Biogenic tellurium nanorods as a novel antivirulence agent inhibiting pyoverdine production in *Pseudomonas aeruginosa*

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

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Key Words:	Shewanella oneidensis, Pseudomonas aeruginosa, pyoverdine, antivirulence, tellurium, nanomaterial

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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

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42 **Introduction**

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

54 An alternative approach to combat bacterial infections is to develop antivirulence
55 drugs that disarm pathogens by targeting the production of virulence traits such as bacterial
56 toxins (Cegelski et al. 2008; Marra 2004; Rasko and Sperandio 2010). Because the
57 production of many virulence factors are controlled by intercellular communication or
58 quorum sensing (QS) (Rutherford and Bassler 2012), QS is one of the most common targets
59 for new antivirulence drugs (Kaufmann et al. 2008; Rasko and Sperandio 2010). For example,
60 a number of QS inhibitors with efficacy against *Pseudomonas aeruginosa* QS have been
61 developed (Rasmussen and Givskov 2006). However, recent studies revealed that bacteria
62 might also evolve resistance to antivirulence compounds such as QS inhibitors (Garcia-
63 Contreras et al. 2013; Maeda et al. 2012; Mellbye and Schuster 2011). Hence, there is an
64 emergent need to develop non-traditional antivirulence drugs that do not lead to bacterial
65 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

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90 **Biosynthesis of TeNRs by *S. oneidensis***

91 Stock cultures of *S. oneidensis* MR-1 were maintained in LB medium with 20% glycerol at -
92 80°C. The bacterial cells were grown in LB medium containing 100 µg/ml potassium tellurite.
93 The cultures were bubbled with nitrogen gas for 5 min and the headspace in the culture was
94 less than 3% of the total volume. After 72 h, cells were removed by centrifugation at 5,000×g
95 for 20 min. Only extracellular TeNRs in the supernatant were harvested and used in this study
96 because harvesting TeNRs from bacterial cells would require cell lysis, which might cause
97 contamination of TeNRs by various biomolecules. The supernatant was then concentrated
98 using Millipore Amicon centrifugal filter units (MWCO ~3 kDa). Te nanorods (TeNRs) were
99 harvested by using ultracentrifugation at 100,000g for 30 min, washed three times with
100 MilliQ water and dried at 50°C under vacuum.

101 **Epifluorescence and Electron Microscopic Imaging**

102 After an overnight exposure to TeO_3^{2-} , *S. oneidensis* cell suspension was placed on a glass
103 microscope slide and dried in an oven at 70°C. The dried sample was then stained using 4'-6-
104 diamidino-2-phenylindole (DAPI). Briefly, 5 µl of DAPI solution (1 mg/mL) was dropped
105 onto the sample and incubated at room temperature, in the dark, for 45 min. The glass slide
106 was then rinsed with ultrapure water to remove excess DAPI. The stained samples were
107 examined using an inverted epifluorescence microscope (Microscope Axio Observer.Z1, Carl
108 Zeiss). Transmission electron microscopy (TEM) images and energy dispersive X-ray
109 spectroscopy (EDS) were obtained following the procedures described previously
110 (Shahjamali et al. 2012).

111 **Quantification of Pyoverdine Production in *P. aeruginosa***

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3 112 *P. aeruginosa* PAO1 wild type and mutant strains (Table S1) were cultivated at 37°C in ABT
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5 113 minimal medium supplemented with 30 mM glucose and 5 g/l casamino acids (ABTGC)
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7 114 (Chua et al. 2013) in the absence or presence of TeNRs at different concentrations.
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9 115 Pyoverdine fluorescence (Ex 398 nm, Em 460 nm) (Greenwald et al. 2007) and optical
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11 116 density at 600 nm (OD₆₀₀) were recorded using a TECAN infinite M200PRO plate reader.
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15 117 **Inductively Coupled Plasma Mass Spectrometry**

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18 118 To determine the dissolution of TeO₃²⁻ from the biogenic TeNPs, the suspension of 100
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20 119 µg/ml TeNPs in ABTGC medium was filtered through a centrifugal filter unit (molecular
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22 120 weight cutoff 3 kDa; Amicon Ultra-4 3K). The total TeO₃²⁻ concentration was quantified
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24 121 using an inductively coupled plasma mass spectrometer (ICP-MS) (ELAN DRC-e, Perkin
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26 122 Elmer; Waltham, MA) as described previously (Kim et al. 2012).
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30 123 **PvdA-gfp Assay**

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34 124 To investigate the impacts of TeNRs on biosynthesis of PVD in *P. aeruginosa*, the expression
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36 125 of a key PVD synthesis gene *pvdA* was monitored using a *pvdA::gfp* fluorescent reporter as
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38 126 described previously (Kaneko et al. 2007; Yang et al. 2009). The *pvdA::gfp* reporter strain
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40 127 was cultivated in ABTGC medium at 37°C in the absence or presence of TeNRs at different
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42 128 concentrations. GFP fluorescence (Ex 395 nm, Em 509 nm) and OD₆₀₀ were measured using
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44 129 a TECAN infinite M200PRO plate reader.
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48 130 **Quantitative Polymerase Chain Reaction (qPCR)**

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52 131 *P. aeruginosa* PAO1 was cultivated in ABTGC medium at 37°C in the absence or presence
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54 132 of TeNRs (50 µg/ml). After 9 h, 0.5 ml of the culture was mixed with 1 ml of RNA protect
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56 133 (Qiagen mini RNA prep) and centrifuged at 6,000g for 10 min. Total RNA content was
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134 extracted using a commercially available kit (Qiagen mini RNA prep) following the
135 instructions from the manufacturer. RNA concentration was determined using a NanoDrop
136 spectrophotometer (Thermo Scientific, DE, USA). Two µg of the total RNA was used for
137 first-strand cDNA synthesis by using a commercially available kit (Fermentas Life Sciences,
138 EU). The resultant cDNA was used in qPCR reactions on ABI StepOnePlus system (Life
139 Technologies, CA). Primers (efficiency >90% for all primers) were obtained from Sigma Life
140 Sciences and their sequences are listed in Table S2. PCR was carried out with 1 µl of first-
141 strand cDNA in a total volume of 20 µl containing 0.2 mM of each primer, 10 µl of PCR
142 KAPA SYBR® FAST qPCR Master Mix ABI (2X) Prism™, 0.4 µl ROX high.
143 Amplification parameters used were: initial activation 3 min at 95°C, then 40 cycles of 3 s at
144 95°C and 30 s at respective appropriate annealing temperature. Experiments were performed
145 in triplicates for all the genes. The amount of each target gene was normalized to a reference
146 gene *tsf* and compared to control samples (Xie et al. 2011). Data analysis was performed
147 using the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = \Delta C_T(\text{treated sample}) - \Delta C_T(\text{untreated sample})$, $\Delta C_T =$
148 $C_T(\text{target gene}) - C_T(tsf)$, and C_T is the threshold cycle value for the amplified gene (Livak
149 and Schmittgen 2001).

150 **iTRAQ-based Proteomic Analysis**

151 Proteomic analysis was conducted using an iTRAQ-coupled two-dimensional LC-MS/MS
152 technique as described previously (Zhou and Chen 2011). Briefly, cells of *P. aeruginosa*
153 PAO1 grown in ABTGC medium in the absence or presence of 50 µg/ml TeNRs at late
154 exponential growth phases were harvested by centrifugation at 3000×g for 20 min at 4°C and
155 lysed in lysis buffer (50 mM NaCl, 5 mM DTT, 1 mM PMSF and 50 mM Tris-HCl, pH 8.0)
156 by intermittent sonication. Unbroken cells were removed by centrifugation at 3000 × g for
157 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 μ g) 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* Δ *motB* and Δ *fliF* 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 μ g/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

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

197 **Results and Discussion**

198 **Biogenic TeNRs from TeO₃²⁻ Reduction by *S. oneidensis***

199 TeNRs have been successfully prepared via physicochemical routes including chemical
200 reduction (Gao et al. 2008; Liu et al. 2004; Zhu et al. 2004) and photolytic preparation
201 (Webber and Brutchey 2009). The unique helical chain conformation of Te crystals typically
202 leads to a highly anisotropic growth to form one-dimensional (1D) structures such as
203 nanorods (Webber and Brutchey 2009). TeNRs can also be produced by bacteria grown on
204 Te oxyanions such as tellurite (TeO₃²⁻) (Baesman et al. 2007; Turner et al. 2012; Wang et al.
205 2011), which offers a “green” approach for the synthesis of Te nanomaterials under facile

206 conditions in the absence of organic solvents. For example, although TeO_3^{2-} is highly toxic to
207 most microorganisms and has been used as an antimicrobial agent for decades (Turner et al.
208 2012), *S. oneidensis* has been reported to be able to reductively transform TeO_3^{2-} to $\text{Te}(0)$ in
209 the form of rod-like nanostructures, *i.e.*, TeNRs, intracellularly and at the cell exterior (Kim
210 et al. 2012; Klonowska et al. 2005).

211 TeNRs used in this study were obtained through microbial reduction of TeO_3^{2-} by *S.*
212 *oneidensis* using lactate as an electron donor. We observed black precipitates, presumably
213 aggregates of TeNRs, at the cell exterior upon the exposure of *S. oneidensis* cells to TeO_3^{2-}
214 (Figure 1A). TEM-EDX showed that the biogenic TeNRs obtained here were about 10-20 nm
215 wide, up to several hundreds of nm long, and were comprised entirely of Te (Figure 1B and
216 C). In this study, we focused on the novel antivirulence activity of the biogenic TeNRs and
217 further detailed material characterization of the TeNRs was not included. In fact, the
218 reduction of TeO_3^{2-} by *S. oneidensis* has been reported in literatures and the $\text{Te}(0)$ nanorods
219 obtained from bacterial reduction of TeO_3^{2-} under similar experimental conditions have been
220 extensively characterized (Kim et al. 2012; Klonowska et al. 2005).

221 **Inhibition of PVD Biosynthesis by Biogenic TeNRs**

222 In an effort to elucidate impacts of the biogenic TeNRs on microorganisms, we grew *P.*
223 *aeruginosa* in the presence of TeNRs. Interestingly, TeNRs (up to 100 $\mu\text{g/ml}$) have no
224 negative impact on bacterial growth, as evidenced by comparable or even slightly higher
225 specific growth rate for all treated cultures, compared to the controls (Table S3), suggesting
226 that the biogenic TeNRs do not exhibit bactericidal activity against *P. aeruginosa*.
227 Chemically synthesized Te nanomaterials with different structures such as nanorice,
228 nanopencil and nanowire have been reported to exhibit excellent antibacterial activity
229 because of the oxidative dissolution of TeO_3^{2-} from the Te nanomaterials (Lin et al. 2012).

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230 Here, our biogenic TeNRs did not show bactericidal activity, possibly due to a high stability
231 and resistance to molecular oxygen of the biogenic TeNRs. To confirm this, we used ICP-MS
232 to quantify the concentration of TeO_3^{2-} in the ABTGC medium with the presence of 100
233 $\mu\text{g/ml}$ TeNRs but no TeO_3^{2-} could be detected after 24 h. The resistance to oxidative
234 dissolution and the lack of bactericidal activity of the biogenic TeNRs open a new window of
235 novel applications other than being used as a bactericidal agent.

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

241 The attenuation of PVD fluorescence by TeNRs suggests a potential for using TeNRs
242 as a novel antivirulence agent by targeting PVD production. To further investigate whether
243 the attenuation of PVD fluorescence by TeNRs was because of the inhibition of the
244 production of PVD in the cultures, we employed a *pvdA::gfp* reporter strain which allowed
245 the biosynthesis of PVD to be monitored based on GFP fluorescence. In the presence of
246 TeNRs, the GFP fluorescence in the cultures of the reporter strain, in both exponential and
247 stationary phases of growth, was significantly lower than for cultures without TeNRs (Figure
248 3A), suggesting that the TeNRs inhibited the expression of *pvdA* gene and, hence, PVD
249 biosynthesis. Quantitative polymerase chain reaction (qPCR) analysis revealed that the
250 expression levels of several other key PVD biosynthesis genes in *P. aeruginosa* exposed to
251 50 $\mu\text{g/ml}$ of TeNRs were also significantly lower than the no-TeNRs controls (Figure 3B),
252 confirming the inhibitory effect of TeNRs on the biosynthesis of PVD in *P. aeruginosa*.

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3 253 The inhibition of PVD biosynthesis by biogenic TeNRs at the transcriptional level
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5 254 was further validated at the protein level through a proteomics approach. Among the proteins
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7 255 differently expressed in *P. aeruginosa* in the presence or absence of TeNRs were
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10 256 siderophore-related proteins PvdA, FpvA, FptA and PchB (Table 1).

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13 257 Consistent with the qPCR data, both PvdA and FpvA, the key proteins involved in
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15 258 PVD biosynthesis and ferri-PVD uptake, respectively, were down-regulated in the presence
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17 259 of biogenic TeNRs. Interestingly, PchB and FptA, the proteins that play an important role in
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19 260 pyochelin biosynthesis and ferri-pyochelin uptake, were also found down-regulated,
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21 261 suggesting that the biogenic TeNRs also inhibit the production of pyochelin in *P. aeruginosa*.
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23 262 In addition to PVD, pyochelin is another major siderophore that *P. aeruginosa* produces to
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25 263 meet its need for iron. Although PVD appears to be more essential than pyochelin in iron
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27 264 depleted conditions, both PVD and pyochelin are considered to be virulence factors of *P.*
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29 265 *aeruginosa*. The inhibitory effects of the biogenic TeNRs on the production of both PVD and
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31 266 pyochelin render them a promising antivirulence agent against *P. aeruginosa*.
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36 267 **Polysaccharides, Amyloids, and Flagellar Motility in Cell-TeNRs Interaction**

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39 268 The inhibitory effect of TeNRs on PVD biosynthesis led us to address how TeNRs at the cell
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41 269 exterior inhibit intracellular gene expression. In *P. aeruginosa*, two major extracellular
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43 270 polysaccharides Pel and Psl as well as functional amyloids Fap have been demonstrated to
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45 271 play important roles protecting bacteria from various unfavourable conditions (Billings et al.
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47 272 2013; Colvin et al. 2011; Dueholm et al. 2010; Hidalgo et al. 2010; Mann and Wozniak 2011).
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49 273 In addition, the polysaccharide Psl has been reported to be able to act as a signal to stimulate
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51 274 biofilm formation of *P. aeruginosa* (Irie et al. 2012). To test whether the extracellular
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53 275 polysaccharides and amyloids are involved in the interaction between TeNRs and *P.*
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55 276 *aeruginosa*, we monitored the production of PVD in mutant strains lacking extracellular
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277 polysaccharides ($\Delta pelA$, $\Delta pslBC$, $\Delta pelA\Delta pslBCD$) or amyloids ($\Delta fapC$) in the presence and
278 absence of TeNRs. Our results showed that the lack of extracellular polysaccharides Pel and
279 Psl or amyloids did not abolish the inhibitory effect of TeNRs on PVD production (Figure
280 S1), suggesting that the inhibition of PVD production by TeNRs is not mediated by
281 extracellular polysaccharides and amyloids.

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

297 **Cytotoxicity of Biogenic TeNRs**

298 We demonstrated that the production of PVD in *P. aeruginosa* could be inhibited by
299 biogenic TeNRs. To further demonstrate the clinical potential of the biogenic TeNRs, we
300 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 *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 *S. oneidensis* and demonstrated that the biogenic TeNRs could effectively inhibit the production of PVD, one of the main virulence factors in *P. aeruginosa*. Our results suggested that extracellular polysaccharides, *i.e.*, Pel and Psl, and amyloids 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, 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 *P. aeruginosa* and possibly other bacterial pathogens.

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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).

Locus tag	Protein	% Cov ^a	Ratio ^b
PA2386	L-ornithine 5-monooxygenase, PvdA	40	0.50
PA2398	Ferri-pyoverdine receptor, FpvA	17	0.32
PA4221	Ferri-pyochelin receptor, FptA	8	0.45
PA4230	Salicylate biosynthesis protein PchB	33	0.41

^aThe percentage of matching amino acids from identified peptides divided by the total number of amino acids in the sequence; ^bThe ratio of protein production level in biogenic TeNRs-incubated cells to control cells without TeNRs.

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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 $\Delta motB$ and $\DeltafliF$ 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 formazon dye. Untreated cells were used as controls. Data shown are mean ± standard deviation (n=3).

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Supplemental Information

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

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Say Chye Loo^{2,3}, Staffan Kjelleberg², Liang Yang^{2,5*}, Bin Cao^{1,2*}

- 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 $\pm$ standard deviation (n=3).

Table S1. List of *P. aeruginosa* mutants used in this study.

$\Delta pelA$	A mutant lacking Pel polysaccharide (Yang et al. 2011)
$\Delta ps/BCD$	A mutant lacking Psl polysaccharide (Yang et al. 2011)
$\Delta pelA\Delta ps/BCD$	A mutant lacking both Pel and Psl polysaccharides (Yang et al. 2011)
$\Delta fapC$	A mutant lacking Fap amyloids (Dueholm et al. 2010)
$\Delta fliF$	Disruption of <i>fliF</i> encoding a flagellar MS-ring protein (this study)
$\Delta motB$	Disruption of <i>motB</i> encoding a flagellar motor protein (this study)

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Table S2. List of primers used in qPCR analysis.

Gene	Forward primer	Reverse primer
<i>tsf</i>	CCTTCAACGAGAAGCTGACC	CTTTCAGGTTGACCACGACA
<i>fpvA</i>	ACACCGGCGAAACCAAGAGCAACT	TAGACCGCGCCGACGTAGGGAATG
<i>pvdE</i>	CACCGCCCCCGTCGCTATCG	GGTCTTGTCCGGGGCCTCCTGTT
<i>pvdN</i>	CGAGCGACGCCGGAATAAATGG	GTTGCACGGCGAAGTAGCGACCTG
<i>pvdQ</i>	GATCTACGGCCCGCTGGTGGTCTG	AGTTGCGCCGCGGGGTGATGC
<i>pvdS</i>	TCGGGGCCGGAGGAAGAAGG	CGGCGGGCGCTGAGATGG
<i>pvdT</i>	AACCGCATCGCCATCCCCTACTCC	TCACGCCGATCCCGCCTACCAG
<i>pvdP</i>	CCGCCGCGCTTCCGTGTGCT	CGCTCATGGGCCAGGGGGAAGTG

Table S3. Influence of TeNRs on growth of *P. aeruginosa* PAO1 in ABTGC media at 37°C. Specific growth rate was determined from $\mu = d \ln(OD_{600})/dt$, where OD_{600} was the optical density at a wavelength of 600 nm. Values are means $\pm$ standard deviation (n=3).

Concentration of TeNRs ($\mu\text{g/ml}$)	Specific growth rate (h^{-1})
0	0.42 ± 0.04
25	0.49 ± 0.05
50	0.49 ± 0.04
100	0.50 ± 0.05