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Pyocyanin biosynthesis protects *Pseudomonas aeruginosa* from nonthermal plasma inactivation

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Summary

***Pseudomonas aeruginosa* is an important opportunistic human pathogen, which raises a worldwide concern for its increasing resistance. Nonthermal plasma, which is also called cold atmospheric plasma (CAP), is an alternative therapeutic approach for clinical infectious diseases. However, the bacterial factors that affect CAP treatment remain unclear. The sterilization effect of a portable CAP device on different *P. aeruginosa* strains was investigated in this study. Results revealed that CAP can directly or indirectly kill *P. aeruginosa* in a time-dependent manner. Scanning electron microscopy and transmission electron microscope showed negligible surface changes between CAP-treated and untreated *P. aeruginosa* cells. However, cell leakage occurred during the CAP process with increased bacterial lactate dehydrogenase release. More importantly, pigmentation of the *P. aeruginosa* culture was remarkably reduced after CAP treatment. Further mechanical exploration was performed by utilizing mutants with loss of functional genes involved in pyocyanin biosynthesis, including *P. aeruginosa* PAO1 strain-derived *phzA1*::Tn, *phzA2*::Tn, Δ*phzA1*/Δ*phzA2*, *phzM*::Tn and *phzS*::Tn, as well as corresponding gene deletion mutants based on clinical PA1 isolate. The results indicated that pyocyanin and its intermediate 5-methyl phenazine-1-carboxylic acid (5-Me-PCA) play important roles in *P. aeruginosa* resistance to CAP treatment. The unique enzymes, such as PhzM in the pyocyanin biosynthetic pathway, could be novel targets for the therapeutic strategy design to control the growing *P. aeruginosa* infections.**

Introduction

Pseudomonas aeruginosa is an important Gram-negative opportunistic pathogen, which causes a variety of diseases from wound infections to healthcare-associated pneumonia, cystic fibrosis and sepsis (Botelho *et al.*, 2019; Shortridge *et al.*, 2019). With the extensive use of antibiotics, approximately 30% of the *P. aeruginosa* clinical isolates are multidrug-resistant and extensively drug-resistant (Grau *et al.*, 2019). Particularly, the carbapenem-resistant *P. aeruginosa* has been listed as one of the six superbugs with high risk to human health and one of the listed antibiotic-resistant members by the World Health Organization with priority for the urgent development of novel antibiotics (Tacconelli *et al.*, 2018). The development of a novel antibiotic against *P. aeruginosa* is time- and labour-consuming, thus demanding alternative therapeutic strategies to control *P. aeruginosa* infection.

Plasma, which represents the fourth fundamental state of matter in nature, is classified as nonthermal (≤ 340 K) and thermal (≥ 15 000 K) plasma according to the temperature of ionized gas (Šimončicová *et al.*, 2019). The nonthermal plasma, also called cold atmospheric plasma (CAP), is a novel approach for microbial disinfection (Rao *et al.*, 2020). The first attempt at CAP-based microbial inactivation was performed in 1957 by using a voltage of 300 V to generate plasma with atmospheric air (Krueger *et al.*, 1957). Since then, CAP has been gradually applied for disinfection in various fields, including the food industry, modern agriculture and medical treatment (Dai *et al.*, 2018; Judée *et al.*, 2018; Sarangapani *et al.*, 2018; Xiong, 2018; Rao *et al.*, 2020). As a gaseous mixture, CAP comprises many active ingredients, such as charged particles, UV radiation and highly reactive species, which enforce complex bactericidal mechanisms to achieve microbial inactivation (Pattison *et al.*, 2012; Han *et al.*, 2016; Lunov *et al.*, 2016; Pai *et al.*, 2018).

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Different microbial species may have distinct sensitivity to CAP treatment, and the microbial factors that affect the antimicrobial efficiency of CAP are currently obscure. Previous studies reported that staphyloxanthin, a golden carotenoid pigment produced by *Staphylococcus aureus,* can protect different *S. aureus* strains from CAP inactivation (Yang *et al*., 2020). Thus, the antioxidant components derived from microorganisms may have considerable potential in microbial resistance to CAP processing.

One of the most important characteristics for *P. aeruginosa* is the production and secretion of pyocyanin, a redox-active green pigment that serves as a fundamental virulence factor contribution to bacterial infections (Huang *et al*., 2012). The effects of pyocyanin biosynthesis on the antimicrobial effectiveness of CAP were investigated in this study. Various *P. aeruginosa* strains were treated with a self-assembled portable CAP device, and CAP was found to sterilize *P. aeruginosa* isolates effectively in a time-dependent manner. With the reduced bacterial survivals after CAP procedure, the CAP-treated bacterial culture exhibited attenuated pigmentation compared with the untreated control group. With various transposon-insertion single gene mutants and gene deletion mutants, the results further demonstrated that inactivation of *phzM* and *phzS* genes presented opposite roles in their contribution to *P. aeruginosa* survivals after CAP treatment, with a reduction effect for the former and an increment role for the later. The addition of pyocyanin and its biosynthetic intermediate 5-methyl phenazine-1-carboxylic acid (5-Me-PCA) to the bacterial suspension displayed substantially protective roles for *P. aeruginosa* under CAP sterilization. Nevertheless, the addition of phenazine-1-carboxylic acid (PCA), another biosynthetic intermediate, exhibited negligible CAP-resistance. Our results had proved that pyocyanin and 5-Me-PCA were crucial factors for the protection of *P. aeruginosa* from CAP inactivation. This finding implied a new way to design antimicrobial strategies for *P. aeruginosa* infection treatments.

Results

CAP effectively sterilized P. aeruginosa *strains*

A small portable CAP device was designed on the principle of dielectric barrier discharge (DBD) to generate steady CAP for testing in tubes (Fig. 1). One *P. aeruginosa* standard strain PAO1 and two clinical strains PA1 and PA3 were selected to evaluate the sterilization effect of CAP treatment. All bacterial strains presented significant reductions in the survival numbers after 30 s of CAP treatment compared with the untreated ones (Fig. 2A–C). Comparatively, clinical strain PA1 showed the most sensitivity to the CAP treatment, followed by PA3, whereas PAO1 exhibited additional survival after exposure to the same CAP process. Moreover, the sterilization effect of CAP on *P. aeruginosa* was time-dependent. The survival numbers of PAO1 and PA1 cells gradually decreased with increasing CAP-treatment time, and all bacterial cells were completely inactivated after 60 s of CAP treatment (Fig. 2D and E).

In addition to the direct treatment duration, the incubation time after CAP treatment also affects the antimicrobial efficiency of plasma (Guo *et al*., 2018a,b). The surviving cells of PAO1 were gradually reduced with the prolonged storage time after the same CAP pretreatment process (Fig. 2F). This reduction indicated a continuous bactericidal effect of the CAP-activated solution. The indirect antibacterial effect of CAP-activated solution on *P. aeruginosa* was then detected. The bacterial cells were suspended by normal saline (NS) buffer, which was first activated by CAP processing for 30 s, followed by incubated at room temperature for increasing times (0, 15, 30 and 60 min) and the surviving bacterial cells were counted. The results showed that the number of survivals was gradually decreased with the incubation

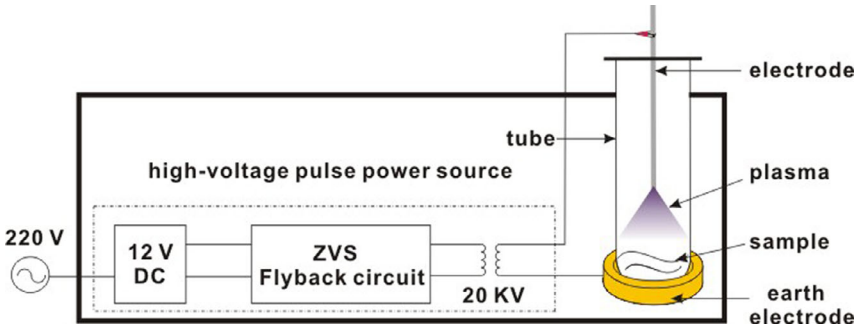

Fig. 1. Schematic of the portable CAP device. It was designed according to the principle of dielectric barrier discharge (DBD) and composed of a direct current (DC)-regulated power supply (GOPHERT CPS6003), azero-voltage switching (ZVS)-flyback circuit, and a pulse transformer with the maximum amplitude of 20 kV for the air gap between a sharp electrode and the earth electrode to generate the plasma inside the test tube with samples.

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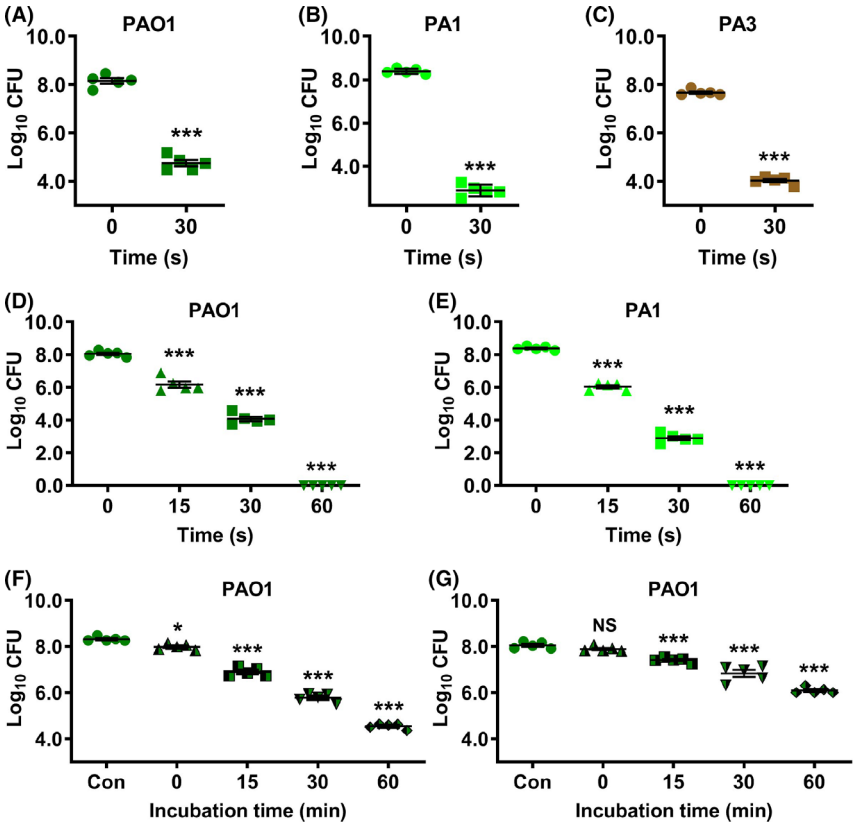

Fig. 2. Sterilization effects of the self-assembled CAP device on *P. aeruginosa*. Strains (A) PAO1, (B) PA1 and (C) PA3 were exposed to CAP for 30 s and followed 1 h incubation time at room temperature. The survival bacterial cells were counted using an agar plate dilution method. (D) *P. aeruginosa* PAO1 and (E) PA1 were exposed to CAP for different duration (0, 15, 30 and 60 s) and followed 1 h of incubation at room temperature. The survivors were counted using an agar plate dilution method. (F) Strain PAO1 was exposed to CAP for 30 s and followed distinct incubation times as indicated. The survival of cells was determined with a plate dilution method. The untreated PAO1 served as control (Con). (G) The NS buffer was exposed to CAP for 30 s, and a volume ratio of 1:1 was then used to treat PAO1 suspension for increasing times as indicated. The survival cells were determined with a plate dilution method. Con represented the untreated PAO1 control. All experiments were conducted for five times. The numbers of survival bacteria are presented as mean ± standard derivations (SD). One-way ANOVA was used for testing multiple groups. *$P < 0.05$ and ***$P < 0.001$; NS represented no statistical significance compared with untreated group.

time increasing, and approximately 2.1 $\log_{10}$ CFU reduction was achieved after 1 h incubation in CAP-activated solution (Fig. 2G). These results demonstrated that the CAP produced by the portable device and the CAP-activated NS solution can effectively sterilize *P. aeruginosa* strains in a time-dependent manner.

CAP treatment impaired the pyocyanin produced by P. aeruginosa

The mechanism underlying microbial inactivation by CAP is complicated. A set of plasma species may attack bacterial cells to facilitate morphological change, some species may break down chemical bonds in the peptidoglycan structure, whereas others can execute lipid peroxidation on the bacterial membrane (Judée *et al.*, 2018). Bacterial morphological variations after CAP treatment were first evaluated. Scanning electron microscopy (SEM) demonstrated that little damage was observed in the cell surface of *P. aeruginosa* PAO1 after CAP treatment (Fig. 3A). Further transmission electron microscopy (TEM) observation revealed that nearly all CAP-treated PAO1 cells maintained intact cell morphology, and only a few cells were broken after CAP treatment (Fig. 3B). Nevertheless, the difference in cellular integrity between cells with and without CAP-treatment was ignorable (Fig. 3A and B). Bacterial lactate dehydrogenase (LDH) release assay was then performed to analyse the cell membrane integrity before and after CAP treatment. The results showed that CAP caused membrane damage due to the increased release of LDH (Fig. 3C). This phenomenon was correlated with the survival rate reduction in CAP-treated cells compared with that in untreated bacteria (Fig. 3D). Interestingly, the pigmentation of *P. aeruginosa* PAO1 culture was decreased after CAP treatment (Fig. 3E). The pyocyanin in the *P. aeruginosa* culture before and after CAP treatment was extracted with chloroform (Fig. S1) and determined

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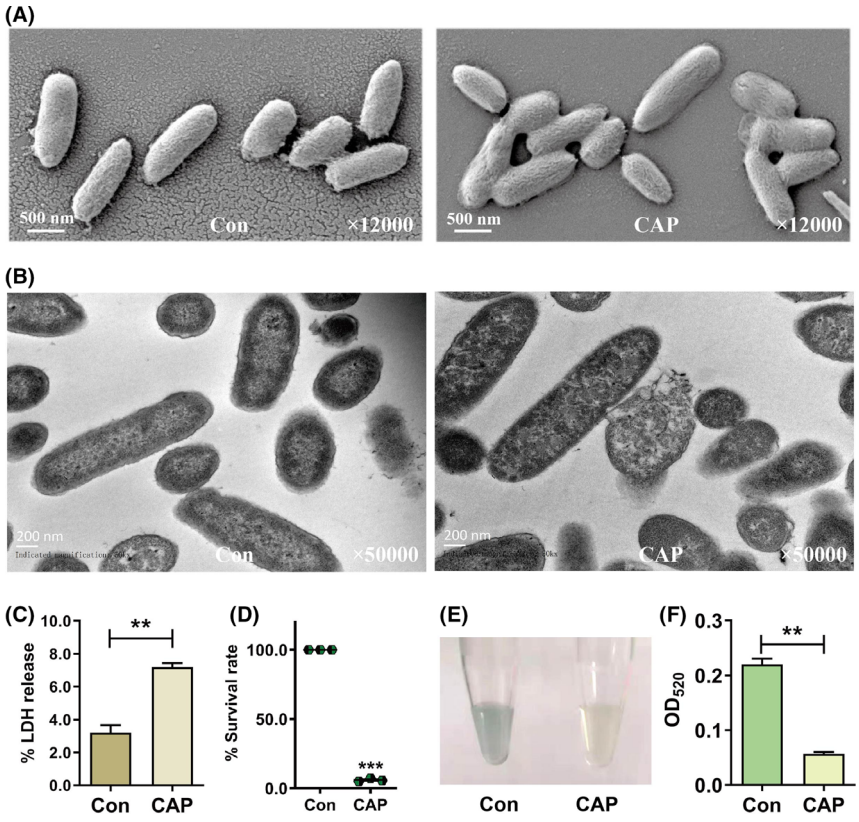

Fig. 3. CAP procedure impaired the pyocyanin produced by *P. aeruginosa*. The *P. aeruginosa* PAO1 was exposed to CAP for 30 s and followed 1 h of incubation at room temperature. Then CAP-treated PAO1 cells (CAP) were observed by (A) SEM and (B) TEM. The untreated PAO1 cells served as control (Con). The magnification was indicated in each panel. The suspension of PAO1 was exposed to CAP for 3 min and followed 1 h incubation time. Then (C) the LDH release in the supernatant with or without CAP treatment was detected and (D) the survival bacterial cells were counted with a plate dilution method. (E) The colour of PAO1 suspension changed after 3 min of CAP treatment. (F) The OD_{520} value of PAO1 suspension before and after 3 min of CAP treatment was detected. The experiments were repeated at least three times. Statistical significance was calculated using Student's *t* test. $^{**}P < 0.01$ and $^{***}P < 0.001$.

as described (Dietrich *et al.*, 2006). The OD_{520} value was significantly decreased in the CAP-treated solution compared with the untreated control extractives (Fig. 3F). These results indicated that CAP treatment impairs *P. aeruginosa* pyocyanin, a redox-active phenazine compound, which may serve as protective factors to facilitate bacterial survival under CAP processing.

The pyocyanin protects P. aeruginosa *from CAP inactivation*

Pyocyanin is a major virulence factor contributing to *P. aeruginosa* pathogenesis, and more than 90% of clinical *P. aeruginosa* isolates produce pyocyanin (Costa *et al.*, 2017). The biosynthesis of green pigment pyocyanin in *P. aeruginosa* starts from the chorismic acid, which is catalysed by seven enzymes encoded by two alternative operons termed *phzA1B1C1D1E1F1G1* and *phzA2B2C2-D2E2F2G2*, to synthesize phenazine-1-carboxylic acid (PCA) (Fig. 4A), followed by two unique enzymes, namely PhzM and PhzS, to convert PCA to pyocyanin (Mavrodi *et al.*, 2001). One double gene deletion mutant Δ*phzA1/*Δ*phzA2* was constructed and four transposon insertion mutants of *P. aeruginosa* strain PAO1, namely *phzA1*::Tn, *phzA2*::Tn, *phzM*::Tn, *phzS*::Tn (Jacobs *et al.*, 2003), were selected and treated singly with CAP to explore the role of pyocyanin biosynthesis in *P. aeruginosa* under CAP sterilization. The pigment phenotypes of these strains were first evaluated. PAO1 mutants Δ*phzA1/*Δ*phzA2, phzM*::Tn and *phzS*::Tn, respectively, displayed white, light yellow and dark red, whereas *phzA1*::Tn and *phzA2*::Tn mutants exhibited the green colour similar to the wild-type PAO1 strain (Fig. 4B). The cultures of *P. aeruginosa* strain PAO1 and its derivatives were showed in Fig. S2. The OD_{520} value detection revealed that the *phzM*::Tn and Δ*phzA1/*Δ*phzA2* mutants displayed a weak pigment, while the *phzS*::Tn strain showed a stronger pigment compared with wild-type PAO1. The *P. aeruginosa* PAO1 and its derivative mutants were separately processed by CAP under the same condition for

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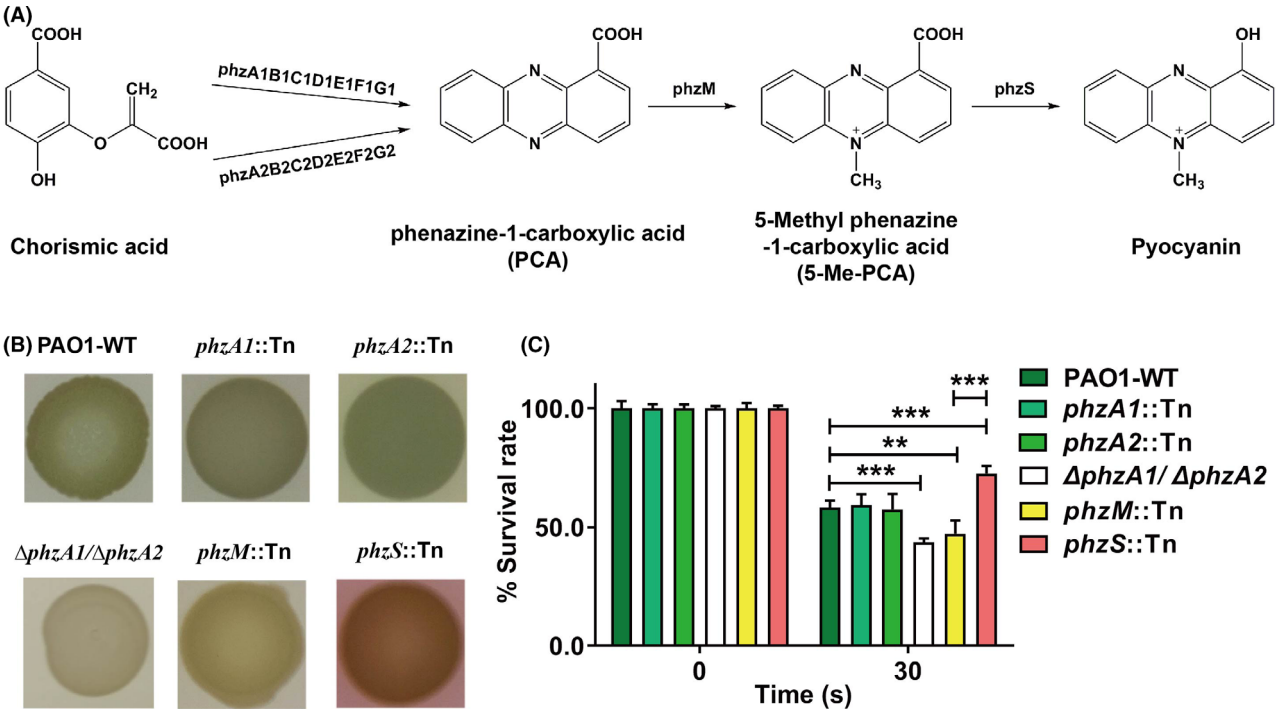

Fig. 4. Inactivation of genes involved in pyocyanin biosynthesis contributed to bacterial survivals of *P. aeruginosa* after CAP treatment.
A. The biosynthetic pathway of pyocyanin pigment in *P. aeruginosa*.
B. The pigment phenotypes of *P. aeruginosa* PAO1 and its derivatives *phzA1*::Tn, *phzA2*::Tn, Δ*phzA1*/Δ*phzA2*, *phzM*::Tn, and *phzS*::Tn mutants. Bacterial colonies were presented after 20 h of culture in Luria-Bertani (LB) agar plates.
C. The *P. aeruginosa* PAO1 and its derivatives were separately exposed to CAP for 30 s and followed 1 h incubation at room temperature. The survival cells were determined with the plate dilution method. Experiments were conducted five times. Statistical significance was calculated using One-way ANOVA. $^{**}P < 0.01$ and $^{***}P < 0.001$.

30 s, followed by 1 h incubation, and then the survival bacterial cells were counted with the plate dilution method (Yang *et al.*, 2020). The results showed that *phzM*::Tn and Δ*phzA1*/Δ*phzA2* was sensitive to CAP treatment, whereas the *phzS*::Tn mutant exhibited more resistance to CAP processing compared with wild-type PAO1 (Fig. 4C). No survival difference was observed in *phzA1*::Tn, *phzA2*::Tn and PAO1 strains after the identical CAP treatment (Fig. 4C). Similarly, there was no difference in the reduction of CFU between the *phzM*::Tn and Δ*phzA1*/Δ*phzA2* strains after the identical CAP treatment (Fig. 4C). Next, different pigmentation mutant strains including Δ*phzA1*, Δ*phzA2*, Δ*phzA1*/Δ*phzA2*, Δ*phzM* as well as Δ*phzS* were constructed on the basis of clinical isolate PA1, and CAP inactivation assay was repeated. Similar to the results of strain PAO1 and its derivatives, the sensitivity of PA1-Δ*phzA1*/Δ*phzA2*, PA1-Δ*phzM* and PA1-Δ*phzS*, to CAP treatment were significantly changed compared with wild-type PA1 strain, nevertheless, the deletion of *phzA1* or *phzA2* gene alone rarely affected the survival rate of PA1 strain after CAP treating (Fig. S3). These data indicated that pyocyanin and its biosynthetic intermediate 5-methyl phenazine-1-carboxylic (5-Me-PCA) may play pivotal roles in the resistance of *P. aeruginosa* to CAP processing, whereas other intermediates, such as PCA, have ignorable protection roles.

Next, the pyocyanin and 5-Me-PCA intermediate were respectively extracted from cultures of *P. aeruginosa* PAO1 and *phzS*::Tn and added to the bacterial suspension before CAP treatment to further determine the effect of pyocyanin biosynthesis on bacterial resistance to CAP disinfection. It could be clearly observed that the addition of pyocyanin and 5-Me-PCA extracts effectively protected bacterial cells (PAO1 and *phzM*::Tn mutant) from CAP disinfection (Fig. 5A and B). Moreover, the pure pyocyanin and PCA were commercially purchased and respectively added to bacterial suspension at different concentrations (0, 10 nM, 1 μM and 100 μM); the commercially available vitamin E was added at the same concentrations and served as a positive control. Bacterial cells of *P. aeruginosa* PAO1 were then processed by CAP device. Results showed that both pyocyanin and vitamin E presented dose-dependent protective effects on *P. aeruginosa* PAO1 against CAP (Fig. 5D and E). However, the addition of PCA did not provide any protection against CAP treatment, even at the concentration of 1 mM (Fig. 5C, Fig. S4). In addition, the toxic effects of PCA, pyocyanin and vitamin E on bacterial cells were

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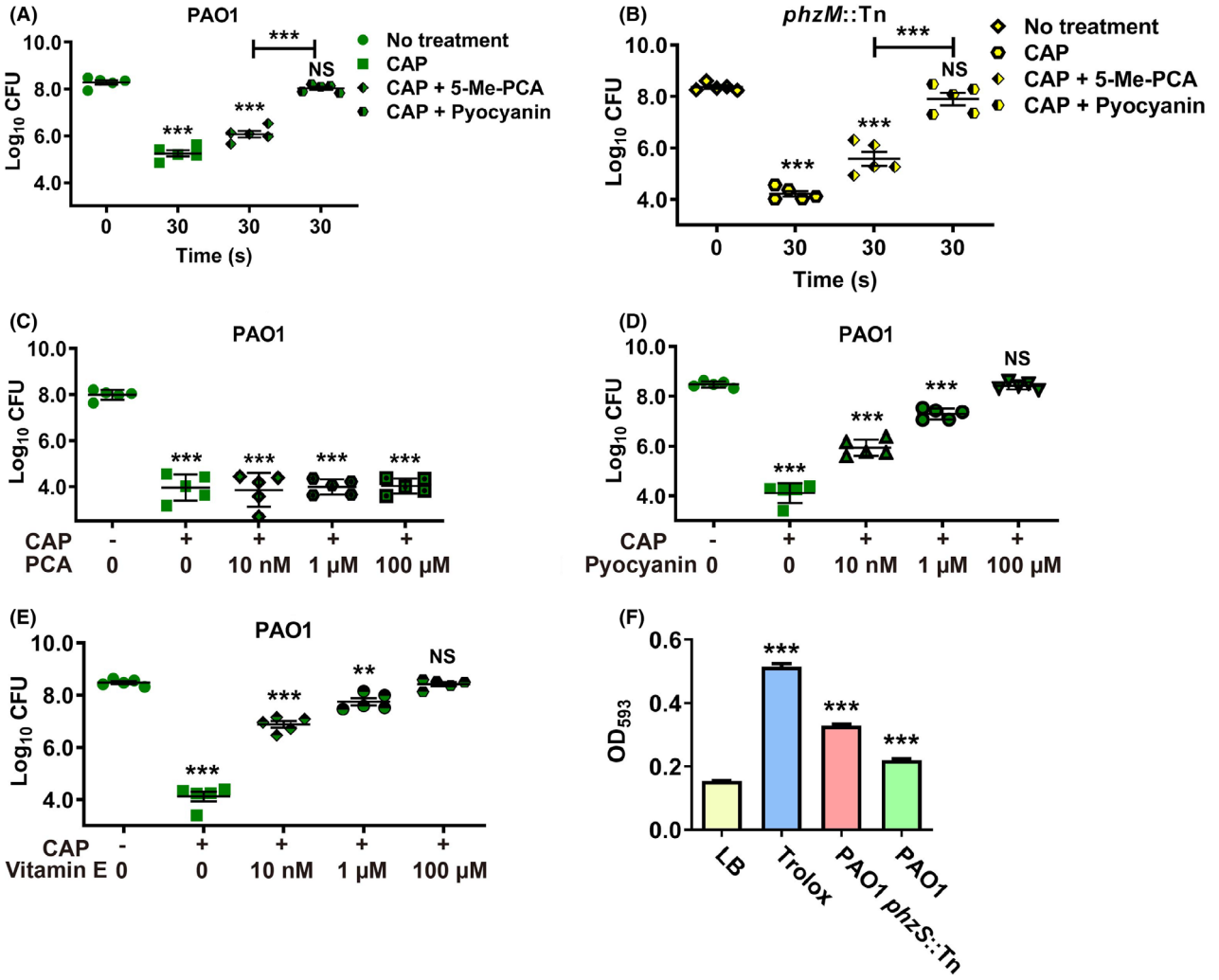

Fig. 5. Addition of pyocyanin or its biosynthetic intermediate 5-Me-PCA protected *P. aeruginosa* from CAP processing. Thirty microliters of extracted pyocyanin and 5-Me-PCA from equal volume of bacterial cultures were added to *P. aeruginosa* (A) PAO1 and (B) *phzM*::Tn mutant, then the suspension were separately exposed to CAP for 30 s and followed 1 h incubation at room temperature. The survival cells were determined with the plate dilution method. Different doses of (C) PCA, (D) pyocyanin and (E) vitamin E were respectively added to the bacterial solution of *P. aeruginosa* PAO1. Then the suspension was exposed to CAP for 30 s and followed 1 h incubation. The survivors were counted and indicated. (F) Total antioxidant capacity of *phzS*::Tn and PAO1 culture were detected by FRAP. Trolox and LB served as positive and negative controls respectively. All experiments were repeated five times. Statistical significance of each tested groups of interest relative to the untreated or LB group was calculated using One-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$; NS represented no statistical significance compared with untreated group.

evaluated. Different doses of reagents were added into PAO1 suspension, respectively; then, the mixtures were incubated for 1 h and the cell numbers were counted. However, no significant influence on bacterial growth was observed (Fig. S5). These data provided additional evidence that pyocyanin and 5-Me-PCA intermediate of *P. aeruginosa*, but not PCA, work well in anti-CAP treatment.

Interestingly, the *phzS*::Tn mutant has more survivors after CAP treatment compared with the wild-type PAO1 (Fig. 4C), which seems to be a contradictory result to support the protection role of pyocyanin in *P. aeruginosa* CAP resistance. Nevertheless, the OD_{520} value of *phzS*::Tn culture was much higher than that of PAO1 (Fig. S2), indicating the accumulation of 5-Me-PCA intermediate due to the loss of *phzS* function in *P. aeruginosa*, which represented stronger antioxidant activity than pyocyanin produced by wild-type PAO1 in the same culture condition. The ferric reducing capability of the plasma (FRAP) method was performed to detect the overall antioxidant capacity of *phzS*::Tn and PAO1 culture. The supernatant of the *phzS*::Tn strain culture exhibited stronger antioxidant activity than that from PAO1 (Fig. 5F). Overall, the results suggested that pigment pyocyanin and its synthetic intermediate 5-Me-PCA were responsible for the resistance of *P. aeruginosa* to CAP inactivation.

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Discussion

The pathogenic phenazine-producing *P. aeruginosa* can cause acute and chronic diseases in patients with immunocompromised health, burn or injury (Mavrodi *et al.*, 2001). The prevalence of multidrug-resistant and extensively drug-resistant *P. aeruginosa* strains, especially the carbapenem-resistant isolates, has worsened the current clinical therapeutic situation (Tacconelli *et al.*, 2018). CAP has been proven to be an efficient strategy for the inactivation of various microorganisms and is widely applied in medical treatments, including wound sterilization and medical disinfection (Mohd Nasir *et al.*, 2016; Hilker *et al.*, 2017; Gherardi *et al.*, 2018; Weiss *et al.*, 2019). CAP comprises various kinds of components, which may independently or cooperatively impose complex mechanisms during microbial inactivation (Alkawareek *et al.*, 2014; Scholtz *et al.*, 2015). For example, the reactive species in CAP can trigger oxidative damages of bacterial DNA, protein, lipids and polysaccharides (Liu *et al.*, 2016; Guo *et al.*, 2018a,b; Itooka *et al.*, 2018; Su *et al.*, 2018; Bauer, 2019); the UV radiation can induce adjacent pyrimidine in DNA to form dimers that intervene the replication and transcription (Klämpfl *et al.*, 2012; Lackmann *et al.*, 2013); the charged particles may cause the formation of pores on bacterial membranes (Kelly-Wintenberg *et al.*, 1998; Maisch *et al.*, 2012). However, the influence factors of the environment and the molecules in certain pathogens that affect the antimicrobial efficiency of CAP remain unknown.

Different *P. aeruginosa* strains were directly treated with a self-designed portable CAP device or indirectly treated with CAP-activated solution. The results indicated that all *P. aeruginosa* cells were sensitive to CAP treatment in strain- and time-dependent manners. The reference *P. aeruginosa* strain PAO1 exhibited more resistance to CAP exposure compared with clinical strains PA1 and PA3, suggesting that potential mechanisms causing CAP resistance might exist according to specific strain background. Different phenotypes of *P. aeruginosa* strain before and after CAP treatment were then detected to demonstrate the underlying mechanism of CAP killing *P. aeruginosa*, as well as to find out possible CAP-resistance associated factors. LDH releasing assay showed increased release of LDH after CAP processing, indicating that CAP treatment might sterilize bacterial cells by inducing cell leakage. This result was consistent with that of Han *et al.*, who revealed that the cell leakage might be one of the mechanisms which CAP inactive Gram-negative *Escherichia coli* (Han *et al.*, 2016). However, our results of the CAP-activated solution and incubation time showed that CAP mainly killed bacteria through reactive nitrogen oxides under low power and short time treating conditions. First, the SEM and TEM observation showed negligible changes in *P. aeruginosa* cells after CAP treatment, excluding the sterilization roles of charged particles which induce membrane perforation. Second, CAP-pretreated NS solution, which lacking UV, charged particles and short-lived reactive groups, could effectively reduce the CFU of bacteria, indicating that some other substances, and most likely the long-lived reactive species (H_2O_2 and NO_2^-), were responsible for the indirect sterilization of *P. aeruginosa* strains by CAP-activated NS solution (Chen *et al.*, 2017). It is well known that RNS can induce cell death through DNA damage and ROS induces cell death through apoptosis and necrosis (Boehm *et al.*, 2016; Kim and Chung, 2016). Third, a considerable reduction in *P. aeruginosa* pigmentation was observed after CAP treatment, suggesting that phenazine components produced by *P. aeruginosa* may act as targets or anti-CAP molecules during plasma processing. All these results above demonstrated that sterilization of CAP might also depend on the effects of reactive nitrogen oxides.

The *P. aeruginosa* synthesized pyocyanin is well known for its antioxidant capacity. Recently, it has been shown that pyocyanin could stimulate quorum sensing-mediated tolerance to oxidative stress and increase persist cell populations in *Acinetobacter baumannii* (Bhargava *et al.*, 2014). Another study revealed that almost half of surviving *P. aeruginosa* detected DNA changes about phenazine biosynthesis genes after CAP treatment, suggesting that the phenazine was a target for CAP-mediated oxidative damage (Mai-Prochnow *et al.*, 2015). The pyocyanin is a dominant phenazine pigment of *P. aeruginosa*, which participates in bacterial pathogenesis (Costa *et al.*, 2017). The biosynthesis of pyocyanin is controlled by *phzABCDEFG* operons, *phzM* and *phzS* genes (Lau *et al.*, 2004). The enzymes encoded by two alternatively homologous operons (*phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*) were responsible for the synthesis of PCA from chorismic acid (Mavrodi *et al.*, 2001). Then the methyltransferase PhzM converts PCA to the dark red intermediate 5-Me-PCA (Mavrodi *et al.*, 2006), and the flavoprotein monooxygenase PhzS controls the final step of pyocyanin synthesis from 5-Me-PCA (Denning *et al.*, 2003). In this study, five mutants with inactivated or deleted genes of *phzA1*, *phzA2*, *phzA1/phzA2*, *phzM* and *phzS*, based on PAO1 strain, were selected to explore the effects of pyocyanin biosynthesis on antimicrobial activity of CAP, and their sensitivities to CAP treatment were evaluated. The *phzA1*::Tn and *phzA2*::Tn mutants produced similar pyocyanin with the wild-type *P. aeruginosa* PAO1, and exhibited comparable susceptibility to CAP procedure. On the contrary, the Δ*phzA1*/Δ*phzA2* double mutant strain completely lost the function of pigmentation, and was much more easily to be

killed by CAP compared with wild-type PAO1 strain. These data indicated that operons *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2* have complementary roles in the biosynthesis of PCA, blocking only one of these two pathways would not influence the biosynthesis of pyocyanin. After CAP processing, we have also observed that the survival cells of *phzM*::Tn strain were remarkably decreased, which was in contrast with those of *phzS*::Tn strains with increased survivors compared to wild-type PAO1 (Fig. 4C). The *phzM*::Tn strain exhibited light yellow phenotype due to the production of PCA. However, there was no statistical significance between *phzM*::Tn strain (light yellow) and Δ*phzA1*/Δ*phzA2* strain (white) in the susceptibility to CAP treatment. This finding indicated that the PCA accumulation in *phzM*-inactivation mutant culture may not contribute to bacterial survivals, suggesting no protective role of PCA in *P. aeruginosa* against CAP procedure, which had been further verified by adding of purified PCA before CAP treatment. Nevertheless, the inactivation of the *phzS*::Tn mutant by CAP was more difficult than the wild-type PAO1. A reasonable interpretation is that both pyocyanin and its biosynthetic intermediate 5-Me-PCA have protection roles in *P. aeruginosa* under CAP treatment, and accumulated 5-Me-PCA due to *phzS* gene inactivation in *phzS*::Tn mutant displayed additional survivors, which was consistent with the finding that the concentration of 5-Me-PCA from *phzS*::Tn over-night culture was much higher than that of pyocyanin from PAO1, and the culture supernatant of *phzS*::Tn mutant had stronger anti-oxidant capability than the same volume of that from PAO1. Similar results were repeated with the same pigment gene mutants generated from PA1 clinical strain, suggesting that the protection roles of pyocyanin and 5-Me-PCA against CAP-processing were universal.

Besides the work of constructing various pigment gene mutants and evaluating their tolerance to CAP treatment, we also prepared pyocyanin and its intermediates, either by extracting from the corresponding culture medium or by purchasing from reagent companies, and evaluated their abilities in protecting *P. aeruginosa* from CAP treatment. Consistent with the results of gene mutants, both of extracted pyocyanin and 5-Me-PCA could effectively protect *P. aeruginosa* from CAP inactivation. Furthermore, the purchased pyocyanin protected PAO1 in a dose-dependent manner while PCA exhibited no protective effect.

The role of pyocyanin and 5-Me-PCA in the protection of *P. aeruginosa* from CAP treatment may be ascribed to their antioxidant capabilities. The 5-Me-PCA has a carboxyl group at its 1-carbon site, and pyocyanin has a hydroxyl group at the same site, which make them easily oxidized (Chatterjee *et al.*, 2017). Since the hydroxyl group is more easily oxidized than the carboxyl group, we speculated that the protective effect of pyocyanin would be stronger if equal amounts of pyocyanin and 5-Me-PCA were added. Regretfully, the intermediate 5-Me-PCA is not commercially available, thus, we could not compare the protective function of pure 5-Me-PCA with pyocyanin by adding them into bacterial suspension at equivalent concentration. Further investigations are needed to determine the different antioxidant capabilities chemically. In addition, we found that the pyoverdine, another pigment of *P. aeruginosa*, also had a certain protective effect against CAP treatment, which is worthy of further study (Fig. S6).

Overall, the CAP generated from a self-assembled small device can effectively kill different *P. aeruginosa* strains in a reactive species-dependent manner. The inactivation of *P. aeruginosa* by CAP may also result in cell leakage. Except for PCA, the phenazine components of pigment biosynthesis, such as pyocyanin and 5-Me-PCA, act as protective molecules in *P. aeruginosa* resistance to CAP inactivation. These findings provide deep insights into the antimicrobial mechanisms of CAP from the treatment targets of bacteria and imply novel targets for the design of therapeutic strategies against *P. aeruginosa* infections.

Experimental procedures

Portable CAP device suitable for tube test

We designed and assembled a portable CAP device on the principle of dielectric barrier discharge (DBD) for this study (Fig. 1). The GOPHERT CPS-6003, a high precision adjustable direct current (DC) stabilized power, was selected as the power supply, whose input voltage was 220V, output voltage ranged from 0 to 60 V and output current ranged from 0 to 3 A. Then, a zero voltage switching (ZVS)-flyback circuit as described and linked a pulse transformer was used to convert current and output high voltage alternating current (AC) pulse with the maximum amplitude of 20 kV (Lin and Dong, 2011). Finally, a sharp metal electrode was connected to generated discharge for atmospheric plasma generation. For sample treatment, 0.5 ml of bacterial sample of interest was added into the test tube, which was then put in CAP device, adjusted the electrode tip to keep 0.5 cm away from the liquid surface. During CAP process, the output-power maintained at a constant power of 8 W with 16 V and 0.5 A, and the exposing time ranged from 0 to 3 min.

Bacterial strains

P. aeruginosa stain PAO1 is a commonly used laboratory-adapted reference strain. *P. aeruginosa* PA1 and PA3 were clinical multidrug-resistant isolates obtained from patients suffering from respiratory-tract infections (Lu *et al.*, 2015). PAO1 mutant strains, *phzA1*::Tn, *phzA2*::Tn, *phzM*::Tn and *phzS*::Tn

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generated by transposon-insertion as previously described (Jacobs *et al.*, 2003). Other pigment gene deleted mutants Δ*phzA1*/Δ*phzA2* (based on PAO1 background), PA1-Δ*phzA1,* PA1-Δ*phzA2,* PA1-Δ*phzA1*/Δ*phzA2,* PA1-Δ*phzM* and PA1-Δ*phzS* were constructed by homologous recombination as previously described (Huang and Wilks, 2017). All primers used in this study are listed in Table S1. Take construction of the PA1-Δ*phzA1* mutant for example. Firstly, we amplified the flanking fragments of *phzA1* by PCR. The upstream and downstream fragments of the *phzA1* gene were cloned to the pEX18Tc suicide plasmid to construct recombinant plasmid pEX18Tc-Δ*phzA1,* which was then transformed into PAO1 competent cells. The PAO1-Δ*phzA1* mutant was screened with 100 μg ml^{-1} tetracycline and verified by DNA sequencing of *phzA1* gene flanking region. All *P. aeruginosa* strains were grown in Luria-Bertani (LB) medium at 37°C with shaking.

CAP direct treatment

To detect the antimicrobial effect of the self-assembled CAP device, *P. aeruginosa* strains were grown overnight in LB broth. The bacterial suspension was diluted to a density of 2×10^8 colony-forming unit (CFU) per mL (0.5 McFarland density) by sterilized normal saline (NS, containing 0.9% NaCl). Then bacterial samples were treated by CAP for different times (from 0 to 3 min), followed by different incubation duration (from 0 to 60 min). After that, the survival of bacterial cells was determined through the plate dilution method as described (Yang *et al.*, 2020).

CAP indirect treatment

To detect the indirect antibacterial effect of CAP-activated solution, *P. aeruginosa* strains were grown overnight in LB broth. The bacterial cells were diluted to a density of 2×10^8 CFU ml^{-1} (0.5 McFarland density) by normal saline (NS) buffer. Then, NS was first treated for 30 s with plasma to achieve a CAP-activated solution, and a volume ratio of 1:1 was then applied to the diluted bacterial suspension of *P. aeruginosa* PAO1. The mixture was incubated for increasing times (0, 15, 30 and 60 min) at room temperature, and the surviving bacterial cells were counted using a plate dilution method (Yang *et al.*, 2020).

Extraction and detection of P. aeruginosa *pigment*

Extraction of 5-Me-PCA and pyocyanin from *P. aeruginosa* cultures was carried out as previously described (Dietrich *et al.*, 2006; Kennedy *et al.*, 2015). Briefly, pyocyanin compounds were extracted from 100 ml culture where PAO1 strain was grown for five days at 28°C. After centrifugation at 10 000 *g* for 10 min at 4°C, the cell-free supernatant was used to extract pyocyanin compounds with 20 ml of chloroform three times. The organic layer was collected and filtered with a 0.22-μm filter (Millipore). The OD_{520} value was read by a spectrophotometer (Thermo Scientific), and the pyocyanin was concentrated under 37°C. The acidized extraction was carried out to identify pyocyanin by the addition of hydrochloric acid, and the solution turned pink if the pyocyanin compounds existed. The pyocyanin derived from 100 ml of bacterial culture was dissolved in 1 ml NS. To extract 5-Me-PCA, the ethyl acetate was used as described (Kennedy *et al.*, 2015). After three times of extraction, the organic layer was filtered and the 5-Me-PCA components were concentrated under 37°C. The 5-Me-PCA extracted from 100 ml of cell culture was dissolved in 1 ml NS.

Pyocyanin biosynthesis resistant to CAP treatment

Pseudomonas aeruginosa PAO1 and *phzS*::Tn strains were grown overnight in LB broth. The bacterial suspension was diluted to a density of 2×10^8 CFU ml^{-1} (0.5 McFarland density) by sterilized NS. The pyocyanin and 5-Me-PCA intermediate were extracted from cultures of *P. aeruginosa* PAO1 and *phzS*::Tn, and dissolved in NS as above. Before CAP treatment, 0–30 μl pigment solution was added into bacterial suspension, and then the suspension was exposed to CAP for 30 s and followed 1 h of incubation at room temperature. After that, the survival of bacterial cells was determined through the plate dilution method as described (Yang *et al.*, 2020).

To further compare the anti-CAP abilities of pyocyanin and its intermediates, the pure pyocyanin and PCA reagents were purchased from Sigma-Aldrich. The vitamin E (MedChemExpress) was purchased and served as a positive control. All reagents were separately prepared as 10mM stock solution with sterilized DMSO or PBS solution and stored at −20°C. During CAP treatment assay, pyocyanin, PCA, or vitamin E solution was added to bacterial suspension at different concentrations (0, 10 nM, 1 μM, 100 μM). Then, the mixture was treated by CAP device, and the survival bacterial cells were determined.

SEM observation

Bacterial cell integrity was observed by SEM. Briefly, the *P. aeruginosa* PAO1 solutions with or without CAP treatment were dropped onto clean cover glasses, fixed overnight with 2.5% glutaraldehyde at 4°C. After washing twice with NS solution, the samples were dehydrated sequentially in ethanol of gradient concentrations (30%, 50%, 60%, 70% and 90%) for 5 min each, followed by rinses in 100% ethanol for three times with 7 min of

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each. Then, cells were treated with tert-butyl alcohol of gradient concentrations (30%, 50%, 70%, 90% and 100%) for replacement of ethanol. Finally, air-dried samples were immediately sputter-coated with gold and observed by the scanning electron microscope (S-3400N II; Hitachi, Tokyo, Japan).

TEM observation

Bacterial cell structure was observed by TEM. Briefly, the *P. aeruginosa* PAO1 solutions with or without CAP treatment were dropped onto clean cover glasses, fixed overnight with 2.5% glutaraldehyde at 4°C. After washing with PBS for three times, the samples were fixed with 1% OsO_4 for 2 h. Then, samples were washed with PBS for three times, and dehydrated sequentially in ethanol of gradient concentrations (50%, 70% and 90%) for 5 min each. Next, the samples were rinsed in 100% ethanol twice for 7 min each. After dehydration, samples were embedded in a resin at 25°C for 4 h. After polymerization at 65°C for 48 h, the samples were sliced with an EM UC7 ultramicrotome device (Leica, Wetzlar, Germany), stained with uranyl acetate for 20 min, followed by alkaline lead citrate for 10 min. Finally, the prepared samples were observed under a TECNAI 10 transmission electron microscope (Philips, Amsterdam, The Netherlands).

LDH release assay

The bacterial cell leakage was evaluated by LDH release using a LDH Cytotoxicity Assay Kit (C0016; Beyotime Biotechnology, Shanghai, China). The *P. aeruginosa* PAO1 was exposed to CAP for 3 min and followed by 1 h of incubation. Then, the sample was centrifuged at 10 000 *g* for 2 min at 4°C, and 120 μl of supernatant was mixed with 60 μl of LDH working solution (a mixture of lactic acid, 2-p-iodophenyl-3-nitrophenyl tetrazolium chloride and diaphorase at a ratio of 1:1:1). The mixture was incubated at dark for 30 min at 25°C, the optical density values were measured at both 490 nm (sample response) and 600 nm (reference) with a spectrophotometer (Thermo Fisher Scientific Oy, Vantaa, Finland).

Antioxidant activities of bacterial pigments detected by FRAP method

The total antioxidant capacity of bacterial pigments in the cultures of *P. aeruginosa* PAO1 or its mutant strain *phzS*::Tn was determined by the Ferric Reducing Ability of Plasma (FRAP) method using a Total Antioxidant Capacity Assay Kit (S0116; Beyotime Biotechnology). Antioxidant Trolox (an analogue of vitamin E) and LB broth was used as positive and negative controls respectively. In brief, strains PAO1 and *phzS*::Tn were cultivated overnight, and the culture was centrifuged at 10 000 *g* for 10 min. Then 5 μl of supernatant was mixed with 180 μl of FRAP working solution (a mixture of diluted solution, tripyridyltriazine and detection buffer at a ratio of 10:1:1). After incubation at 37°C for 5 min, the optical density value was measured at 593 nm in a spectrophotometer (Thermo Scientific).

Statistics analysis

Data were presented as mean ± SD. Statistical analysis was performed using GraphPad Prism version 5.0. Statistical evaluation was performed by the paired student's *t*-test to treat samples between two groups. One-way ANOVA was used to compare the data difference between groups. The results were considered statistically significant at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

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Conflict of interest

We declare that we have no competing financial interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Extraction and identification of pigments in the culture of *P. aeruginosa* PAO1. (A) The pigments in the suspension of *P. aeruginosa* PAO1 after CAP treatment (CAP) were extracted with chloroform, the pigments from untreated bacterial culture served as control (Con). (B) The pigments were identified with acidized method by the addition of hydrochloric acid. The solution turned pink indicated the existence of pyocyanin.

Fig. S2. The pigment phenotypes of *P. aeruginosa* PAO1 and its mutant cultures. The *P. aeruginosa* wild-type PAO1 and its derivative mutants (*phzA1*::Tn, *phzA2*::Tn, *phzM*::Tn, and *phzS*::Tn) were cultured in LB broth for five days at 28°C, and the cultures were centrifuged 15 000 *g* for 5 min at 4°C. Then, supernatants in the Eppendorf tubes were photographed, and the OD_{520} value of the supernatants was read in a spectrophotometer.

Fig. S3. Effects of CAP on *P. aeruginosa* PA1 and its derivatives. The *P. aeruginosa* PA1 and its derivatives as indicated were separately exposed to CAP for 30 s and followed 1 h incubation at room temperature. The survival cells were determined with the plate dilution method. Experiments were conducted five times. Statistical significance was calculated using One-way ANOVA. $*P < 0.05$, and $***P < 0.001$ relative to that of PA1-WT.

Fig. S4. Protective role of the pure PCA in *P. aeruginosa* under CAP treatment. High dose of pure PCA (1 mM) was added to the bacterial solution of *P. aeruginosa* PAO1. Then the suspension was exposed to CAP for 30 s and followed 1 h incubation. The survivors were counted. Experiments were conducted five times. Statistical significance was calculated using One-way ANOVA. $***P < 0.001$.

Fig. S5. Evaluation of the toxic effects of PCA, pyocyanin and vitamin E on PAO1 cells. *P. aeruginosa* PAO1 solution were added with different doses of PCA, pyocyanin or vitamin E (0, 10 nM, 1 μM and 100 μM) and were incubated for 1 h. The survivors were determined with the plate dilution method. Experiments were conducted five times. Statistical significance was calculated using One-way ANOVA, NS represented no statistical significance compared with untreated PAO1.

Fig. S6. Protective role of the pure pyoverdine in *P. aeruginosa* under CAP treatment. The different doses of pyoverdine (0, 10 nM, 1 μM and 100 μM) were added to the bacterial solution of *P. aeruginosa* PAO1. Then the suspension was exposed to CAP for 30 s and followed 1 h incubation. The survivors were counted. Experiments were conducted five times. Statistical significance was calculated using One-way ANOVA. $***P < 0.001$ relative to untreated PAO1; NS represented no statistical significance compared with untreated PAO1.

Table S1. All primers used in this study.

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