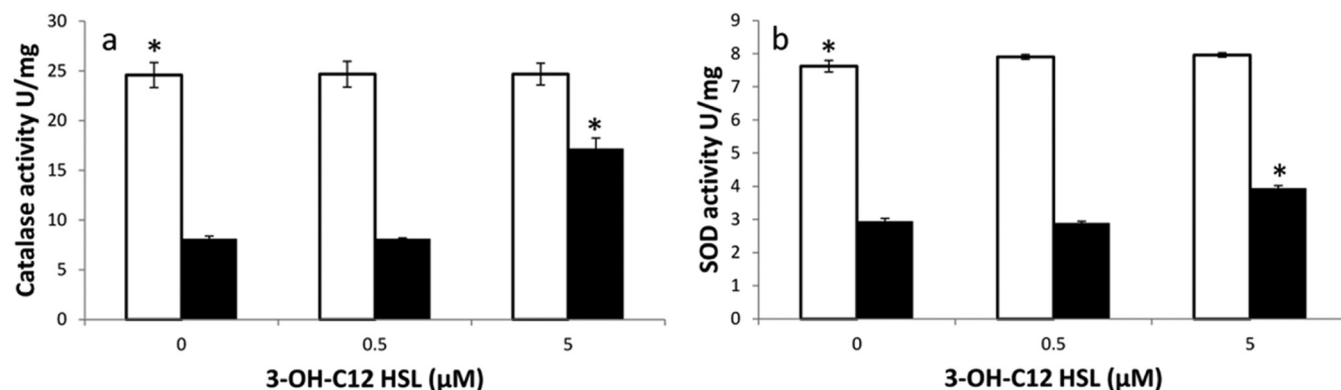


FIG 6 Catalase (a) and SOD (b) activities in *A. baumannii* M2 (□) and its autoinducer synthase mutant *A. baumannii* M2 (*abaI::Km*) (■) grown in the presence of 3-OH-C<sub>12</sub>-HSL. Data are means from three experiments. \*,  $P \leq 0.001$ .

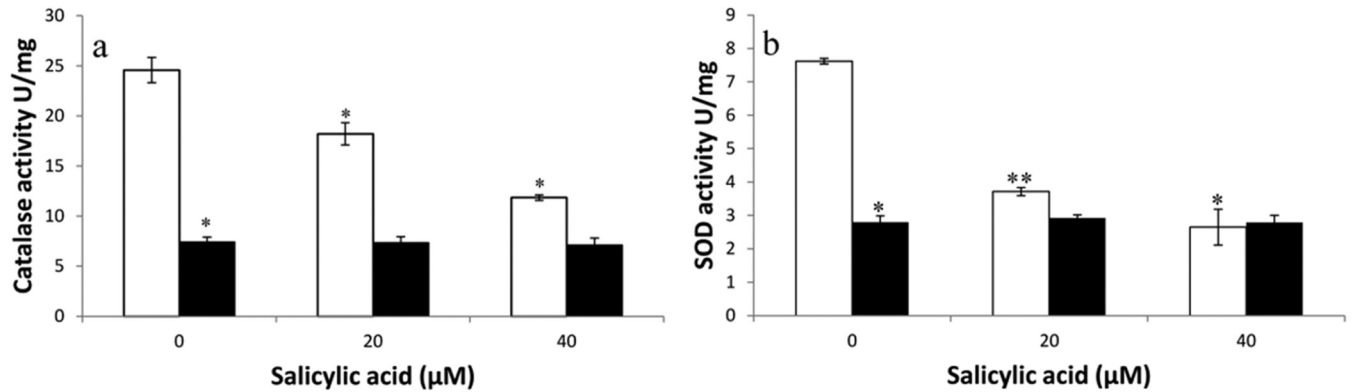


FIG 7 Catalase (a) and SOD (b) activities in *A. baumannii* M2 (□) and its autoinducer synthase mutant *A. baumannii* M2 (*abaI::Km*) (■) grown in the presence of salicylic acid. Data are means from three experiments. \*,  $P \leq 0.01$ ; \*\*,  $P \leq 0.001$ .

exogenously added 5  $\mu\text{M}$  3-OH- $\text{C}_{12}$  homoserine lactone (HSL), the quorum-sensing signal of *A. baumannii* (Fig. 6a and b), although a lower concentration (0.5  $\mu\text{M}$ ) was not effective. The wild-type strain did not show any appreciable change in the catalase and SOD activities on addition of 3-OH- $\text{C}_{12}$ -HSL, as it has a quorum sensing system that produces 3-OH- $\text{C}_{12}$ -HSL. Likewise, salicylic acid, a known quencher of quorum sensing, showed a significant ( $P < 0.01$ ) decrease in catalase and SOD activities of *A. baumannii* M2 in a concentration-dependent manner (20 and 40  $\mu\text{M}$ ), while there was no effect on the quorum-sensing mutant strain, indicating that the expression of catalase and SOD activities is under the control of the quorum-sensing system in *A. baumannii* (Fig. 7a and b).

**Exogenous pyocyanin increases the persister cell population of *A. baumannii*.** Persister cells are a bacterial subpopulation that is tolerant to killing by antibiotics, while the majority of the sensitive cells are killed. Exposure of a logarithmic-phase culture of *A. baumannii* M2 to the MICs of amikacin (4  $\mu\text{g}/\text{ml}$ ) and carbenicillin (50  $\mu\text{g}/\text{ml}$ ) in a time-dependent assay showed biphasic killing. A rapid decline in the antibiotic-sensitive population was followed by a plateau formed by antibiotic-tolerant persister cells. Amikacin and carbenicillin exposure led to the generation of 0.07 and 0.02% persister cells, which significantly ( $P \leq 0.05$ ) increased 4- and 3-fold, respectively, in the presence of pyocyanin (Fig. 8a and b). A similar biphasic killing pattern was observed

when *A. baumannii* M2 was exposed to amikacin and carbenicillin at concentrations well above their MICs (3 $\times$  and 5 $\times$ ), and once again, pyocyanin increased the number of persister cells significantly (data not shown). Since multidrug tolerance is an epigenetic, nonheritable change, the persister cells formed in response to amikacin and carbenicillin were shown to retain their sensitivity to these antibiotics when regrown in LB. This was confirmed by performing the assay for three consecutive cycles (Fig. 9a and b).

## DISCUSSION

Mixed-species biofilms are ubiquitous and are not uncommon in hospital environments. *P. aeruginosa* is a major cause of chronic lung infection in cystic fibrosis patients and may form mixed-species biofilms with coinfecting pathogens. *A. baumannii* is an emerging human pathogen having overlapping sites of infection with *P. aeruginosa* and has been shown previously in our lab to stably coexist with it under planktonic growth conditions (10). The present study shows a stable *in vitro* association of the two in a static mixed-species biofilm using fluorescent-dye-tagged *A. baumannii* and *P. aeruginosa* cultures.

*P. aeruginosa* secretes pyocyanin, a redox-active compound which generates ROS. Pyocyanin in its reduced form is highly unstable and reacts with molecular  $\text{O}_2$  to form superoxide ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), or hydroxyl radical ( $\text{OH}^-$ ), all of

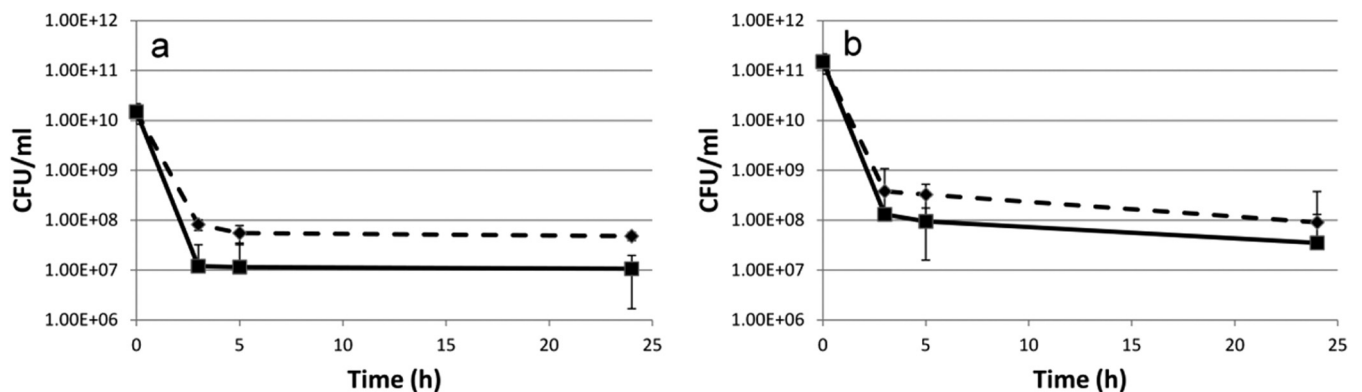


FIG 8 Effect of pyocyanin on the *A. baumannii* M2 persister cell population. The culture was grown to mid-log phase at 37°C with aeration and challenged with (a) amikacin and (b) carbenicillin at MIC in the presence (◆) and absence (■) of pyocyanin.

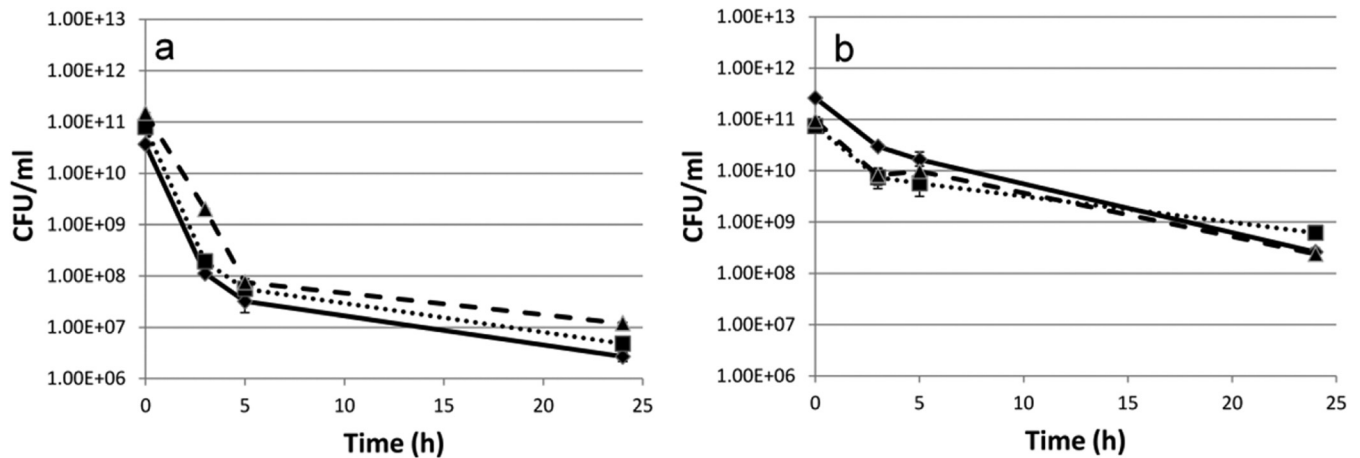


FIG 9 Nonheritability of the *A. baumannii* M2 persister phenotype on exposure to (a) 4 µg/ml of amikacin or (b) 50 µg/ml of carbenicillin. An overnight culture of *A. baumannii* M2 was diluted 1:20 with LB and exposed to antibiotics for 24 h. The surviving cells were then regrown in fresh antibiotic-free LB until stationary phase and exposed to antibiotic again for 24 h. The procedure was repeated three times. ◆, passage 1; ■, passage 2; ▲, passage 3.

which affect the growth and viability of a number of organisms (31). This benefits *P. aeruginosa* by reducing competition from coinfecting pathogens during infection. However, pyocyanin showed no growth inhibition of *A. baumannii* (MIC > 18 mM), as it counteracted the pyocyanin-mediated oxidative stress by enhanced production of catalase and SOD. *A. baumannii* has been reported to combat H<sub>2</sub>O<sub>2</sub>-generated oxidative stress by increasing its catalase activity (32).

Pyocyanin exerts oxidative stress on *P. aeruginosa* cells also, but it survives because of limited redox cycling of pyocyanin and increased levels of catalase and SOD (7). The production of antioxidant enzymes is controlled by the quorum-sensing system in *P. aeruginosa* (13), but no such relationship has been reported for *A. baumannii*. In the present study, we report for the first time that catalase and SOD are under the control of a quorum-sensing system in *A. baumannii*. A quorum-sensing mutant of *A. baumannii*

M2, M2 (*abaI::Km*), lacking the autoinducer synthase gene, had significantly low levels of catalase and SOD enzymes even on exposure to pyocyanin, compared to its wild type. Supplementation of the mutant with 3-OH-C<sub>12</sub>-HSL, the quorum-sensing signal molecule in *A. baumannii*, increased catalase and SOD enzyme activities significantly. Likewise, interfering with quorum sensing in *A. baumannii* M2 by salicylic acid, a known quencher of quorum sensing (33), reduced the catalase and SOD levels. Greater resistance of *A. baumannii* M2 to H<sub>2</sub>O<sub>2</sub> than its quorum-sensing mutant further supports the idea that quorum sensing is involved in tolerance of oxidative stress. Hence, a combination of a quorum-sensing quencher and ROS-generating agents like H<sub>2</sub>O<sub>2</sub> would be more effective in disinfection of fomites to control *A. baumannii*, which can persist in the hospital environment for a long time.

Bacterial tolerance to antibiotics has been linked with the

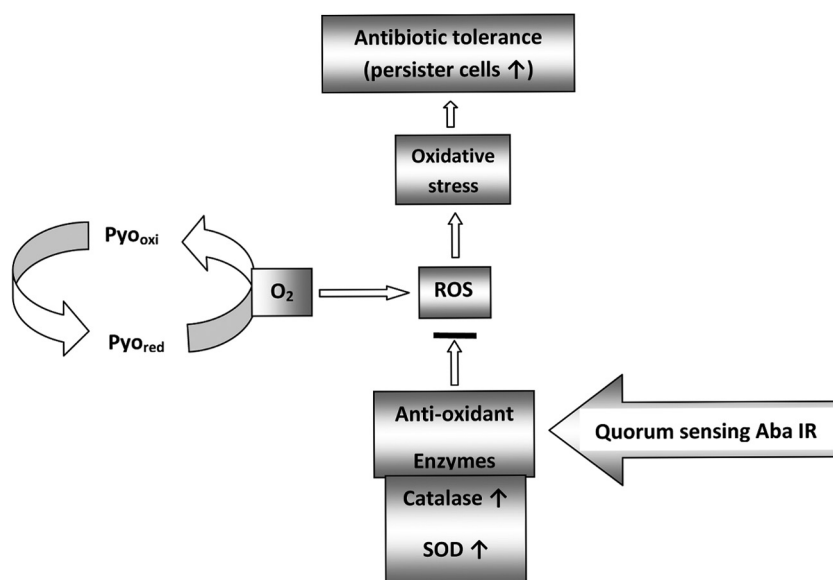


FIG 10 Proposed mechanism of *A. baumannii* survival of pyocyanin-induced oxidative stress.

emergence of persister cells, the phenotypic variants that are multidrug tolerant and are responsible for chronicity of infections. Persistence against antibiotics is genus or even strain specific, as *P. aeruginosa* has been reported to produce significantly fewer persisters than *E. coli* and *Staphylococcus aureus* under standard laboratory conditions (30), and persister cell formation in *A. baumannii* showed a highly heterogeneous pattern, ranging from 0.0007 to 10.1% (polymyxin B) and 0.0003 to 11.84% (tobramycin) in different clinical strains of *A. baumannii* (1). The present study shows persister cell formation in *A. baumannii* in response to carbenicillin (an extended-spectrum  $\beta$ -lactam) and amikacin (an aminoglycoside). These studies indicate that the persistence phenomenon is widely distributed among clinical *A. baumannii* strains, probably due to the antibiotic stress prevalent in hospital settings that may also be responsible for the selection of genetic determinants at different intensities in different strains. However, the genes involved in the persistence phenotype in *A. baumannii* are yet to be decoded. Since carbenicillin kills dividing cells only and amikacin kills both dividing and nondividing cells, it seems that for every antibiotic, specific sets of genes are induced for persistence. Many antibiotics have been shown to induce respiratory stress by generating ROS for killing the cells. Increased levels of catalase and SOD in *A. baumannii* in response, as shown in this work for pyocyanin-induced stress as well, may protect the cells which have developed persistence as a mechanism to survive (Fig. 10). Pyocyanin significantly ( $P \leq 0.05$ ) enhanced the persister cell formation induced by amikacin and carbenicillin. A 90-fold increase in the fraction of persister cells in *P. aeruginosa* in response to pyocyanin or 3-oxo-C<sub>12</sub> HSL (its quorum-sensing signal molecule) has been reported (30). These observations have clinical relevance, as pyocyanin has been detected in the sputum and pulmonary secretions of cystic fibrosis patients with *P. aeruginosa* infections (16). These studies warrant further investigations, as coinfection with *A. baumannii* and *P. aeruginosa* could have serious implications for disease management.

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We declare that no competing interests exist.

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