C-di-GMP regulates *Pseudomonas aeruginosa* stress response to tellurite during both planktonic and biofilm modes of growth

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Stress response plays an important role on microbial adaptation under hostile environmental conditions. It is generally unclear how the signaling transduction pathway mediates a stress response in planktonic and biofilm modes of microbial communities simultaneously. Here, we showed that metalloid tellurite (TeO\(_{2}^--\)) exposure induced the intracellular content of the secondary messenger cyclic di-GMP (c-di-GMP) of *Pseudomonas aeruginosa*. Two diguanylate cyclases (DGCs), SadC and SiaD, were responsible for the increased intracellular content of c-di-GMP. Enhanced c-di-GMP levels by TeO\(_{2}^-\) further increased *P. aeruginosa* biofilm formation and resistance to TeO\(_{2}^-\). *P. aeruginosa ΔsadC ΔsiaD* and PAO1/p lac-yhjH mutants with low intracellular c-di-GMP content were more sensitive to TeO\(_{2}^-\) exposure and had low relative fitness compared to the wild-type PAO1 planktonic and biofilm cultures exposed to TeO\(_{2}^-\). Our study provided evidence that c-di-GMP level can play an important role in mediating stress response in microbial communities during both planktonic and biofilm modes of growth.

Microorganisms display a striking ability to adapt to unfavorable conditions such as exposure to UV radiation, heavy metals and antibiotic treatments, by inducing stress responses and forming surface-attached biofilms\(^1,2\). Biofilms consist of microbial cells embedded in their self-produced extracellular polymeric substances (EPS). The EPS can account for up to 90% of the biofilm biomass and serve as physical barriers to protect biofilm cells\(^3\). Hence, biofilms dramatically increase the tolerance of bacterial cells towards environmental stress and immune attack during the course of infections\(^4,5\). Extensive intercellular communication and interactions have been observed within biofilms, and cells with distinct physiology may play different roles under stress conditions\(^6,8\).

Bis-(3\(^\prime\)-5\(^\prime\))'-cyclic dimeric guanosine monophosphate (C-di-GMP) plays an important role in biofilm formation of a wide range of bacteria\(^9\). Bacterial intracellular c-di-GMP content is determined by diguanylate cyclases (DGCs) that catalyze the formation of c-di-GMP and phosphodiesterases (PDEs), which degrade c-di-GMP\(^9\). When intracellular c-di-GMP content is high, bacterial cells reduce motility and...
increase synthesis of EPS matrix, resulting in biofilm formation. In contrast, biofilm cells increase their motility and disperse from biofilms when the intracellular c-di-GMP content is low. C-di-GMP signaling can be induced by stress conditions such as antimicrobial exposure. The impact of c-di-GMP on mediating stress response by microbial communities during both planktonic and biofilm modes of growth remains unclear.

Anthropogenic activities have resulted in serious metal(loid) pollution, especially in industrialized countries and regions. The natural ecosystems are often direct or indirect recipients of toxic metal(loid)s such as TeO$_3^{2−}$. Many environmental bacteria including Pseudomonas aeruginosa are capable of surviving in the presence of TeO$_3^{2−}$ at low concentrations by reducing TeO$_3^{2−}$ to Te(0) nanomaterials, as a result of either detoxification, redox maintenance or respiration. Although the toxic effects of metal(loid)s on environmental microorganisms at individual cell levels have been extensively studied, little is known about the impacts of metal(loid)s on bacterial social behaviors.

In the present study, we investigated the role of c-di-GMP in mediating stress responses by the opportunistic pathogen Pseudomonas aeruginosa to a toxic metalloid, tellurite (TeO$_3^{2−}$). TeO$_3^{2−}$ is highly toxic to most microbes and had been previously described by Alexander Fleming as an antimicrobial agent. Bacterial cells take up TeO$_3^{2−}$ and subsequently reduce it to tellurium nanoparticles, which can be easily tracked by the black precipitates on the bacterial cell surface. Quantification of intracellular c-di-GMP and proteomic analysis indicated that c-di-GMP levels were induced by TeO$_3^{2−}$ exposure, which enhanced P. aeruginosa TeO$_3^{2−}$ resistance and biofilm formation. SadC and SiaD were found to be important in the induction of c-di-GMP by TeO$_3^{2−}$ exposure. We showed that mutants with low intracellular c-di-GMP content could be outcompeted by the wild-type strain from biofilm and planktonic cultures under metalloid stress condition.

Results

Stress responses of P. aeruginosa to TeO$_3^{2−}$ induced c-di-GMP signaling. Cultivation of different bacterial species in the presence of sub-lethal concentrations of antimicrobial agents is a widely employed method to investigate their stress responses. The MIC of P. aeruginosa to TeO$_3^{2−}$ is 100 μg/ml in ABTGC medium. Large aggregates (approximately 1-3 mm) were formed when P. aeruginosa was grown in ABTGC media containing 10 μg/ml TeO$_3^{2−}$ at 37°C (Fig. 1a). Further analysis of the TeO$_3^{2−}$-induced aggregates by field emission scanning electron microscopy (FE-SEM) and energy-dispersive X-ray spectroscopy (EDX) revealed the presence of tellurium-containing precipitates around the bacterial cells (Fig. 1b,c). No tellurium-containing precipitates were observed for P. aeruginosa cells growing in medium without TeO$_3^{2−}$. Thus, the tellurium-containing precipitates might generate conditions of membrane-associated stress for P. aeruginosa cells.

TeO$_3^{2−}$ and oxyanions such as selenite/selenite are well known to exert their toxic effects on microorganisms via generation of reactive oxygen species (ROS). We measured the generation of ROS by P. aeruginosa cells exposed to sub-lethal concentrations of TeO$_3^{2−}$ as well as SeO$_3^{2−}$ and SeO$_2^{2−}$ by using the OxiSelect™ in vitro ROS/RNS assay kit. As anticipated, exposure of P. aeruginosa cells to the TeO$_3^{2−}$, SeO$_3^{2−}$ and SeO$_2^{2−}$ significantly increased their cytoplasmic ROS content regardless of the nutrient conditions (Fig. 1d).

Proteomic analysis of TeO$_3^{2−}$ stressed P. aeruginosa cells. Oxidative stress response by P. aeruginosa leading to aggregate formation, recently reported to resemble the biofilm physiology has not been documented. We thus investigated the overall impact of TeO$_3^{2−}$ on P. aeruginosa cells using a comparative proteomic approach for cells cultivated with and without 10 μg/ml TeO$_3^{2−}$.

Using a p-value cut-off of 0.05 and a fold change cut-off of 5 (as described in the Materials and Methods), 129 proteins were significantly affected by TeO$_3^{2−}$ exposure with 64 proteins upregulated (Supplementary table 1) and 65 proteins being down-regulated (Supplementary table 2).

The expression of several outer membrane associated proteins was induced by TeO$_3^{2−}$ treatment, including OprQ (PA2760, 28.8-fold), Oprl precursor (PA2853, 15-fold), probable outer membrane protein precursor (PA2391, 10.9-fold), OprM (PA0427, 10.5-fold), Oprl precursor (PA0973, 9.8-fold), OprD precursor (PA0958, 9.8-fold), OprB (PA3186, 9.7-fold) and OprC (PA3790, 8.1-fold) (Supplementary table 1). The membrane transporter CdrB of the large extracellular protein CdrA was induced 25.8-fold by exposure to TeO$_3^{2−}$ (Supplementary table 1). CdrAB expression has been used as a c-di-GMP indicator and reported to promote biofilm formation and auto-aggregation in a Psl polysaccharide dependent manner, and co-immunoprecipitation experiments have clearly shown that CdrA binds to Psl. HPLC analysis showed that P. aeruginosa PA01 cultivated in ABTGC medium with 10 μg/ml TeO$_3^{2−}$ treatment had a higher relative intracellular c-di-GMP concentration compared to untreated control samples (approximately 2.5-fold) (Fig. 1e).

SadC and SiaD contribute to c-di-GMP induction by TeO$_3^{2−}$. CdrAB belongs to a family of bacterial proteins secreted by the two-partner secretion system. Recently, two other members of this family, XacFhaB from Xanthomonas axonopodis pv. Citri and FHA from Bordetella pertussis have also been implicated in biofilm formation. These large inter-bacterial adhesins may play a key role in establishing structured biofilm communities under stress conditions. The cdrA promoter is positively regulated by the c-di-GMP concentration, and the expression of P$_{cdrA}^\text{gfp}$ has been recently used as a biosensor...
of the intracellular content of c-di-GMP in *P. aeruginosa*. We tested the expression of the *P. aeruginosa* reporter in *P. aeruginosa* cultures with and without the presence of TeO$_3^{2−}$ and found that TeO$_3^{2−}$ exposure significantly increased the expression of fluorescence in a dose dependent manner (Fig. 2a). This result is in accordance with our HPLC quantification and indicates that TeO$_3^{2−}$ exposure increases the intracellular content of c-di-GMP and that TeO$_3^{2−}$ induced aggregates might carry physiological traits similar to those of biofilms.

Recently, both SadC and SiaD, were shown to be able to transduce an extracellular signal generated by the toxic detergent SDS and catalyze synthesis of c-di-GMP for facilitating biofilm formation by *P. aeruginosa*. The defect environmental signaling ΔsadC and ΔsiaD mutants were severely impaired in expression of the *P. aeruginosa* reporter in the presence of TeO$_3^{2−}$ (Fig. 2a). SiaD appears to be more important than SadC for *P. aeruginosa* induction by TeO$_3^{2−}$ since the ΔsadC mutant still displayed a slight induction of *P. aeruginosa* by TeO$_3^{2−}$ (Fig. 2a).

Exopolysaccharides are the major EPS components of *P. aeruginosa* biofilms and are well known to be induced by high intracellular c-di-GMP content in *P. aeruginosa*. We examined the expression of a lacZ-based biosensor of the Pel synthesis operon (mini-CTX-pel-lacZ) in *P. aeruginosa* strains under TeO$_3^{2−}$ stress. As with *P. aeruginosa* fusion, the expression of the pel-lacZ fusion was induced by TeO$_3^{2−}$ treatment, with SiaD essential for this induction (Fig. 2b). However, there was a slight induction of the pel-lacZ fusion by tellurite even in the ΔsadCΔsiaCD double mutant (Fig. 2b).

Consistent with our observation of TeO$_3^{2−}$-induced aggregation, *P. aeruginosa* grown in the presence of TeO$_3^{2−}$ formed more biofilms than cells grown without TeO$_3^{2−}$ (Fig. 3). The induction of biofilm formation was dependent on the presence of Pel and Psl polysaccharides (Fig. 3).

**Induction of c-di-GMP confers a growth advantage under tellurite exposure during planktonic cultures.** Since c-di-GMP signaling was induced by TeO$_3^{2−}$ exposure, we examined whether induction of c-di-GMP signaling would confer a growth advantage of *P. aeruginosa* under TeO$_3^{2−}$ exposure. There was no growth defect of ΔsadC, ΔsiaD and ΔsadCΔsiaD mutants under normal growth condition as compared to PAO1 control (Fig. 4a). However, the *P. aeruginosa* ΔsadC, ΔsiaD single or double mutants were more sensitive to TeO$_3^{2−}$ (Fig. 4b). Similarly, the PAO1/p_lac_yhjH mutant, which
contains a PBBRMCS-2 plasmid with a constitutively expressed phosphodiesterase gene \( yhjH \) fused to and expressed by the \( lac \) promoter and thus has a low intracellular of c-di-GMP content\(^{12}\), was also more sensitive to \( \text{TeO}_3^{2-} \) (Fig. 4). These results showed that intracellular c-di-GMP content determines the tolerance of \( P.\ aeruginosa \) to \( \text{TeO}_3^{2-} \) exposure during planktonic cultures.

**Low intracellular c-di-GMP mutants lose fitness under stress during both planktonic and biofilm modes of growth.** When \( cfp \)-tagged PAO1 and \( yfp \)-tagged \( \Delta \text{sadC}\Delta \text{siaD} \) mutant strains were combined 1:1 (vol/vol) for planktonic co-cultivation experiments, the wild-type showed higher survival rates and gained a higher level of relative fitness than the \( \Delta \text{sadC}\Delta \text{siaD} \) mutant in the presence of \( \text{TeO}_3^{2-} \) than without \( \text{TeO}_3^{2-} \) (Fig. 5a). Since diverse phenotypic and genotypic variants coexist in bacterial biofilms\(^{37,38}\), we tested whether \( \text{TeO}_3^{2-} \) exposure-induced biofilm formation by high c-di-GMP containing cells would lead to protection of mutants with low intracellular c-di-GMP content in co-cultures. Here, PAO1 displayed a higher relative fitness than the \( \Delta \text{sadC}\Delta \text{siaD} \) mutant in biofilm co-cultures with and without the presence of \( \text{TeO}_3^{2-} \) (Fig. 5b). However, the relative fitness of \( \Delta \text{sadC}\Delta \text{siaD} \) compared to PAO1 in biofilm co-cultures was slightly higher with the presence of \( \text{TeO}_3^{2-} \) than in its absence (Fig. 5b). This suggests \( \text{TeO}_3^{2-} \) could potentially induce expression of other DGC harboring proteins in the \( \Delta \text{sadC}\Delta \text{siaD} \) mutant and partly restore the intracellular c-di-GMP levels and biofilm formation.
When we mixed cfp-tagged PAO1 and yfp-tagged PAO1/p\(\text{lac}\)-yhjH strains 1:1 (vol:vol) for planktonic co-cultivation experiments, the wild-type PAO1 strain gained a higher level of relative fitness than the c-di-GMP depleted PAO1/p\(\text{lac}\)-yhjH strain with and without exposure to TeO\(_2^\text{2−}\) (Fig. 6a). Moreover, PAO1/p\(\text{lac}\)-yhjH was fully outcompeted by PAO1 in biofilm co-cultures supplemented with TeO\(_2^\text{2−}\) (Fig. 6b). These results suggest that variants with low intracellular c-di-GMP content are unlikely to be protected and maintained by both \(P.\ aeruginosa\) planktonic and biofilm communities when c-di-GMP is required for stress response.

**Discussion**

Bacterial cells face various types of stress during the colonization of natural environments and hosts. A series of stress response mechanisms has evolved in bacteria to cope with these harmful conditions. One well characterized stringent stress response mechanism is SpoT-mediated ppGpp accumulation, which can be provoked by nutritional stress caused by harmful conditions such as antibiotic treatment and UV irradiation\(^{39}\). ppGpp is able to bind directly to the bacterial RNA polymerase and further regulate transcriptional activity of many genes.

In addition to the stringent stress response, bacteria employ a wide range of social behaviors for surviving under unfavorable environmental conditions and these responses also contribute to bacterial pathogenesis\(^{40}\). For example, the \(Staphylococcus aureus\) agr quorum-sensing system is involved in the oxidative stress response\(^{41}\). Biofilm formation is evoked as a stress response mechanism by a wide range of bacteria\(^{42}\). It involves encasing bacterial cells inside the densely packed EPS matrix components and attaching firmly to biotic and abiotic surfaces. Biofilms are up to 1,000 times more resistant to antimicrobial agents compared to their planktonic counterparts\(^{43}\).

Recently, bacteria were found to form floating biofilm-resembling aggregates that are resistant to antimicrobials and phagocytosis\(^{28}\). Our work here showed that TeO\(_2^\text{2−}\) exposure can elevate the c-di-GMP level in \(P.\ aeruginosa\) and lead to the formation of floating aggregates. TeO\(_2^\text{2−}\)-induced floating aggregate formation requires Pel and Psl polysaccharides as well as extracellular DNA (eDNA) (Fig. S1), in accordance with the Psl polysaccharide-eDNA interaction enabling the formation of skeleton of \(P.\ aeruginosa\).
biofilms. In addition to serving as matrix scaffolds, the polysaccharides could also induce synthesis of iron siderophore pyoverdine via the Gac/Rsm pathway in the floating aggregates, as we had previously demonstrated. The formation of stress-induced biofilm-resembling aggregates might contribute to the dissemination of infection in the host.

The results presented here demonstrate that *P. aeruginosa* mutants with low c-di-GMP content were more sensitive to TeO$_3^{2-}$ exposure in planktonic cultures and thus their growth was negatively affected by TeO$_3^{2-}$ exposure, as compared to c-di-GMP containing wild-type strain (Fig. 4). Consistent with this finding, a recent study on biodegradation of 3-chloroaniline by *Comamonas testosteroni* reported that, compared with the wild type, the strain with an elevated c-di-GMP level exhibited a better growth on the toxic substrate at high concentrations. In addition to TeO$_3^{2-}$, the detergent Na-dodecylsulfate (SDS) also raised the c-di-GMP levels and caused aggregation of *P. aeruginosa*. In accordance with the TeO$_3^{2-}$ findings, the ΔsiaD mutant with low intracellular c-di-GMP content was more sensitive to SDS during planktonic growth. Together, these studies highlight that c-di-GMP signaling is involved in multiple stress response mechanisms, which might due to multiple DGCs and PDEs being encoded by many bacterial species.

Finally, we found that wild-type PAO1 strain biofilms prevented the attachment of mutants with low intracellular c-di-GMP content in both normal and TeO$_3^{2-}$ stress co-cultures. Our previous study revealed that the polysaccharides in *P. aeruginosa* biofilms could not be shared, for structural or functional benefits, by mutants that are defective in their synthesis. These latter findings corroborate with the results presented here, and c-di-GMP mediated synthesis of polysaccharides may form another strategy to repress the proliferation and maintenance of c-di-GMP defective variants in biofilms. Considering
that polysaccharides with similar structure to the *P. aeruginosa* polysaccharides are widely distributed in natural bacterial species, our results might reflect a conserved strategy employed by a range of bacterial species to repress the spreading of variants which cannot respond to environmental conditions by moderating their own c-di-GMP levels.

**Methods**

**Bacterial strains and growth medium.** The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Escherichia coli* DH5α strain was used for standard DNA manipulations. LB medium was used to cultivate *E. coli* strains. Batch cultivation of *P. aeruginosa* was carried out at 37 °C in ABT minimal medium supplemented with 5 g glucose l⁻¹ (ABTG) or 2 g glucose l⁻¹ and 2 g casamino acids l⁻¹ (ABTGC). For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg ampicillin (Ap) ml⁻¹, 15 μg gentamicin (Gm) ml⁻¹, 15 μg tetracycline (Tc) ml⁻¹, or 8 μg chloramphenicol (Cm) ml⁻¹. For marker selection in *P. aeruginosa*, 30 μg Gm ml⁻¹, 50 μg Tc ml⁻¹, and 200 μg carbenicillin (Cb) ml⁻¹ were used, when appropriate. Antibiotics were not added to *P. aeruginosa* cultures for c-di-GMP,
stress response and biofilm assays as the plasmids we used were highly stable for these short-term experiments.

**Construction of *P. aeruginosa* mutants.** The ΔpelA, ΔpslBCD and ΔpelAΔpslBCD mutants defective for Pel and/or Psl polysaccharide biogenesis were constructed by allelic displacement as previously described. The ΔsadC, ΔsiaD and ΔsadCΔsiaD mutants defective for SadC and/or SiaD diguanylate cyclase were constructed by allelic displacement as previously described.

**Quantification of static biofilms.** The microtitre tray biofilm formation assay was performed as described by O’Ttoole & Kolter. Briefly, overnight cultures grown in ABTG medium were diluted to OD₆₀₀ = ~0.001 with fresh ABTG medium and transferred to the wells of polystyrene 96-well microtitre trays (200 μl per well) and incubated for 24 h at 37°C. Liquid culture was removed from each well and the wells were washed twice with 0.9% NaCl followed by staining with 0.1% crystal violet and washing twice with 0.9% NaCl. The crystal violet-stained biofilms were then resuspended in 96% ethanol, and the absorbance of biofilm-associated dye was measured at 600 nm. Experiments were performed in triplicate, and the results are shown as the mean ± sd.
Field emission scanning electron microscopy (FE-SEM) and energy-dispersive X-ray spectroscopy (EDX). The aggregates were dried and coated with platinum (Pt) using a vacuum electric sputter coater (JEOL JFC-1300, JEOL Asia Pte Ltd, Singapore). SEM images were taken using a field emission scanning electron microscope (FE-SEM, JSM-7600, JEOL Asia Pte Ltd, Singapore) at a voltage of 2.0-5.0 kV and EDX spectra were obtained using an energy-dispersive X-ray spectroscope (AZtecEnergy, Oxford Instruments, Oxfordshire, UK) as previously described\textsuperscript{50}. Experiments were performed in triplicate, and representative images were shown.

Table 1. Strains and plasmids used in this study.

| Strain(s) or plasmid | Relevant characteristic(s) | Source or reference |
|----------------------|-----------------------------|---------------------|
| *P. aeruginosa* strains | | |
| PAO1 | Prototypic wild-type strain | \textsuperscript{55} |
| ΔpelA | Gm\textsuperscript{r}; pelA derivative of PAO1 constructed by allelic exchange | \textsuperscript{38} |
| ΔpslBCD | Gm\textsuperscript{r}; pslBCD derivative of PAO1 constructed by allelic exchange | \textsuperscript{38} |
| ΔpelAΔpslBCD | Gm\textsuperscript{r}; pelA/pslBCD derivative of PAO1 constructed by allelic exchange | \textsuperscript{38} |
| ΔsadC | Gm\textsuperscript{r}; sadC derivative of PAO1 constructed by allelic exchange | This study |
| ΔsiaD | Gm\textsuperscript{r}; siaD derivative of PAO1 constructed by allelic exchange | This study |
| ΔsadCΔsiaD | Gm\textsuperscript{r}; sadC/siaD derivative of PAO1 constructed by allelic exchange | This study |
| PAO1/p\textsubscript{cdrA-gfp} | Gm\textsuperscript{r}; PAO1 carrying the p\textsubscript{cdrA-gfp} report | \textsuperscript{30} |
| ΔsadC/p\textsubscript{cdrA-gfp} | Gm\textsuperscript{r}; ΔsadC carrying the p\textsubscript{cdrA-gfp} report | This study |
| ΔsiaD/p\textsubscript{cdrA-gfp} | Gm\textsuperscript{r}; ΔsiaD carrying the p\textsubscript{cdrA-gfp} report | This study |
| ΔsadCΔsiaD/p\textsubscript{cdrA-gfp} | Gm\textsuperscript{r}; ΔsadCΔsiaD carrying the p\textsubscript{cdrA-gfp} report | This study |
| PAO1/p\textsubscript{lac-yhjH} | Te\textsuperscript{c}; PAO1 containing the p\textsubscript{lac-yhjH} vector | \textsuperscript{12} |
| PAO1/p\textsubscript{pel-lacZ} | Te\textsuperscript{c}; PAO1 carrying the mini-CTX-pelA-lacZ report | This study |
| ΔsadC/p\textsubscript{pel-lacZ} | Te\textsuperscript{c}; ΔsadC carrying the mini-CTX-pelA-lacZ report | This study |
| ΔsiaD/p\textsubscript{pel-lacZ} | Te\textsuperscript{c}; ΔsiaD carrying the mini-CTX-pelA-lacZ report | This study |
| ΔsadCΔsiaD/p\textsubscript{pel-lacZ} | Te\textsuperscript{c}; ΔsadCΔsiaD carrying mini-CTX-pelA-lacZ report | This study |
| *E. coli* strain | | |
| DH5α | F\textsuperscript{−}, ø80dLacZΔM15, Δ[lacZYA-argF]U169, deoR, recA1, endA1, hsdR17(rK\textsuperscript{−}, mK\textsuperscript{+}), phoA, supE44, λ\textsuperscript{−}, thi\textsuperscript{−}1, gyrA96, relA1 | Laboratory collection |
| Plasmids | | |
| pUCP22 | Ap\textsuperscript{r}; Gm\textsuperscript{r}; Broad-host-range cloning vector | \textsuperscript{56} |
| pMPELA | Ap\textsuperscript{r}; Gm\textsuperscript{r}; pelA allelic replacement vector | \textsuperscript{57} |
| pMPSL-KO1 | Ap\textsuperscript{r}; Gm\textsuperscript{r}; pslBCD allelic replacement vector | \textsuperscript{58} |
| pEX18Gm::ΔsadC | Ap\textsuperscript{r}; sadC allelic replacement vector | \textsuperscript{34} |
| pEX18Gm::ΔsiaD | Gm\textsuperscript{r}; siaD allelic replacement vector | \textsuperscript{34} |
| pFLP2 | Ap\textsuperscript{r}; Source of FLP recombinase | \textsuperscript{59} |
| p\textsubscript{psa}gfp | Ap\textsuperscript{r}; Gm\textsuperscript{r}; pUCP22 carrying the p\textsubscript{psa}gfp fusion | \textsuperscript{36} |
| pRK600 | Cm\textsuperscript{r}; ori ColEl RK2-Mob\textsuperscript{+} RK2-Tra\textsuperscript{+}; helper vector for conjugation | \textsuperscript{80} |
| p\textsubscript{psa}yhjH | Te\textsuperscript{c}; pBBR1MC53 carrying the yhjH gene | \textsuperscript{12} |
| Mini-CTX-pel-lacZ | Te\textsuperscript{c}; mini-CTX vector carrying the pel-lacZ fusion | \textsuperscript{36} |
Reactive oxygen species (ROS) assay. PAO1 cultures were grown in ABTG or LB medium controls and media with 10 μg ml⁻¹ TeO₃²⁻, SeO₃²⁻ and SeO₂²⁻, respectively. The ROS content of 1 ml stationary phase bacterial cells were then measured by using the OxiSelect™ in vitro ROS/RNS assay kit (Green Fluorescence), accordingly to manufacturer's instructions. 2',7'-dichlorodihydrofluorescein (DCF) was used as a standard and the concentrations of ROS from PAO1 cultures were estimated according to the DCF standard curve. The fluorescence of the samples was read by the Tecan Infinite 2000 Microplate Reader at 480 nm excitation/530 nm emission. Experiments were performed in triplicate, and the results are shown as the mean ± sd. Student’s t-test was performed for testing differences between groups.

iTRAQ-based proteomics analyses. P. aeruginosa PAO1 was grown in ABTG medium with and without 10 μg/ml TeO₃²⁻ at 37°C with shaking until stationary phase was reached. Cells were harvested and iTRAQ-based proteomics analyses were carried out as previously described³³.

Determination of minimal inhibitory concentration (MIC). The MIC assays employed a microtiter broth dilution method as previously described in the NCSLA guidelines⁵¹. Briefly, fresh ~16 h cultures of P. aeruginosa were diluted in ABTG medium. For determination of MIC, potassium tellurite was dissolved in water at a concentration 10 times higher than the required range by serial dilutions from a stock solution. 10 μl of each concentration were added to each corresponding well of a 96-well microtiter plate (polypropylene, Costar Corp.) and 90 μl of bacterial culture (~1 × 10⁵ cells) in ABTG medium were added. The plate was incubated at 37°C for 16-18h. MIC was taken as the lowest concentration where no visual growth (based on OD₆₀₀ of bacteria) was detected. Experiments were performed in triplicate and representative results were shown.

TeO₃²⁻ tolerance assay. Overnight cultures of different P. aeruginosa strains were inoculated into ABTG medium containing 20 μg/ml TeO₃²⁻ and cultivated overnight (24h). Overnight cultures were serially diluted and plated onto LB agar media. LB plates were incubated at 37°C overnight before CFU calculation. Experiments were performed in triplicate, and the results are shown as the mean ± sd.

Beta-galactosidase activity assay. A classical β-galactosidase assay³² was used to measure expression of the Ppel-lacZ fusion in P. aeruginosa strains transformed with the mini-CTX-pel-lacZ fusion³⁶, which carries the pel promoter fused to the E. coli lacZ gene. Experiments were performed in triplicate, and the results are shown as the mean ± sd. Student’s t-test was performed for testing differences between groups.

Gfp reporter fusion assay. The expression of the c-di-GMP Pabot-gfp biosensor⁵⁰ in P. aeruginosa strains in the presence and absence of TeO₃²⁻ was monitored by using a Tecan Infinite 2000 Microplate Reader. Monitoring strains were cultivated in 24-well microtiter plate with ABTG medium with different concentrations of TeO₃²⁻ at 37°C with shaking. OD₆₀₀ and GFP fluorescence (in relative fluorescence units, rfu) were measured every hour until the culture reach stationary growth phase. Experiments were performed in triplicate, and the results are shown as the mean ± sd. Student’s t-test was performed for testing differences between groups.

Quantification of c-di-GMP concentration. Extraction of c-di-GMP was conducted as previously described⁴⁵. 10 ml of P. aeruginosa cells in the early stationary phase from the ABTG medium with and without 10 μg/ml TeO₃²⁻ were washed twice with 1 mM ammonium acetate. Cells were lysed with 0.6 M HClO₄ on ice for 30min. Cell debris was removed by centrifugation and supernatant was neutralized to pH 6.0 with the addition of 2.5 M KHCO₃. The precipitated KClO₄ was removed by centrifugation and the supernatant was used for relative quantification of c-di-GMP. The concentration was measured by High Performance Liquid Chromatography (HPLC), the injection volume is 20 μl with 254 nm as detection wavelength. Reverse-phase C18 Targa column (2.1 x 40 mm, 5 μm) (catalog number: TR-0421-C185) was used with solvent A (10 mM ammonium acetate in water) and solvent B (10 mM ammonium acetate in methanol) at a flow rate of 0.2 ml min⁻¹. Eluent gradient is as follows: 0 to 8 min, 1% B; 8 to 14 min, 15% B; 14 to 16 min, 19% B; 16 to 24 min, 100% B; 24 to 32 min, 100% B; 32 to 40 min, 1% B; 40 to 42 min, 1% B. The retention time of c-di-GMP is around 14.0 min. The c-di-GMP concentration was normalized by total protein concentration. The relative c-di-GMP concentrations of cells treated with 10 μg/ml TeO₃²⁻ tellurite against cells in ABTGC only were shown. Experiments were performed in triplicate, and the results are shown as the mean ± sd. Student’s t-test was performed for testing differences between groups.

Competition assay. Competition assays were performed in both planktonic and biofilm co-cultures. In planktonic co-cultures, cfp-tagged wild-type PAO1 was mixed 1:1 (vol/vol) with yfp-tagged PAO1/pΔΔsadCΔsiaD and the mixtures inoculated into fresh ABTG medium with and without the presence of 10 μg/ml TeO₃²⁻. For relative fitness calculation, co-cultures were plated in LB agar plates after 24h cultivation at 37°C with shaking. Colony-forming units (CFUs) Nₜ were determined from three individual experiments and the number of PAO1 and PAO1/pΔΔsadCΔsiaD colonies were determined based on their specific fluorescence at times t = 0 and at t = T. Relative fitness was
determined as $r_g = [N(T) - N(0)]/[N(T) - N(0)]$ as previously described with modification, resulting in a fitness of $'I$ when competing organisms are equally fit. Experiments were performed in triplicate, and the results are shown as the mean ± sd. Student's t-test was performed for testing differences between groups.

In biofilm co-cultures, cpf-tagged wild-type PAO1 cells were mixed with yfp-tagged PAO1/ΔsiaD cells at 1:1 (vol/vol) and the mixtures were inoculated into fresh ABTGC medium with and without the presence of 10 μg/ml TeO$_2$-. Static biofilms were cultivated on cover slides at 37°C for 24 h as previously described. Biofilms were imaged with a Zeiss LSM780 confocal laser scanning microscope (CLSM) equipped with detectors and filter sets for monitoring of CpF and Yfp fluorescence. Images were obtained using a 40 ×/1.4 objective. Simulated three-dimensional images and sections as well as biovolumes were generated using the Imaris software package (Bitplane AG). The biovolume $V_j$ of each strain in the biofilm mode was determined from three individual experiments based on their fluorescence at times $t = 0$ and at $t = T$. Relative fitness was determined as $r_j = [V_j(T) - V_j(0)]/ [V_j(T) - V_j(0)]$ as previously described with modification, resulting in a fitness of $'I$ when competing organisms are equally fit. Experiments were performed in triplicate, and the results are shown as the mean ± sd. Student's t-test was performed for testing differences between groups.

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Author Contributions

T.T.N., B.C., S.K. and L.Y. designed the project. S.L.C., M.T.R., J.B.A., M.J.Y. and K.S. performed the experiments. T.E.N., M.G., B.C. and L.Y. interpreted data. B.C. and L.Y. wrote the main manuscript text. All authors reviewed the manuscript.

Additional Information

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