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Impact of Sublethal Levels of Single-Wall Carbon Nanotubes on Pyoverdine Production in *Pseudomonas aeruginosa* and Its Environmental Implications

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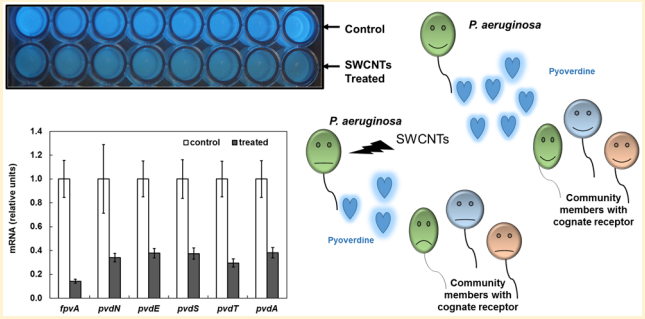
Supporting Information

ABSTRACT: Although bactericidal activities of nanomaterials against environmental bacteria have been extensively studied, little is known about the sublethal impacts of nanomaterials, which is a critical gap in our comprehensive understanding of the impacts of nanomaterials on microbial ecosystems. Using *Pseudomonas aeruginosa* as a model organism, we report for the first time that a sublethal level of single-wall carbon nanotubes (SWCNTs) (40 or 80 μg/mL) inhibited the production of pyoverdine, an important metabolite that is involved in interactive behavior of microbial communities. Transcriptional assay and quantitative polymerase chain reaction analysis revealed a decrease (up to 85%) in the level of expression of genes involved in biosynthesis and transport of pyoverdine in the presence of SWCNTs. Pyoverdine produced by certain bacteria in environmental microbial communities can be exploited by other bacteria in the local communities and has been implicated as playing an important role in establishing intercellular interactions. Our results of the inhibition of pyoverdine production in *P. aeruginosa* by SWCNTs at sublethal concentrations imply an important sublethal impact of SWCNTs on cell−cell interactions in microbial communities that often exist and play critical roles in maintaining the health of ecosystems in various natural and engineered environments.



INTRODUCTION

Carbon nanotubes (CNTs) are long, hollow cylindrical nanostructures wrapped up by graphene sheets.[1] On the basis of the number of graphene sheets present, CNTs can be catalogued as single-wall CNTs (SWCNTs) and multiwall CNTs (MWCNTs). CNTs have many potential applications in various fields because of their unique physical, electrical, and mechanical properties. For example, CNTs can be used in environmental applications as sensors, sorbents, and filters for removing pathogens and viral particles,[2−4] in biomedical applications such as detection, imaging, and drug delivery,[5−7] and in high-strength composites and energy storage devices.[8] SWCNTs have been demonstrated to exhibit antimicrobial activities stronger than those of MWCNTs.[9−13]

The potential use of SWCNTs in commercial and industrial settings along with their strong antibacterial activities has raised serious concerns about their impacts on human health and the environment. Previous studies of the toxicity of SWCNTs mainly focused on human health, and very recently, some important work on their environmental impacts has been conducted.[14] The SWCNTs in the environment will potentially impact organisms at all levels of the food chain. The impacts exerted on bacteria are of particular interest because these organisms are at low trophic level and play critical roles in natural and engineered ecosystems.[15] Most studies working on bacterium−SWCNT interactions focused on the toxicity to individual bacteria.[10,11] However, SWCNTs in the environment are expected to be present at low concentrations that may not significantly affect bacterial viability or growth.[16] Little is known about the sublethal impacts of SWCNTs on bacterial functions, which is a critical gap toward a comprehensive understanding of the impacts of SWCNTs on ecosystems.

In various natural and engineered environments, microorganisms are often present in polymicrobial communities in which cells actively interact with each other. Microorganisms in communities are functionally linked to each other, and any changes in functions of specific bacteria may cause restructuring of the whole microbial communities and affect the ecosystems. In microbial communities, certain bacteria excrete metabolites

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that are beneficial not only to themselves but also to other bacteria in the local community, known as "public goods", allowing cell−cell interactions.[17,18] Primary examples of such "public goods" in environmental microbial communities are siderophores, a group of organic compounds produced by bacteria to facilitate the uptake of iron, an essential nutrient for bacterial growth. Siderophores produced by bacteria growing on plant roots also enhance plant−microbe interactions[19] and drive microbial evolution.[20,21] *Pseudomonas aeruginosa* is a ubiquitous environmental bacterium that produces siderophores,[22,23] and pyoverdine (PVD) is one of its most important siderophores that is involved in intraspecific and/or interspecific cell−cell interactions and the establishment of infections.[24,25] Any changes in PVD production in *P. aeruginosa* may change the interactive behavior of whole microbial communities.

The objective of this study was to explore sublethal impacts of SWCNTs on environmental bacteria. Specifically, using *P. aeruginosa* as a model environmental bacterium, we examined the impacts of SWCNTs on the production of PVD, a "public good" that has been implicated to play a critical role in environmental microbial communities.

MATERIALS AND METHODS

Synthesis of SWCNTs. SWCNTs were synthesized by the arc-discharge method (AP-SWNT, tube diameter of ∼1.4 nm, Carbon Solutions, Inc.) and purified by a centrifugation-based method.[26] Surface functional groups were added by refluxing purified nanotubes in concentrated nitric acid (68%, Merck) for 3 h. The functionalized nanotubes were recovered by filtration and washed with a 0.2 M sodium hydroxide solution (Merck) to remove small carbon debris generated during acid treatment.[27] The solid was then washed with ∼500 mL of distilled (DI) water to remove any adsorbed ions and dried in a vacuum oven overnight. A suspension of 1 mg/mL nanotubes was prepared by tip sonication performed at 20 W for 1 h in an ice−water bath (Sonics, VCX-130). The ζ potential of the nanotube suspension was measured using a ZetaPALS particle size analyzer (Brooks Instrument). At pH 6.3, the ζ potential was -39.30 ± 1.02 mV.

Cell Viability Assay. Bacterial viability was tested by the drop plate method.[28−30] *P. aeruginosa* PAO1 (ATCC 15692) cultures were grown in the presence of SWCNTs (0, 40, or 80 μg/mL) at 37 °C for 24 h in a shaking incubator (200 rpm, initial OD_{600} of ∼0.08). For the viability assay, samples were withdrawn at regular intervals and diluted 10−10^8-fold, and 10 μL of each dilution was dropped onto LB agar plates. Eight replicates for each dilution were used. After being incubated for 24 h, colonies were enumerated as colony-forming units (CFUs).[28]

Quantification of PVD in *P. aeruginosa* Cultures. ABT minimal medium [15 mM $(NH_4)_2SO_4$, 40 mM Na_2HPO_4, 20 mM KH_2PO_4, 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.01 mM $FeCl_3$] supplemented with 30 mM glucose and 5 g/L casamino acids (ABTGC) was used to grow *P. aeruginosa* at 37 °C[31] in the absence or presence of SWCNTs at different concentrations (0, 40, or 80 μg/mL). The PVD fluorescence (excitation at 398 nm, emission at 460 nm)[32] and optical density at 600 nm (OD_{600}) were recorded using a TECAN infinite M200PRO plate reader. The cell density was determined by a drop plate-based CFU count method.[30] All the fluorescence measurements were taken together with cell-free controls to rule out the abiotic effects of SWCNTs. In addition, conditioned media (cell-free supernatant of overnight cultures) supplemented with SWCNTs (40 or 80 μg/mL) were used as another set of controls to check for the interference of SWCNTs on the fluorescence measurements.

Transcriptional Assay for *pvdA*. To investigate the effect of SWCNTs on the gene involved in PVD precursor biosynthesis, a *pvdA::gfp* reporter strain[33,34] was used, where the expression of gene *pvdA* can be monitored by GFP fluorescence. Gene *pvdA* is a key gene involved in PVD precursor biosynthesis in cytoplasm. The reporter strain was cultivated in ABTGC medium at 37 °C with and without SWCNTs at different concentrations (0, 40, or 80 μg/mL). A TECAN infinite M200PRO plate reader was used to monitor GFP fluorescence (excitation at 485 nm, emission at 535 nm) and OD_{600}.

Flow Cell Biofilms. The influence of SWCNTs on biofilms was evaluated using multichannel flow cells (BioCentrum-DTU). The dimensions and assembly of the flow cell systems have been described elsewhere.[35,36] Each channel of the flow cells was inoculated using 0.4 mL diluted overnight cultures of GFP-tagged *P. aeruginosa* PAO1 in M9 minimal medium (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$ supplemented with 0.4% glucose) (OD_{600} of 0.15). After inoculation, medium flow was stopped to allow the cells to attach (∼1 h) to the glass coverslips used in the flow cells. Then air-saturated medium was continuously supplied with a flow rate of 8 mL/h for biofilm growth in each channel. After 96 h, SWCNTs were introduced to the biofilms along with the medium at a concentration of 20 μg/mL.

Confocal Laser Scanning Microscopy (CLSM) Imaging. Biofilms grown in the flow cell systems were imaged (20× magnification) using a confocal laser scanning microscope (Carl Zeiss Microscopy LSM 780) equipped with detectors and filter sets. The GFP fluorescence of the cells was observed with excitation at 488 nm and emission at 509 nm. PVD fluorescence was observed with excitation and emission at 405 and 461 nm, respectively. IMARIS version 7.6.4 (Bitplane, Zurich, Switzerland) was used to analyze the confocal images.[36,37]

Quantification of Cell Detachment. Detachment of cells from the biofilms was quantified using a drop plate method.[28] Briefly, the effluents from the flow cells were collected in regular intervals and serially diluted. Then, six replicates of 10 μL from each of selected dilutions were plated on LB agar medium. Colony-forming units (CFU) were enumerated after overnight incubation at 37 °C.

Inhibition of PVD Biosynthesis by SWCNTs in *Pseudomonas fluorescence*. *P. fluorescence* strain OE28.3[38] was grown in ABTGC medium at 30 °C in the presence of varying concentration of SWCNTs. PVD fluorescence (excitation at 398 nm, emission at 460 nm)[32] and optical density at 600 nm (OD_{600}) were recorded using a TECAN infinite M200PRO plate reader.

Quantitative Polymerase Chain Reaction (qPCR). *P. aeruginosa* PAO1 was grown in ABTGC medium at 37 °C in the absence or presence of SWCNTs (40 μg/mL). After 9 h, cells were harvested and total RNA was extracted using a commercially available kit (Qiagen mini RNA prep) following the instructions from the manufacturer. Method details and the primers designed and used in this study (Table S1 of the Supporting Information) are available in the Supporting Information. Experiments were performed in triplicate for all

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the genes. Genes *tsf* and *rpoD* were used as reference genes for normalization.[39] Data analysis was performed using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_T$(treated sample) − ΔC_T(untreated sample), $\Delta C_T = C_T$(target gene) − C_T(*tsf*), and C_T is the threshold cycle value for the amplified gene.[40]

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Cells were grown in the presence and absence of 40 μg/L SWCNTs in ABTGC medium for 24 h and harvested by centrifugation at 6000*g* for 15 min. The cell pellets were lysed at 4 °C in lysis buffer (0.1% SDS, 0.5 M TEAB, and 50 mM protease inhibitor tablet) with intermittent vortexing and sonication for 15−20 min on an MSE-Soniprep-150 Sonicator (Sanyo Gallenkamp, Leicestershire, U.K.) for 5 s with a 10 s cooling interval between each pulse. The lysate was filtered through a 0.2 μm syringe filter. The iron concentration was quantified using an ICP-OES instrument (PerkinElmer Optima DV2000). To determine the leaching of iron from SWCNTs, iron in the suspension of 40 μg/L SWCNTs in the ABTGC medium was also measured.

Statistical Analysis. The IBM SPSS (www.ibm.com/software/sg/analytics/spss/) was used for statistical analyses, including two-way analysis of variance (ANOVA). Details of the statistical analyses can be found in the respective result sections.

RESULTS AND DISCUSSION

The fluorescence of PVD is often used to reflect PVD production in *P. aeruginosa* cultures because PVD is a water-soluble fluorescent siderophore.[31] In the presence of SWCNTs, the signal of PVD fluorescence (normalized by growth as indicated by cell density in CFU per milliliter) in *P. aeruginosa* cultures was attenuated significantly in a SWCNT concentration-dependent manner (Figure 1). We quantified the PVD fluorescence of conditioned medium (cell-free culture supernatant) before and after supplementation with SWCNTs and found no interference of SWCNTs with PVD fluorescence measurement. At a concentration of 40 or 80 μg/mL, SWCNTs caused a 51−70 or 85−96% decrease, respectively, in PVD production capability of *P. aeruginosa* cells in exponential (6−12 h) and stationary (15−24 h) growth stages. A two-way

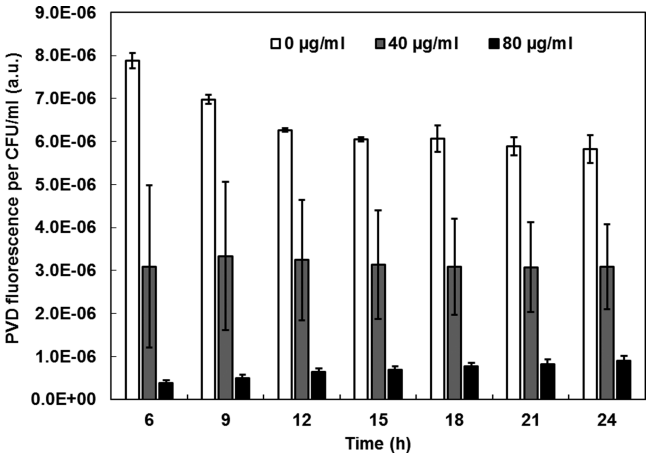

Figure 1. Influence of SWCNTs on PVD fluorescence (normalized by cell density) in *P. aeruginosa* cultures growing in ABTGC medium. Data shown are means ± the standard deviation (n = 3). A two-way ANOVA shows that no significant effect from time or the time−concentration interaction can be found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

Table 1. Densities of Viable Cells in *P. aeruginosa* Cultures with or without SWCNTs[a]

SWCNTs (μg/mL)	cell density (CFU/mL) 0 h	24 h	ratio (24 h vs 0 h)
0	(1.30 ± 0.19) × 10^7	(1.15 ± 0.26) × 10^{11}	8.8 × 10^3
40	(1.77 ± 0.27) × 10^7	(1.25 ± 0.33) × 10^9	71
80	(1.80 ± 0.21) × 10^7	(1.18 ± 0.24) × 10^9	65

[a]Data shown are means ± the standard deviation (n = 8).

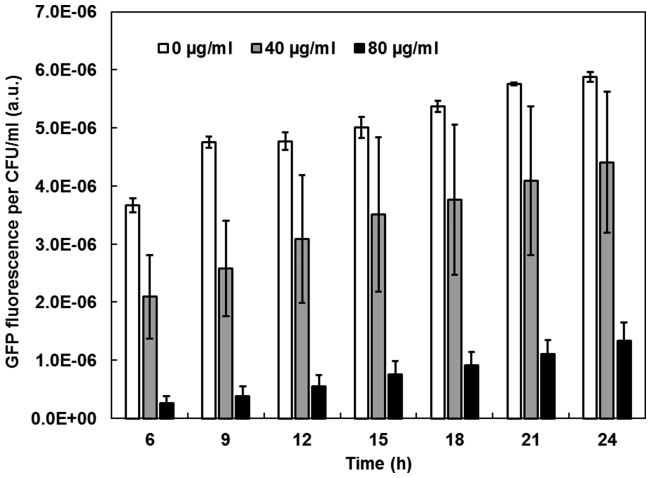

Figure 2. Influence of SWCNTs on GFP fluorescence in *P. aeruginosa* reporter strain P_{pvdA}-*gfp* growing in ABTGC medium. Data shown are means ± the standard deviation (n = 3). A two-way ANOVA shows that no significant effect from time or the time−concentration interaction can be found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

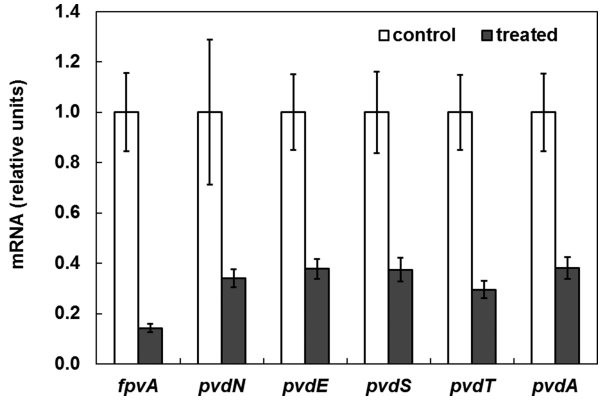

Figure 3. Influence of SWCNTs (40 μg/mL) on the expression level of key genes involved in PVD production at 9 h in the midexponential growth phase. Values were normalized to the housekeeping gene *tsf*. The C_T values for *tsf* remained unchanged for the control and the treated cells (24.68 ± 0.92 and 23.84 ± 0.30, respectively), suggesting that the expression of *tsf* is not affected by SWCNTs. Abbreviations: PvdS, transcriptional regulator; PvdE, export ABC transporter to transport PVD precursors across the inner membrane; PvdN, periplasmic enzymes involved in chromophore formation; PvdT, subunit of the ATP-dependent efflux pump for PVD secretion; FpvA, outer membrane transporter to import ferri-pyoverdine; pvdA, lysine/ornithine *N*-monooxygenase. Data shown are means ± the standard deviation (n = 3). Statistical significance determined by a paired Student's t test ($p < 0.001$).

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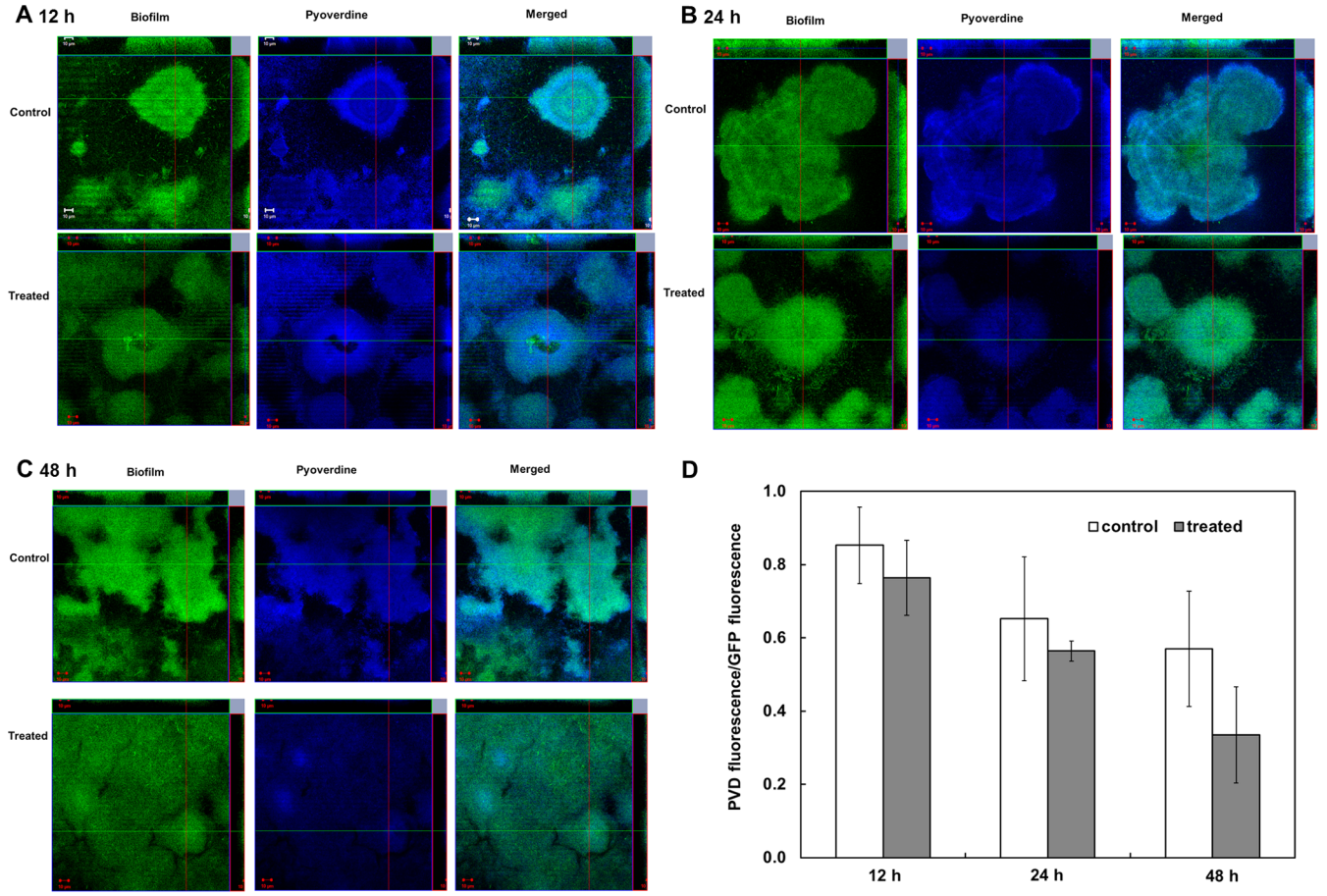

Figure 4. CLSM images of mature *P. aeruginosa* biofilms upon exposure to SWCNTs (20 μg/L) for (A) 12, (B) 24, and (C) 48 h. The control biofilms were grown in parallel with the SWCNT-treated biofilms. Controls in panels A–C were biofilms grown in the absence of SWCNTs for 108 h (96 h + 12 h), 120 h (96 h + 24 h), and 144 h (96 h + 48 h), respectively. (D) Biovolume ratio of PVD-producing biomass to total biofilm biomass, indicating the amount of PVD in the unit biovolume of the biofilms. CLSM images contain top-down views (x–y planes) and side views (x–z and y–z planes). The scale bar is 10 μm. Results are representative of three separate experiments.

ANOVA was performed to investigate the effect of time and concentration. No significant effect from time or the time–concentration interaction was found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

We also observed a reduced rate of rowth of *P. aeruginosa* in the presence of SWCNTs (Table 1), which is consistent with previous reports demonstrating an antibacterial activity of SWCNTs against *P. aeruginosa*.[3,11] The minimal inhibitory concentration (MIC) of SWCNTs under our experimental conditions was ~200 μg/mL. No cell growth could be observed at a lethal dose (>200 μg/mL). In the presence of 40 or 80 μg/mL SWCNTs, cell growth was still observed, resulting in a final cell density of $(1.25 \pm 0.33) \times 10^9$ or $(1.18 \pm 0.24) \times 10^9$, respectively. Hence the question of whether there are any sublethal impacts of SWCNTs on the cells arises. In particular, in this study, we examined whether SWCNTs affect the PVD production of viable cells. To address this question, a biomarker strain that allows the monitoring of the expression of a key gene involved in PVD biosynthesis was employed.

Gene *pvdA* encodes an enzyme L-ornithine N5-oxygenase responsible for the hydroxylation of L-ornithine, which represents an early step in the biosynthesis of PVD in *P. aeruginosa*.[41] To monitor PVD biosynthesis in viable cells, a reporter strain P_{pvdA}-*gfp* in which a gene encoding green fluorescent protein (GFP) was under control of the promoter of gene *pvdA* was employed, and the GFP fluorescence was used as an indicator to monitor the biosynthesis of PVD. Figure 2 shows the GFP fluorescence (per CFU per milliliter) of biomarker strain P_{pvdA}-*gfp* in planktonic cultures with or without SWCNTs. From a two-way ANOVA using the factors time and concentration, no significant effect from time or the time–concentration interaction was found (both $p > 0.9995$), while the effect of concentration is significant ($p < 0.0005$).

In the presence of SWCNTs, GFP fluorescence was markedly lower than that of the cultures without SWCNTs, which suggests that the expression of gene *pvdA*, and hence, PVD biosynthesis, was significantly inhibited by SWCNTs. To confirm this, the expression of several key genes involved in PVD biosynthesis was quantified by using qPCR. Relative gene expression levels as impacted by SWCNTs at a concentration of 40 μg/mL are shown in Figure 3.

Genes responsible for the biosynthesis of PVD precursors (*pvdA*), transferring PVD precursors from cytoplasm to the periplasmic space (*pvdE*), maturation of PVD precursors in the periplasmic space (*pvdN*), and exporting mature PVD out of the cells (*pvdT*) were found to be downregulated in the cells treated with SWCNTs (Figure 3). In addition, the level of expression of two genes encoding a transcriptional regulator (*pvdS*) and a siderophore-binding protein (*fpvA*) also decreased in the cells treated with SWCNTs (Figure 3).

In *P. aeruginosa*, there are three well-characterized quorum-sensing systems, namely, *las*, *rhl*, and *pqs*,[42] that interact with

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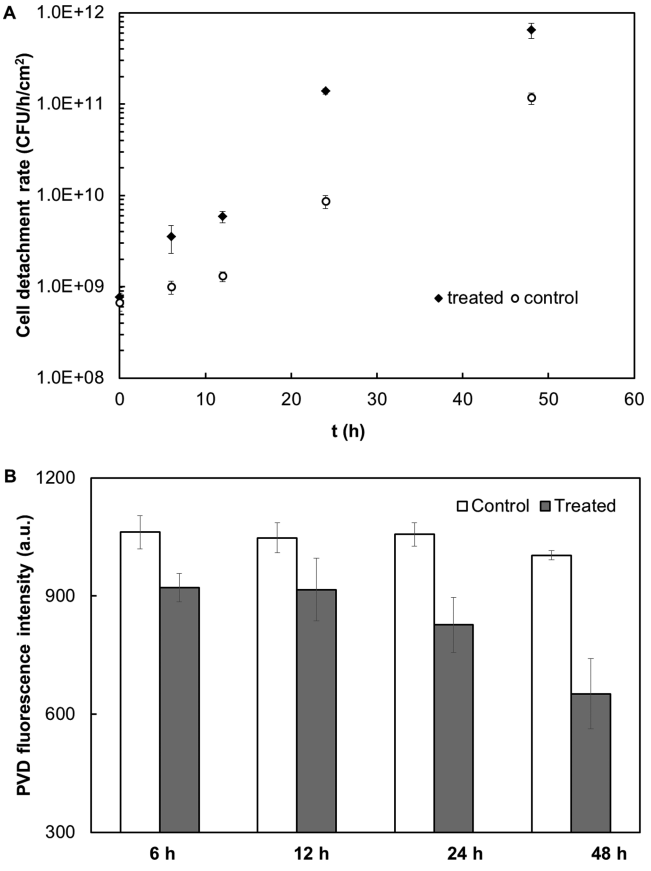

Figure 5. (A) Cell detachment and (B) PVD fluorescence in effluents from mature *P. aeruginosa* biofilms in the absence or presence of SWCNTs (20 μg/L). Experiments were conducted in triplicate.

each other and coordinate the expression of a number of genes, including those responsible for PVD production.[43−45] To further examine whether the inhibitory effect of SWCNTs on PVD production was caused by an influence on bacterial quorum sensing signaling, we quantified by using qPCR the expression of the key quorum-sensing genes *lasA*, *rhlA*, and *pqsA* in the bacterial cultures with and without SWCNTs treatment. The results show that the presence of SWCNTs did not inhibit the expression of these quorum-sensing genes (Figure S1 of the Supporting Information). Compared with the cultures with no SWCNTs, the quorum-sensing systems in the treated cultures were slightly enhanced, suggesting that the influence of SWCNTs on quorum sensing is not the primary cause of the decreased level of PVD production.

An intriguing question one would ask is how SWCNTs at the cell exterior affect gene expression inside the cells. In previous studies, Dimpka et al. reported that CuO nanoparticles inhibited PVD production in *P. chlororaphis* O6, where Cu ion release has been implicated to play a key role.[46,47] In this study, we show that SWCNTs inhibit PVD production at the gene transcriptional level. Could it be because SWCNTs release ions, in particular, iron to the growth medium? The iron level in growth media is known to be a key factor influencing PVD biosynthesis. A high iron level of inhibits PVD production. In this study, the synthesis of SWCNTs did not involve iron, and the absence of iron in the SWCNTs was also confirmed by elemental mapping using transmission electron microscopy−energy-dispersive X-ray spectroscopy (TEM−EDX) (data not shown). We further employed ICP-OES to quantify iron concentrations in the medium with or without SWCNTs and found that the presence of SWCNTs slightly decreased the iron levels in the medium [0.89 ± 0.01 or 1.00 ± 0.02 μM, respectively ($p < 0.05$)], which could be due to the adsorption of iron onto the SWCNTs. Hence, the possibility of an additional supply of iron from SWCNTs to the cultures can be ruled out. Interestingly, although SWCNTs inhibit PVD production, the iron level in the cells grown in the presence of SWCNTs (350 ± 1 pmol/mg of protein) was found to be slightly higher than that of the control cells (322 ± 5 pmol/mg of protein) ($p < 0.05$). These results suggest that, in addition to PVD and, hence, the PVD-mediated iron uptake system, SWCNTs may also affect other iron uptake systems.[48,49]

In a recent study, we have shown that biogenic tellurium nanorods could inhibit PVD production by interfering with flagellar motility.[50] On the basis of the data obtained in this study and previous work, we hypothesize that certain extracellular appendages of the bacteria such as surface proteins, exopolysaccharides, pili, and flagella might be interacting with the SWCNTs and transmit the signals across cell membranes. However, the exact mechanism for PVD inhibition by SWCNTs requires further investigation.

In natural environments, microorganisms are often found as surface- or interface-associated assemblies encased in a self-produced polymeric matrix known as biofilms.[30,51,52] In addition, it has been shown that PVD is an important factor influencing biofilm formation of *P. aeruginosa*.[53] Hence, we further examined the influence of SWCNTs on *P. aeruginosa* biofilms using a GFP-tagged strain in flow cell biofilm reactors. After 96 h, mushroom-shaped multicellular towers, typical three-dimensional structures of mature *P. aeruginosa* biofilms, could be observed (Figure S2 of the Supporting Information). SWCNTs were then introduced into the pregrown mature biofilms, and PVD production in the biofilms was evaluated on the basis of PVD fluorescence obtained from CLSM image analysis. Quantitative image analysis revealed that the PVD fluorescence (blue) in the mature biofilms (green) in the SWCNTs-treated (up to 48 h) and the control biofilms was comparable (Figure 4), suggesting no significant influence of SWCNTs on PVD production in mature *P. aeruginosa* biofilms (all $p > 0.05$). Intriguingly, in the presence of SWCNTs, the rate of detachment of the cell from the biofilms was higher and PVD fluorescence in the effluents from the SWCNT-treated biofilms was lower than that of the control (Figure 5). Taken together, our results show that the exposure to SWCNTs for up to 48 h did not significantly influence PVD production in cells encased in mature biofilms; however, it promoted detachment of the cell from the biofilms and decreased the level of PVD production of the detached cells.

Production of siderophores is a highly conserved characteristic in many environmental bacteria, especially *Pseudomonas* species.[54] For example, *P. fluorescence* is a plant root colonizer commonly found in soils, and it protects plant roots from fungal infection by scavenging iron with the help of siderophores.[55,56] SWCNTs could also inhibit siderophore production in *P. fluorescence* in a concentration-dependent manner (Figure S3 of the Supporting Information), suggesting that the inhibitory effect of SWCNTs on siderophore production is not limited to the model organism, i.e., *P. aeruginosa*, used in this study and similar effects may be observed for many other environmental bacteria.

Implications. Environmental bacteria in natural settings communicate, cooperate, or compete with each other. These

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interactions are often mediated by extracellular metabolites such as siderophores. Siderophores are produced by certain environmental bacteria and can be effectively shared among closely related species and can give competitive advantage to some species over others in any ecological niche. This study reports for the first time that SWCNTs at sublethal concentrations inhibit siderophore production in an environmental bacterium. Siderophores are secreted by certain environmental bacteria that can be exploited by local community members with cognate receptors and implicated as key components in establishing microbial communities. Our results reveal an important sublethal impact of SWCNTs on siderophore-enabled cell−cell interactions in microbial communities, which often exist and play critical roles in maintaining the health of ecosystems in various natural and engineered environments.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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