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| Key Words: | Shewanella oneidensis, Pseudomonas aeruginosa, pyoverdine, antivirulence, tellurium, nanomaterial |
Biogenic Tellurium Nanorods as a Novel Antivirulence Agent Inhibiting Pyoverdine Production in Pseudomonas aeruginosa

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Abstract

While antibiotic resistance in bacteria is rapidly increasing, the development of new antibiotics has decreased in recent years. Antivirulence drugs disarming rather than killing pathogens have been proposed to alleviate the problem of resistance inherent to existing biocidal antibiotics. Here, we report a nontoxic biogenic nanomaterial as a novel antivirulence agent to combat bacterial infections caused by *Pseudomonas aeruginosa*. We synthesized, in an environmentally benign fashion, tellurium nanorods (TeNRs) using the metal-reducing bacterium *Shewanella oneidensis*, and found that the biogenic TeNRs could effectively inhibit the production of pyoverdine, one of the most important virulence factors in *P. aeruginosa*. Our results suggest that amyloids and extracellular polysaccharides Pel and Psl are not involved in the interactions between *P. aeruginosa* and the biogenic TeNRs, while flagellar movement plays an important role in the cell-TeNRs interaction. We further showed that the TeNRs (up to 100 µg/ml) did not exhibit cytotoxicity to human bronchial epithelial cells and murine macrophages. Thus, biogenic TeNRs hold promise as a novel antivirulence agent against *P. aeruginosa*.

**Keywords:** tellurium, nanomaterial, *Shewanella oneidensis*, *Pseudomonas aeruginosa*, pyoverdine, antivirulence
Introduction

Bacteria mediated infectious disease is one of the leading causes of death worldwide (Mellbye and Schuster 2011). Traditional antibiotics often kill bacteria or inhibit bacterial growth by targeting functions essential for bacterial viability, such as cell wall biosynthesis, protein synthesis, transcription or DNA replication (Walsh 2003). Hence, bacteria may undergo mutations and rapidly develop highly resistant sub-populations upon exposure to antibiotics (Werner et al. 2008). Indeed, drug resistance has become a major challenge to the treatment of infectious diseases (Levy and Marshall 2004; Wright 2007), with the emergence of multi-drug resistant strains (MDRs) in hospitals across the globe being a serious threat. Development and commercialization of new antibiotics have not been able to meet the growing demand for effective antimicrobials, and there are currently no new broad spectrum antibiotics in the pharmaceutical pipelines (Fernandes 2006; Projan 2003; Talbot 2006).

An alternative approach to combat bacterial infections is to develop antivirulence drugs that disarm pathogens by targeting the production of virulence traits such as bacterial toxins (Cegelski et al. 2008; Marra 2004; Rasko and Sperandio 2010). Because the production of many virulence factors are controlled by intercellular communication or quorum sensing (QS) (Rutherford and Bassler 2012), QS is one of the most common targets for new antivirulence drugs (Kaufmann et al. 2008; Rasko and Sperandio 2010). For example, a number of QS inhibitors with efficacy against *Pseudomonas aeruginosa* QS have been developed (Rasmussen and Givskov 2006). However, recent studies revealed that bacteria might also evolve resistance to antivirulence compounds such as QS inhibitors (Garcia-Contreras et al. 2013; Maeda et al. 2012; Mellbye and Schuster 2011). Hence, there is an emergent need to develop non-traditional antivirulence drugs that do not lead to bacterial resistance. Preferably the agents should have multidimensional non-lethal actions targeting...
different parts and/or biological processes including virulence factors so that the possibility of
developing resistance is considerably diminished. Such a solution may be found in
nanomaterials. Although a variety of nanomaterials have been demonstrated to exhibit
excellent bactericidal (killing) or bacteriostatic (growth-inhibiting) activities (Klaine et al.
2008; Marambio-Jones and Hoek 2010), nanomaterials that can effectively attenuate bacterial
virulence at sublethal concentrations have never been reported.

*Pseudomonas aeruginosa* is an opportunistic bacterial pathogen causing infections in
wounds, burns, ventilator-associated pneumonia and cystic fibrosis. *P. aeruginosa* needs iron
at a concentration of $10^{-6}$ M to grow and establish infections; however, iron concentration in
human serum is at an order of $10^{-24}$ M (Meyer et al. 1996). One strategy used by *P.
aeruginosa* to acquire iron is to produce iron-binding siderophores such as pyoverdine (PVD)
to facilitate its iron uptake. PVD also regulates the production of other virulence factors such
as the endoprotease exotoxin A (Lamont et al. 2002). PVD is considered one of the most
important virulence factors in *P. aeruginosa* and it is required for full expression of virulence
in *P. aeruginosa* infection (Takase et al. 2000). Hence, PVD production can be used as a
promising target for potential antivirulence agents against *P. aeruginosa* (Smith et al. 2012).

Herein, we describe for the first time a biogenic nanomaterial that can significantly
inhibit the production of PVD, one of the most important virulence factors secreted by *P.
aeruginosa* for persistent infections. Specifically, we obtained tellurium(0) nanorods (TeNRs)
through bioreduction of tellurite by a metal-reducing bacterium *Shewanella oneidensis* MR-1
and demonstrated that the biogenic TeNRs could effectively inhibit the production of PVD in
*P. aeruginosa*. We further showed that the TeNRs (up to 100 µg/ml) did not exhibit
cytotoxicity to mammalian cells.

**Materials and Methods**
Biosynthesis of TeNRs by *S. oneidensis*

Stock cultures of *S. oneidensis* MR-1 were maintained in LB medium with 20% glycerol at -80°C. The bacterial cells were grown in LB medium containing 100 µg/ml potassium tellurite. The cultures were bubbled with nitrogen gas for 5 min and the headspace in the culture was less than 3% of the total volume. After 72 h, cells were removed by centrifugation at 5,000×g for 20 min. Only extracellular TeNRs in the supernatant were harvested and used in this study because harvesting TeNRs from bacterial cells would require cell lysis, which might cause contamination of TeNRs by various biomolecules. The supernatant was then concentrated using Millipore Amicon centrifugal filter units (MWCO ~3 kDa). Te nanorods (TeNRs) were harvested by using ultracentrifugation at 100,000g for 30 min, washed three times with MilliQ water and dried at 50°C under vacuum.

**Epifluorescence and Electron Microscopic Imaging**

After an overnight exposure to TeO$_3^{2-}$, *S. oneidensis* cell suspension was placed on a glass microscope slide and dried in an oven at 70°C. The dried sample was then stained using 4′,6-diamidino-2-phenylindole (DAPI). Briefly, 5 µl of DAPI solution (1 mg/mL) was dropped onto the sample and incubated at room temperature, in the dark, for 45 min. The glass slide was then rinsed with ultrapure water to remove excess DAPI. The stained samples were examined using an inverted epifluorescence microscope (Microscope Axio Observer.Z1, Carl Zeiss). Transmission electron microscopy (TEM) images and energy dispersive X-ray spectroscopy (EDS) were obtained following the procedures described previously (Shahjamali et al. 2012).

**Quantification of Pyoverdine Production in *P. aeruginosa***
P. aeruginosa PAO1 wild type and mutant strains (Table S1) were cultivated at 37°C in ABT minimal medium supplemented with 30 mM glucose and 5 g/l casamino acids (ABTGC) (Chua et al. 2013) in the absence or presence of TeNRs at different concentrations. Pyoverdine fluorescence (Ex 398 nm, Em 460 nm) (Greenwald et al. 2007) and optical density at 600 nm (OD$_{600}$) were recorded using a TECAN infinite M200PRO plate reader.

**Inductively Coupled Plasma Mass Spectrometry**

To determine the dissolution of TeO$_3^{2-}$ from the biogenic TeNPs, the suspension of 100 µg/ml TeNPs in ABTGC medium was filtered through a centrifugal filter unit (molecular weight cutoff 3 kDa; Amicon Ultra-4 3K). The total TeO$_3^{2-}$ concentration was quantified using an inductively coupled plasma mass spectrometer (ICP-MS) (ELAN DRC-e, Perkin Elmer; Waltham, MA) as described previously (Kim et al. 2012).

**PvdA-gfp Assay**

To investigate the impacts of TeNRs on biosynthesis of PVD in *P. aeruginosa*, the expression of a key PVD synthesis gene *pvdA* was monitored using a *pvdA::gfp* fluorescent reporter as described previously (Kaneko et al. 2007; Yang et al. 2009). The *pvdA::gfp* reporter strain was cultivated in ABTGC medium at 37°C in the absence or presence of TeNRs at different concentrations. GFP fluorescence (Ex 395 nm, Em 509 nm) and OD$_{600}$ were measured using a TECAN infinite M200PRO plate reader.

**Quantitative Polymerase Chain Reaction (qPCR)**

*P. aeruginosa* PAO1 was cultivated in ABTGC medium at 37°C in the absence or presence of TeNRs (50 µg/ml). After 9 h, 0.5 ml of the culture was mixed with 1 ml of RNA protect (Qiagen mini RNA prep) and centrifuged at 6,000g for 10 min. Total RNA content was
extracted using a commercially available kit (Qiagen mini RNA prep) following the instructions from the manufacturer. RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, DE, USA). Two µg of the total RNA was used for first-strand cDNA synthesis by using a commercially available kit (Fermentas Life Sciences, EU). The resultant cDNA was used in qPCR reactions on ABI StepOnePlus system (Life Technologies, CA). Primers (efficiency >90% for all primers) were obtained from Sigma Life Sciences and their sequences are listed in Table S2. PCR was carried out with 1 µl of first-strand cDNA in a total volume of 20 µl containing 0.2 mM of each primer, 10 µl of PCR KAPA SYBR® FAST qPCR Master Mix ABI (2X) Prism™, 0.4 µl ROX high.

Amplification parameters used were: initial activation 3 min at 95°C, then 40 cycles of 3 s at 95°C and 30 s at respective appropriate annealing temperature. Experiments were performed in triplicates for all the genes. The amount of each target gene was normalized to a reference gene tsf and compared to control samples (Xie et al. 2011). Data analysis was performed using the $2^{\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_T(\text{treated sample}) - \Delta C_T(\text{untreated sample})$, $\Delta C_T = C_T(\text{target gene}) - C_T(tsf)$, and $C_T$ is the threshold cycle value for the amplified gene (Livak and Schmittgen 2001).

**iTRAQ-based Proteomic Analysis**

Proteomic analysis was conducted using an iTRAQ-coupled two-dimensional LC-MS/MS technique as described previously (Zhou and Chen 2011). Briefly, cells of *P. aeruginosa* PAO1 grown in ABTGC medium in the absence or presence of 50 µg/ml TeNRs at late exponential growth phases were harvested by centrifugation at 3000xg for 20 min at 4°C and lysed in lysis buffer (50 mM NaCl, 5 mM DTT, 1 mM PMSF and 50 mM Tris-HCl, pH 8.0) by intermittent sonication. Unbroken cells were removed by centrifugation at 3000 $\times$ g for 10 min at 4 °C. The supernatants containing the cytoplasmic proteins were collected by
centrifugation at 120,000×g for 60 min at 4°C. Protein concentration was determined by Bradford assay using γ-globulin as a control. Proteins from each sample were precipitated by cold acetone at −20 °C. The proteins (~100 µg) were reduced, cysteine blocked, digested and labeled with respective isobaric tags using iTRAQ reagent Multiplex kit (Applied Biosystems Inc., CA, USA) according to manufacturer’s protocol. The analysis of iTRAQ-labeled peptide mixtures were performed on a combination of an Agilent 1200 nanoflow LC system (Agilent Technologies Inc., USA) and a 6530 Q-TOF mass spectrometer (Agilent Technologies Inc., USA). The identification and quantification of the proteins were performed using Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Software Revision A.03.03.084 SR4). Each MS/MS spectrum was searched for species P. aeruginosa against the UniProt protein database. The following criteria were required to consider a protein for further statistical analysis: two or more distinct peptides had to be identified and the fold change had to be greater than 2 or less than 0.5.

**Transposon Mutagenesis Library and Mutant Screening**

The P. aeruginosa ΔmotB and ΔfliF mutants were identified from a transposon mutagenesis library screen, which was constructed by using the Mariner transposon vector pBT20, as previously described (Wang et al. 2013). Both motB and fliF encode proteins involved in flagella-mediated motility. Transconjugants carrying transposon insertion were picked from the selective plates and inoculated into microtiter plate wells containing LB medium. Mutants that produced PVD at a comparable level in the presence and absence of 50 µg/ml TeNRs were selected and saved for further analysis. For identification of the transposon insertion site, the sequence flanking the Mariner transposon in selected mutants was identified by arbitrary polymerase chain reaction (PCR), as previously described (Friedman and Kolter 2004).

**Cytotoxicity Assay of Biogenic TeNRs**
Human bronchial epithelial cells (BEAS-2B; ATCC, Manassas, VA, USA) and murine macrophages (RAW264.7; ATCC, Manassas, VA, USA) were used to test cytotoxicity of the biogenic TeNRs. Both cell types were cultured in complete DMEM (high glucose Dulbecco’s Modified Eagle’s Medium; PAA Laboratories Inc., MA, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% Antibiotics/Antimycotics (PAA Laboratories) at standard culture conditions (37°C, 5% CO₂) and sub-cultured in the ratio of 1:2 to 1:4 at 90% confluency. All cells were passaged at least 3 times before using for the cytotoxicity experiments. Cytotoxicity was measured using the Cell Counting Kit-8 (CCK-8/WST-8; Dojindo Molecular Laboratories Inc., Japan) following the protocols provided by the manufacturer. Cells were seeded in 96-well plates at a seeding density of 10⁴ cells per well. After 24 h incubation, the culture medium was replaced by freshly prepared TeNRs suspension in the cell culture medium and incubated for another 22 h. Ten µl of CCK-8 was then added to each well and the cells were further incubated for 2 h after which the absorbance at 450 nm was measured. Wells containing cells and TeNRs suspension without CCK-8 was used as background.

Results and Discussion

Biogenic TeNRs from TeO₃²⁻ Reduction by S. oneidensis

TeNRs have been successfully prepared via physicochemical routes including chemical reduction (Gao et al. 2008; Liu et al. 2004; Zhu et al. 2004) and photolytic preparation (Webber and Brutchey 2009). The unique helical chain conformation of Te crystals typically leads to a highly anisotrophic growth to form one-dimensional (1D) structures such as nanorods (Webber and Brutchey 2009). TeNRs can also be produced by bacteria grown on Te oxyanions such as tellurite (TeO₃²⁻) (Baesman et al. 2007; Turner et al. 2012; Wang et al. 2011), which offers a “green” approach for the synthesis of Te nanomaterials under facile
conditions in the absence of organic solvents. For example, although TeO$_3^{2-}$ is highly toxic to most microorganisms and has been used as an antimicrobial agent for decades (Turner et al. 2012), S. oneidensis has been reported to be able to reductively transform TeO$_3^{2-}$ to Te(0) in the form of rod-like nanostructures, i.e., TeNRs, intracellularly and at the cell exterior (Kim et al. 2012; Klonowska et al. 2005).

TeNRs used in this study were obtained through microbial reduction of TeO$_3^{2-}$ by S. oneidensis using lactate as an electron donor. We observed black precipitates, presumably aggregates of TeNRs, at the cell exterior upon the exposure of S. oneidensis cells to TeO$_3^{2-}$ (Figure 1A). TEM-EDX showed that the biogenic TeNRs obtained here were about 10-20 nm wide, up to several hundreds of nm long, and were comprised entirely of Te (Figure 1B and C). In this study, we focused on the novel antivirulence activity of the biogenic TeNRs and further detailed material characterization of the TeNRs was not included. In fact, the reduction of TeO$_3^{2-}$ by S. oneidensis has been reported in literatures and the Te(0) nanorods obtained from bacterial reduction of TeO$_3^{2-}$ under similar experimental conditions have been extensively characterized (Kim et al. 2012; Klonowska et al. 2005).

**Inhibition of PVD Biosynthesis by Biogenic TeNRs**

In an effort to elucidate impacts of the biogenic TeNRs on microorganisms, we grew *P. aeruginosa* in the presence of TeNRs. Interestingly, TeNRs (up to 100 µg/ml) have no negative impact on bacterial growth, as evidenced by comparable or even slightly higher specific growth rate for all treated cultures, compared to the controls (Table S3), suggesting that the biogenic TeNRs do not exhibit bactericidal activity against *P. aeruginosa*. Chemically synthesized Te nanomaterials with different structures such as nanorice, nanopensil and nanowire have been reported to exhibit excellent antibacterial activity because of the oxidative dissolution of TeO$_3^{2-}$ from the Te nanomaterials (Lin et al. 2012).
Here, our biogenic TeNRs did not show bactericidal activity, possibly due to a high stability and resistance to molecular oxygen of the biogenic TeNRs. To confirm this, we used ICP-MS to quantify the concentration of $\text{TeO}_3^{2-}$ in the ABTGC medium with the presence of 100 µg/ml TeNRs but no $\text{TeO}_3^{2-}$ could be detected after 24 h. The resistance to oxidative dissolution and the lack of bactericidal activity of the biogenic TeNRs open a new window of novel applications other than being used as a bactericidal agent.

Although the biogenic TeNRs did not exhibit bactericidal activity against $P. \text{aeruginosa}$, the fluorescence of pyoverdine (PVD) was significantly lower in the cultures with TeNRs compared to those without TeNRs at both exponential and stationary phases of growth, and the attenuation of PVD fluorescence by TeNRs occurred in a concentration dependant manner (Figure 2).

The attenuation of PVD fluorescence by TeNRs suggests a potential for using TeNRs as a novel antivirulence agent by targeting PVD production. To further investigate whether the attenuation of PVD fluorescence by TeNRs was because of the inhibition of the production of PVD in the cultures, we employed a $pvdA::gfp$ reporter strain which allowed the biosynthesis of PVD to be monitored based on GFP fluorescence. In the presence of TeNRs, the GFP fluorescence in the cultures of the reporter strain, in both exponential and stationary phases of growth, was significantly lower than for cultures without TeNRs (Figure 3A), suggesting that the TeNRs inhibited the expression of $pvdA$ gene and, hence, PVD biosynthesis. Quantitative polymerase chain reaction (qPCR) analysis revealed that the expression levels of several other key PVD biosynthesis genes in $P. \text{aeruginosa}$ exposed to 50 µg/ml of TeNRs were also significantly lower than the no-TeNRs controls (Figure 3B), confirming the inhibitory effect of TeNRs on the biosynthesis of PVD in $P. \text{aeruginosa}$. 
The inhibition of PVD biosynthesis by biogenic TeNRs at the transcriptional level was further validated at the protein level through a proteomics approach. Among the proteins differently expressed in *P. aeruginosa* in the presence or absence of TeNRs were siderophore-related proteins PvdA, FpvA, FptA and PchB (Table 1).

Consistent with the qPCR data, both PvdA and FpvA, the key proteins involved in PVD biosynthesis and ferri-PVD uptake, respectively, were down-regulated in the presence of biogenic TeNRs. Interestingly, PchB and FptA, the proteins that play an important role in pyochelin biosynthesis and ferri-pyochelin uptake, were also found down-regulated, suggesting that the biogenic TeNRs also inhibit the production of pyochelin in *P. aeruginosa*.

In addition to PVD, pyochelin is another major siderophore that *P. aeruginosa* produces to meet its need for iron. Although PVD appears to be more essential than pyochelin in iron depleted conditions, both PVD and pyochelin are considered to be virulence factors of *P. aeruginosa*. The inhibitory effects of the biogenic TeNRs on the production of both PVD and pyochelin render them a promising antivirulence agent against *P. aeruginosa*.

**Polysaccharides, Amyloids, and Flagellar Motility in Cell-TeNRs Interaction**

The inhibitory effect of TeNRs on PVD biosynthesis led us to address how TeNRs at the cell exterior inhibit intracellular gene expression. In *P. aeruginosa*, two major extracellular polysaccharides Pel and Psl as well as functional amyloids Fap have been demonstrated to play important roles protecting bacteria from various unfavourable conditions (Billings et al. 2013; Colvin et al. 2011; Dueholm et al. 2010; Hidalgo et al. 2010; Mann and Wozniak 2011). In addition, the polysaccharide Psl has been reported to be able to act as a signal to stimulate biofilm formation of *P. aeruginosa* (Irie et al. 2012). To test whether the extracellular polysaccharides and amyloids are involved in the interaction between TeNRs and *P. aeruginosa*, we monitored the production of PVD in mutant strains lacking extracellular
polysaccharides (ΔpelA, ΔpslBC, ΔpelAΔpslBCD) or amyloids (ΔfapC) in the presence and absence of TeNRs. Our results showed that the lack of extracellular polysaccharides Pel and Psl or amyloids did not abolish the inhibitory effect of TeNRs on PVD production (Figure S1), suggesting that the inhibition of PVD production by TeNRs is not mediated by extracellular polysaccharides and amyloids.

We further tested the interaction of TeNRs with the mutants in a transposon mutagenesis library. Interestingly, inhibition of PVD production could not be observed for mutant strains ΔmotB and ΔfliF (Figure 4). MotB (a flagellar motor protein) and FliF (a flagellar MS-ring protein) are proteins involved in flagella-mediated motility and it has been reported that the mutants lacking MotB or FliF has a significantly compromised motility (Chevance and Hughes 2008; Doyle et al. 2004; Grunenfelder et al. 2003). As an important appendage, bacterial flagella not only generate motility but can also sense physical properties of the local environment. For example, flagella have been reported to be used by Vibrio parahaemolyticus to sense surface contact, which triggers a programme of gene expression promoting colonization and virulence (Gode-Potratz et al. 2011). Here, the abolishment of the inhibitory effect of TeNRs on PVD production in the motility-deficient mutants implies an involvement of flagellar movement in the cell-TeNRs interaction. The molecular mechanism by which the interaction between biogenic TeNRs and flagellar movement negatively influences PVD biosynthesis, however, remains to be identified (Figure 5), which merits further investigations.

Cytotoxicity of Biogenic TeNRs

We demonstrated that the production of PVD in P. aeruginosa could be inhibited by biogenic TeNRs. To further demonstrate the clinical potential of the biogenic TeNRs, we carried out a CCK-8 viability assay to evaluate their cytotoxicity by using human bronchial
epithelial cells BEAS-2B and murine macrophages RAW264.7. The presence of TeNRs (up to 100 µg/ml) did not influence the metabolic rate of BEAS-2B and RAW264.7 cells, suggesting a lack of cytotoxicity by the TeNRs against mammalian cells (Figure 6). Thus, the biogenic TeNRs hold a promise as a novel no-biocidal antivirulence agent against \textit{P. aeruginosa} and possibly other bacterial pathogens.

**Conclusion**

In conclusion, we synthesized biogenic TeNRs through an environmentally friendly approach by using a metal-reducing bacterium \textit{S. oneidensis} and demonstrated that the biogenic TeNRs could effectively inhibit the production of PVD, one of the main virulence factors in \textit{P. aeruginosa}. Our results suggested that extracellular polysaccharides, \textit{i.e.}, Pel and Psl, and amyloids are not involved in the interactions between \textit{P. aeruginosa} and the biogenic TeNRs, while flagellar movement plays an important role in the cell-TeNRs interaction, which alters the expression of genes involved in PVD biosynthesis and ferri-PVD uptake through a molecular mechanism that remain to be identified. We further evaluated the cytotoxicity of the TeNRs to mammalian cells and did not observe adverse effects. Taken together, this study reports for the first time a nontoxic biogenic nanomaterial that can be potentially used as a novel antivirulence agent targeting siderophore production to combat bacterial infections caused by \textit{P. aeruginosa} and possibly other bacterial pathogens.

**Acknowledgements**

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Technological University, Singapore.

References


Table 1. Down-regulation of proteins involved in siderophore production and uptake in *P. aeruginosa* PAO1 in the presence of biogenic TeNRs (50 µg/ml).

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<sup>a</sup>The percentage of matching amino acids from identified peptides divided by the total number of amino acids in the sequence; <sup>b</sup>The ratio of protein production level in biogenic TeNRs-incubated cells to control cells without TeNRs.
Figure Captions

Figure 1. (A) Extracellular precipitates of TeNRs (black) in a suspension of cells (blue) visualized by overlay of bright-field and fluorescence imaging. Cells were stained with DAPI. (B) TEM image and (C) EDX spectrum of the biogenic TeNRs.

Figure 2. Influence of TeNRs (0, 25, 50, 100 µg/ml) on PVD fluorescence (normalized by cell density) in P. aeruginosa PAO1 cultures growing in ABTGC media in the exponential (6 h) and stationary phase of growth (12 h). Data shown are mean ± standard deviation (n=3).

Figure 3. (A) Influence of TeNRs (0, 25, 50, 100 µg/ml) on GFP fluorescence (normalized by cell density) in P. aeruginosa reporter strain pvdA-gfp growing in ABTGC media at the exponential phase (6 h) and stationary phase (12 h) of growth. (B) Influence of TeNRs (50 µg/ml) on the expression level of key genes involved in PVD production and uptake of ferri-PVD. Values were normalised to the housekeeping gene tsf. PvdS: a transcriptional regulator; PvdE: an export ABC transporter to transport PVD precursors across the inner membrane; PvdN and PvdP: periplasmic enzymes involved in chromophore formation; PvdQ: a periplasmic enzyme for removal of fatty acid chains from PVD precursors; PvdT: a subunit of the ATP-dependent efflux pump for PVD secretion; FpvA: an outer membrane transporter for import of ferri-pyoverdine. Data shown are mean ± standard deviation (n=3).

Figure 4. PVD fluorescence (normalized by cell density) in 12 h cultures of P. aeruginosa PAO1 wild type and motility-deficient mutants ∆motB and ∆fliF in ABTGC media with (50 µg/ml) or without TeNRs. Data shown are mean ± standard deviation (n=3).

Figure 5. Schematic illustration of the interactions between P. aeruginosa and biogenic TeNRs. The biogenic TeNRs interfere with the flagellar movement of P. aeruginosa cells, which negatively influences, through an unknown mechanism, the expression of genes involved in PVD production and ferri-PVD uptake. The genes that were found down-regulated in the presence of biogenic TeNRs in this study are highlighted in bold.

Figure 6. CCK-8 viability assay for RAW cells and BEAS-2B cells in the presence of TeNRs at different concentrations: 0, 25, 50, 100 µg/ml. Metabolic rate is shown as the dehydrogenase activity which reduces WST-8 salt to formazan dye. Untreated cells were used as controls. Data shown are mean ± standard deviation (n=3).
Biosynthesis of PVD precursor

221x236mm (150 x 150 DPI)
Supplemental Information

Biogenic Tellurium Nanorods as a Novel Antivirulence Agent Inhibiting Pyoverdine Production in *Pseudomonas aeruginosa*

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Figure S1
Table S1
Table S2
Table S3
Figure S1. PVD fluorescence (normalized by cell density) in 12 h cultures of *P. aeruginosa* PAO1 wild type and mutants lacking polysaccharides or amyloids in ABTGC media with (50 µg/ml) or without TeNRs. Data shown are mean ± standard deviation (n=3).
Table S1. List of *P. aeruginosa* mutants used in this study.

<table>
<thead>
<tr>
<th>Table #</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta \text{pelA})</td>
<td>A mutant lacking Pel polysaccharide (Yang et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>(\Delta \text{pslBCD})</td>
<td>A mutant lacking Psl polysaccharide (Yang et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>(\Delta \text{pelA}\Delta \text{pslBCD})</td>
<td>A mutant lacking both Pel and Psl polysaccharides (Yang et al. 2011)</td>
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<tr>
<td>(\Delta \text{fapC})</td>
<td>A mutant lacking Fap amyloids (Dueholm et al. 2010)</td>
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<tr>
<td>(\Delta \text{fliF})</td>
<td>Disruption of <em>fliF</em> encoding a flagellar MS-ring protein (this study)</td>
<td></td>
</tr>
<tr>
<td>(\Delta \text{motB})</td>
<td>Disruption of <em>motB</em> encoding a flagellar motor protein (this study)</td>
<td></td>
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</tbody>
</table>


Table S2. List of primers used in qPCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
<tr>
<td>tsf</td>
<td>CCTTCAACGAGAAGCTGACC</td>
<td>CTTTCAGGTTGACCACGACA</td>
</tr>
<tr>
<td>fpvA</td>
<td>ACACCGGCCAACAAGAGCAACT</td>
<td>TAGACCGCGCCGCAGTAGGGGAATG</td>
</tr>
<tr>
<td>pvdE</td>
<td>CACCGCCCGTCGCTATCG</td>
<td>GGTCTTTGTCGCGGGGCTCTGT</td>
</tr>
<tr>
<td>pvdN</td>
<td>CGAGCGACGCCGCAATAAAATGG</td>
<td>GTTGCAAGGCAAAGTAGCGAGCTG</td>
</tr>
<tr>
<td>pvdQ</td>
<td>GATCTACGGCCCGCTGGTGCTGTCG</td>
<td>AGTGGGCGCCGCCGGGGGTGATG</td>
</tr>
<tr>
<td>pvdS</td>
<td>TCGGGGCGGAGGAAGAAGG</td>
<td>CGCGGCGGCCTGAGATGG</td>
</tr>
<tr>
<td>pvdT</td>
<td>AACGCATCGCCATCCCCTACTCC</td>
<td>TCACGCGCGATTCCGCTACCAG</td>
</tr>
<tr>
<td>pvdP</td>
<td>CCGCGCCTCCGTGTGCTC</td>
<td>GCCTCATGGGCGAGGGGAAGTG</td>
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</tbody>
</table>
Table S3. Influence of TeNRs on growth of *P. aeruginosa* PAO1 in ABTGC media at 37°C. Specific growth rate was determined from $\mu = \frac{d \ln(OD_{600})}{dt}$, where $OD_{600}$ was the optical density at a wavelength of 600 nm. Values are means ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>Concentration of TeNRs (µg/ml)</th>
<th>Specific growth rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>25</td>
<td>0.49 ± 0.05</td>
</tr>
<tr>
<td>50</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>100</td>
<td>0.50 ± 0.05</td>
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</tbody>
</table>