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ORIGINAL ARTICLE

Pseudomonas moraviensis subsp. stanleyae, a bacterial endophyte of hyperaccumulator *Stanleya pinnata*, is capable of efficient selenite reduction to elemental selenium under aerobic conditions

L.C. Staicu[1,2,3], C.J. Ackerson[4], P. Cornelis[5], L. Ye[5], R.L. Berendsen[6], W.J. Hunter[7], S.D. Noblitt[4], C.S. Henry[4], J.J. Cappa[2], R.L. Montenieri[7], A.O. Wong[4], L. Musilova[8], M. Sura-de Jong[8], E.D. van Hullebusch[3], P.N.L. Lens[2], R.J.B. Reynolds[1] and E.A.H. Pilon-Smits[1]

1 Biology Department, Colorado State University, Fort Collins, CO, USA
2 UNESCO-IHE Institute for Water Education, Delft, The Netherlands
3 Université Paris-Est, Laboratoire Géomatériaux et Environnement, UPEM, Marne-la-Vallée, Cedex 2, France
4 Chemistry Department, Colorado State University, Fort Collins, CO, USA
5 VIB Department of Structural Biology, Department of Bioengineering Sciences, Research Group Microbiology, Vrije Universiteit, Brussels, Belgium
6 Plant–Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, Utrecht, The Netherlands
7 USDA–ARS, Fort Collins, CO, USA
8 Biochemistry and Microbiology Department, Institute of Chemical Technology in Prague, Prague, Czech Republic

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Correspondence
Elizabeth Pilon-Smits, Biology Department, Colorado State University, 80523, Fort Collins, CO, USA.
E-mail: epsmits@lamar.colostate.edu

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Abstract

Aims: To identify bacteria with high selenium tolerance and reduction capacity for bioremediation of wastewater and nanoselenium particle production.
Methods and Results: A bacterial endophyte was isolated from the selenium hyperaccumulator *Stanleya pinnata* (Brassicaceae) growing on seleniferous soils in Colorado, USA. Based on fatty acid methyl ester analysis and multi-locus sequence analysis (MLSA) using *16S rRNA*, *gyrB*, *rpoB* and *rpoD* genes, the isolate was identified as a subspecies of *Pseudomonas moraviensis* (97·3% nucleotide identity) and named *P. moraviensis* stanleyae. The isolate exhibited extreme tolerance to SeO_3^{2-} (up to 120 mmol l^{-1}) and SeO_4^{2-} (>150 mmol l^{-1}). Selenium oxyanion removal from growth medium was measured by microchip capillary electrophoresis (detection limit 95 nmol l^{-1} for SeO_3^{2-} and 13 nmol l^{-1} for SeO_4^{2-}). Within 48 h, *P. moraviensis* stanleyae aerobically reduced SeO_3^{2-} to red Se(0) from 10 mmol l^{-1} to below the detection limit (removal rate 0·27 mmol h^{-1} at 30°C); anaerobic SeO_3^{2-} removal was slower. No SeO_4^{2-} removal was observed. *Pseudomonas moraviensis* stanleyae stimulated the growth of crop species *Brassica juncea* by 70% with no significant effect on Se accumulation.
Conclusions: *Pseudomonas moraviensis* stanleyae can tolerate extreme levels of selenate and selenite and can deplete high levels of selenite under aerobic and anaerobic conditions.
Significance and Impact of the Study: *Pseudomonas moraviensis* subsp. stanleyae may be useful for stimulating plant growth and for the treatment of Se-laden wastewater.

Introduction

Selenium (Se) is an element found in fossil fuels, phosphate deposits, sulphide minerals and seleniferous soils (Lemly 2004). Its complex biochemistry allows Se to be cycled through different environmental compartments (Chapman *et al.* 2010). The two oxyanions of Se, selenate (Se[VI], SeO_4^{2-}) and selenite (Se[IV], SeO_3^{2-}), are

water-soluble, bioavailable and toxic (Simmons and Wallschlaeger 2005). Because of the toxicity posed by Se oxyanions, the U.S. Environmental Protection Agency has set a limit of 50 μg l^{-1} for Se in drinking water (USEPA 2003). In its elemental state, Se(0), Se is water-insoluble and less bioavailable (Chapman *et al.* 2010). Various industrial sectors produce wastewaters containing toxic Se oxyanions that can be cleaned up using a microbial treatment system, provided the bacterial inoculum can reduce Se oxyanions to solid Se(0), that can be further removed from wastewater (Sobolewski 2013; Staicu *et al.* 2015). Considering the future trends in energy production based on fossil fuel combustion, it is expected that Se will increase its presence and toxicity in the environment (Lenz and Lens 2009). To cope with this challenge, biotechnological Se removal processes can be employed as a cheaper and more efficient alternative over physical-chemical clean-up technologies (NAMC 2010).

Several *Pseudomonas* species have been reported to metabolize Se oxyanions (*Pseudomonas seleniipraecipitatus* in Hunter and Manter 2011; *Pseudomonas stutzeri* NT-I in Kuroda *et al.* 2011) but with different degrees of success. The genus *Pseudomonas* encompasses a wide array of genetically and metabolically diverse bacterial species. Since Walter Migula coined and introduced the name *Pseudomonas* in 1894, the genus has undergone a dramatic rearrangement to the 218 species indexed at the time of writing (http://www.bacterio.cict.fr/p/pseudomonas.html). Different *Pseudomonas* species have been shown to be opportunistic human pathogens of clinical relevance (*Pseudomonas aeruginosa*), plant pathogens (*Pseudomonas syringae*), denitrifiers (*P. stutzeri*) or plant growth promoters (*Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas chlororaphis*) (Spiers *et al.* 2000; Zago and Chugani 2009). *Pseudomonas* representatives are rod-shaped, Gram-negative γ Proteobacteria, motile by one or more polar flagella, aerobic, catalase positive and chemoorganotrophic, but all these characteristics do not allow an absolute differentiation (Palleroni 2005; Peix *et al.* 2009). The determination of sequence similarity in ribosomal RNA is accepted as a solid classification argument. However, in the case of the genus *Pseudomonas*, the discriminative power of 16S sequencing is rather low, and now a multilocus sequencing analysis approach combining different housekeeping genes is preferred (Mulet *et al.* 2010).

In the current work, we describe the isolation and characterization of a bacterial endophyte (*P. moraviensis* stanleyae) that dwells in the roots of *Stanleya pinnata,* a selenium hyperaccumulator plant species. Hyperaccumulators are plants that accumulate one or more chemical elements to levels typically two orders of magnitude higher than those in the surrounding vegetation (Terry *et al.* 2000). *Stanleya pinnata* is native to Se-rich soils throughout the western USA and has been shown to reach up to 2000 mg kg^{-1} Se (0·2%) in its root tissues, and even higher levels in leaves and flowers (Freeman *et al.* 2006; Galeas *et al.* 2007). The bacterial strain, derived from this extreme environment and reported herein, was tested for selenite and selenate tolerance and reduction under aerobic and anaerobic conditions.

Materials and methods

Media and culture conditions

Luria Broth (LB) was purchased from Fisher Scientific (Waltham, MA, USA). When the growth media was amended with salts of Se oxyanions, the aliquots were added from filter-sterilized stock solutions (1 mol l^{-1}). Sodium selenate, Na_2SeO_4, ≥98%, and sodium selenite, Na_2SeO_3, ≥99%, were purchased from Sigma Aldrich. All other reagents were of analytical grade unless otherwise stated. The incubations were performed at 30°C, pH 7·5, 200 rev min^{-1}, under aerobic and anaerobic conditions. For anaerobic incubations, rubber septa serum bottles containing LB media were used and the headspace of the sealed bottles was replaced and purged with N_2 for 15 min prior to incubation at 30°C, pH 7·5, and 200 rev min.

Isolation of strain #71

The strain, with accession #71, was isolated from the root tissue of Se hyperaccumulator *S. pinnata* (Brassicaceae), growing on the seleniferous soils of Pine Ridge Natural Area (Colorado, USA) on the west side of Fort Collins (40°32·70N, 105°07·87W, elevation 1510 m). Plants were harvested in the field during August 2012 and brought to the lab for storage at 4°C before further processing.

After washing, the plants were surface-sterilized for 15 min on a rotary shaker using 2% sodium hypochlorite (NaClO) and 0·5 ml l^{-1} Tween 20. This step was followed by three washings with sterile H_2O. The last rinsing water was plated on half-strength LB media as a control measure, to test for surface sterility. The tissues were transferred to 10 ml of sterile 10 mmol l^{-1} $MgSO_4$ and then ground at room temperature under sterile conditions using a micropestle and microcentrifuge tubes. The homogenate was allowed to settle and separate gravitationally and the supernatant sampled. Aliquots of 100 μl of the supernatant were placed on solid half-strength LB media. The incubation was performed at room temperature for 7 days. Individual colonies were subcultured on new media to gain pure bacterial monocultures. To test for SeO_3^{2-} reduction capacity, bacterial monocultures were subcultured at 30°C on the same solid

media containing 10 mmol l^{-1} sodium selenite. Among monocultures, isolate #71 was selected for this study based on its apparent high Se tolerance and ability to reduce selenite to red elemental Se.

Identification of strain #71

Whole-cell fatty acid methyl ester analysis

For fatty acid methyl esters (FAME) analysis, 48-h pure cultures cultivated on slant LB agar were supplied to MIDI Labs Inc. (Newark, DE, USA). The results were analysed using the Sherlock Microbial Identification System 6.2 (MIDI Inc., Newark, DE, USA).

Multi-locus sequence analysis

The taxonomic position of the isolate was investigated using a Multi-locus sequence analysis, as described by Mulet *et al.* (2010). The concatenated partial sequences of four housekeeping genes (*16S rRNA*, *gyrB*, *rpoB* and *rpoD*) of the strain were aligned with those of 107 *Pseudomonas* type strains. A phylogenetic tree was generated based on the alignment by neighbour-joining using the CLC main workbench 6.7.2 (CLC bio, Aarhus, Denmark). Using universal primers 16F27 and 16R1492 the 16S rRNAs were amplified (Table S1). Housekeeping genes were amplified using the following primers: UP1E, APrU, M13R and M13(-21) (for *gyrB*); LAPS5 and LAPS27 (for *rpoB*); and PsEG30F and PsEG790R (for *rpoD*) (Table S1). The PCR conditions were as follows: 94°C for 5 min; then 94°C for 1 min, 57°C for 45 s, 72°C for 2 min for 30 cycles; then 72°C for 10 min for final extension followed by cool down to 4°C. The DNA fragments were analysed in 1% agarose gel.

Growth test

Isolate #71 was grown aerobically in liquid LB media and the samples were analysed every 3 h during the first day. The growth curve was constructed based on plate colony counts (Colony Forming Units, CFU) and optical density (OD) measurements at 600 nm absorbance using a Beckman DU530 spectrophotometer. When grown in the presence of 10 mmol l^{-1} Na_2SeO_3, to avoid the spectral interference of red elemental Se, an indirect method was employed. Red Se(0) absorbs at a wavelength around 612 nm (Kumar *et al.* 2014), therefore the production of Se(0) particles interferes with the spectrophotometric ($OD_{600\ nm}$) bacterial growth measurements. Following a procedure adapted from Di Gregorio *et al.* (2005), a control experiment was used to measure the OD_{600} and 200 μl of a 10^{-6} dilution of each time point (6, 9, 12, 24 and 48 h) was plated on solid LB media and incubated at 30°C for 24 h. The experiment containing Na_2SeO_3 was also 10^{-6} diluted and plated together with the diluted control under the same conditions. After 24 h, the CFU number that developed on the control and selenite-amended agar plates was counted and the results (Table S2) were statistically processed (mean and standard deviation) using SigmaPlot (Systat Software Inc., San Jose, CA, USA). The growth curve of the selenite-containing treatment (in triplicate) was built by back calculation using the correlated OD_{600}-CFU of the control treatment.

Selenium oxyanion measurements

Inorganic selenite concentrations were measured using microchip capillary electrophoresis (MCE) in a poly(dimethylsiloxane) (PDMS) device with contact conductivity detection (Noblitt and Henry 2008). The separation background electrolyte was recently developed to specifically target Se oxyanions with high selectivity and sensitivity (Noblitt *et al.* 2014). Detection limits are 53 and 280 nmol l^{-1} for selenate and selenite respectively. Samples were diluted 250-fold in background electrolyte prior to analysis, yielding respective detection limits of 13 and 95 nmol l^{-1}. The specific selenite reduction rate was calculated from the slope of the linearized time course.

Selenium tolerance

Na_2SeO_3 and Na_2SeO_4 were added to LB media at increasing concentrations from 0·1 to 150 mmol l^{-1}. The first concentrations used were 0·1, 0·5, 1, 5 and 10 mmol l^{-1}. Between 10 and 150 mmol l^{-1}, the Se oxyanions concentrations were increased by 10 mmol l^{-1}-increments. Each test tube was inoculated with 1% (v/v) of the same stock culture of *P. moraviensis* stanleyae. The Se tolerance was determined by the highest SeO_3^{2-} and SeO_4^{2-} concentration at which growth was detected by spectrophotometric measurement at $OD_{600\ nm}$. In the case of SeO_3^{2-}, growth was accompanied by the production of red Se(0).

Transmission electron microscopy

For transmission electron microscopy (TEM), 24-h-grown cultures in LB containing either 10 mmol l^{-1} Na_2SeO_3 or no Na_2SeO_3 (control) were sampled, processed and fixed in a solution containing 2·5% glutaraldehyde and 2% formaldehyde (Mishra *et al.* 2011). Aliquots of five micro litre were pipetted onto 400 mesh carbon coated copper TEM grids (EM Sciences). Excess liquid was wicked off with filter paper after 1 min. The resulting samples were examined in a JEOL (Peabody, MA, USA) JEM-1400 TEM operated at 100 kV and spot size 1. The Se(0) particle size was determined by TEM image processing using ImageJ™ 1.47v software (National Institutes of Health free software, http://imagej.nih.gov/ij/).

Inoculation experiment

Indian mustard (*Brassica juncea* L.) plants were grown from surface-sterilized seeds on soil collected from Pine Ridge Natural Area (for soil properties see Galeas *et al.* 2007). The soil was collected in the field and mixed with Turface® gravel in a 2 : 1 soil:Turface® ratio. Polypropylene (Magenta) boxes were filled to a height of 2 cm with this mixture, and autoclaved for 40 min. Seeds were surface-sterilized by rinsing for 30 min in 15% household bleach (1·5% NaClO) followed by five 5 min rinses in sterile water, and then sown in the Magenta boxes at a density of three seeds per box and six boxes per treatment. One week after germination, the seedlings were thinned to one plant per box and inoculated with *P. moraviensis* stanleyae (#71); there was an uninoculated parallel control treatment. Before inoculation, the bacteria were grown in half-strength LB for 24 h at 25°C, harvested by centrifugation and resuspended in 10 mmol l^{-1} $MgSO_4$ to an OD_{600} of 1·0. One millilitre of inoculum was delivered using a pipette to the base of each seedling; the controls received 1 ml of 10 mmol l^{-1} $MgSO_4$. The plants were allowed to grow for 6 weeks. The boxes were watered with autoclaved water every 2 weeks (twice total) in a laminar flow sterile hood. The plants were then harvested, separating the root and shoot. Small shoot and root samples from each plant were placed in 10 mmol l^{-1} $MgSO_4$ for re-isolation of bacterial endophytes, to verify successful inoculation. These were ground using sterile micropestles in microcentrifuge tubes, and 100 μl of the extract was streaked onto LB agar plates, which were monitored after 24 h and compared visually with the inoculum. The remainder of the root and shoot material was dried and weighed. Root and shoot samples were digested in nitric acid according to Zarcinas *et al.* (1987) and analysed for elemental composition using inductively coupled plasma–optical emission spectrometry according to Fassel (1978).

Statistical analysis

The results were statistically processed and plotted using the data analysis software SigmaPlot 12.0v. When the standard deviation was <5%, the error bars are not presented in the figures. All experiments were performed in triplicate unless otherwise stated.

Results

Phylogenetic analysis

After isolation of endophytic bacteria from surface-sterilized *S. pinnata* roots, strain #71 was selected for further study based on its apparent tolerance to selenite and production of red elemental Se. To identify the phylogenetic position of strain #71, we performed a multiphase analysis. First, the cellular fatty acid content of the strain was analysed (Table 1). The major fatty acids were $C_{16:1}$ w7c/16:1 w6c, $C_{16:0}$ and $C_{18:1}$ w7c.

To further establish the taxonomic position of strain #71, we amplified four housekeeping genes (16S rRNA, *gyrB*, *rpoB* and *rpoD*) and compared the concatenated sequences of these genes to those of 107 *Pseudomonas* type strains as described by (Mulet *et al.* 2010). Figure 1 shows the phylogenetic position of strain #71.

Growth

The aerobic growth of *P. moraviensis* stanleyae in LB media with and without the Se oxyanions selenite and selenate is presented in Fig. 2. The control (no Se) exhibited a lag phase of around 4 h, followed by a steep logarithmic growth phase. After 9 h of growth, the culture entered the stationary phase. A similar growth curve was observed with 10 mmol l^{-1} sodium selenate-amended media. In contrast, when 10 mmol l^{-1} sodium selenite was present, the lag phase was prolonged to around 6 h, followed by a 6-h long exponential growth phase. The stationary phase of the SeO_3^{2-} treatment started after around 12 h of incubation.

SeO_3^{2-} reduction

The aerobic reduction of selenite was further investigated by means of MCE. During the first 9 h of incubation, the initial 10 mmol l^{-1} selenite did not decrease (Fig. 3). After 9 h, SeO_3^{2-} was depleted linearly with time, at a reduction rate of 0·27 mmol h^{-1} (R^2 = 0·999). No selenite was detected in the supernatant after 48 h of incubation. The depletion of selenite was paralleled by a change

Table 1 Fatty acid methyl esters profile of *Pseudomonas moraviensis* stanleyae

Fatty acid	(%)
3-OH $C_{10:0}$	3·42
$C_{10:0}$	1·61
2-OH $C_{12:0}$	6·18
3-OH $C_{12:0}$	5·03
$C_{14:0}$	0·55
$C_{16:0}$	29·85
$C_{16:1}$ w7c/16:1 w6c	35·87
$C_{17:0}$ cyclo	3·00
$C_{18:0}$	0·42
$C_{18:0}$ ante/18:2 w6, 9c	0·23
$C_{18:1}$ w7c	13·84

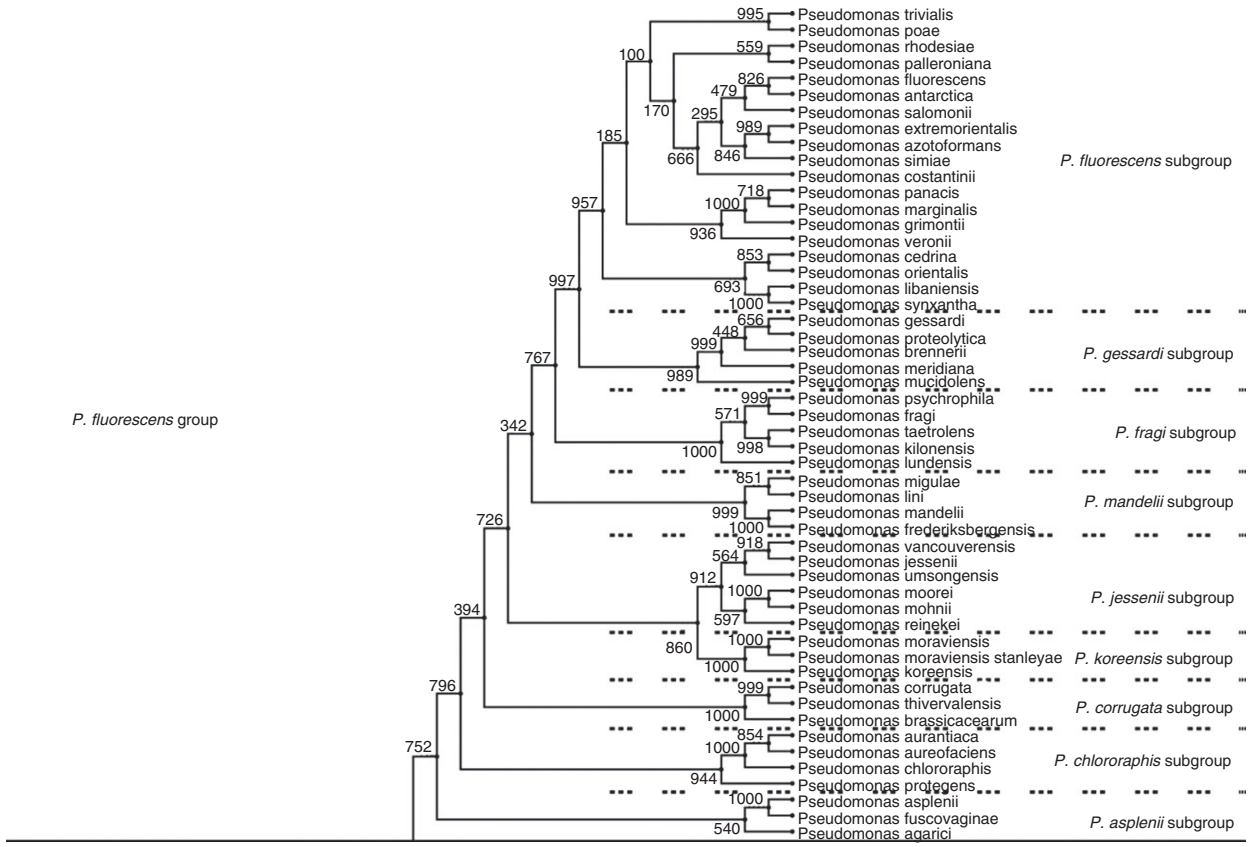

Figure 1 Neighbour-joining tree based on the 16S rRNA, *gyrB*, *rpoB* and *rpoD* gene sequences of *Pseudomonas moraviensis* stanleyae and 107 *Pseudomonas* type strains. The phylogenetic position of *P. moraviensis* stanleyae within the *Pseudomonas fluorescens* group is shown.

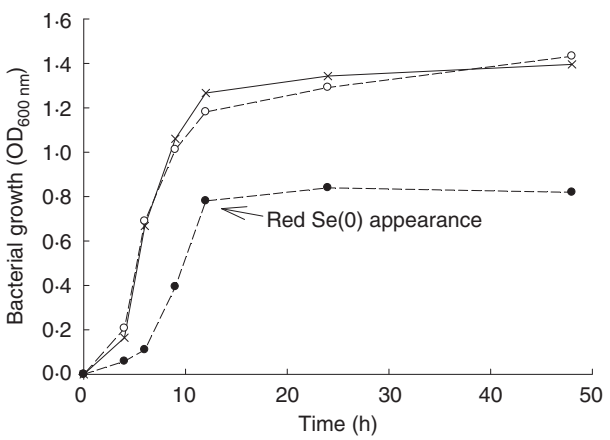

Figure 2 Aerobic growth of *Pseudomonas moraviensis* stanleyae in Luria Broth media without Se oxyanions (×) and in the presence of 10 mmol l^{-1} SeO_3^{2-} (●) and 10 mmol l^{-1} SeO_4^{2-} (○). Note that the arrow indicates the time of red Se(0) appearance.

in colour of the culture from off-white to pink to deep red (results not shown). In contrast, the selenate concentration did not decrease even after 48 h of incubation (Fig. 3) and also no red elemental Se was observed.

Selenium tolerance

The *P. moraviensis* strain described in this study showed extremely high tolerance to Se oxyanions. It grew and produced elemental Se in medium containing up to 120 mmol l^{-1} selenite; however, above 30 mmol l^{-1} concentration there was an increasing lag time. During the first 48 h, the strain showed growth and formation of red Se(0) in selenite concentrations up to 30 mmol l^{-1}. During day 3, bacterial growth was detected in incubations containing up to 50 mmol l^{-1} SeO_3^{2-}. From day 4 to day 10, bacterial growth was detected in incubations containing progressively higher selenite concentrations (from 60 mmol l^{-1} for day 4 to 120 mmol l^{-1} at day 10 respectively). At SeO_3^{2-} concentrations higher than 120 mmol l^{-1}, no bacterial growth was recorded at all, until day 15. In the case of SeO_4^{2-}, bacterial growth was recorded during the tested period up to the highest concentration used, 150 mmol l^{-1}.

Production of red elemental selenium

The production of red Se(0) was strongly correlated with bacterial growth (Table 2). Red Se(0) was produced both

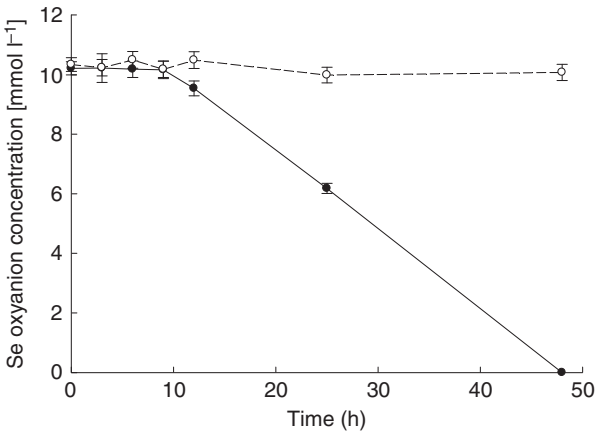

Figure 3 Aerobic reduction of SeO_4^{2-} (○) and SeO_3^{2-} (●) by *Pseudomonas moraviensis* stanleyae as a function of time.

Table 2 Growth of *Pseudomonas moraviensis* stanleyae and formation of red Se(0) in Luria Broth media under different growth conditions

Condition	Growth	Red Se(0) formation
Aerobic		
4°C	−	−
28°C	+++	+++
41°C	+	+
NaCl 5%	+	+
NaCl 7%	−	−
Anaerobic		
28°C	+	+
Heat−killed inoculum*	−	−

−, no growth or red Se(0) production; +, growth and red Se(0) production; +++, optimal growth and red Se(0) production.
*Autoclaved at 121°C for 15 min.

under aerobic and anaerobic conditions by the reduction of selenite (Figs. 4a and S1), likely as a detoxification reaction (Kessi *et al.* 1999). Although the strain could grow in the presence of 10 mmol l^{-1} SeO_3^{2-} or in the presence of 5% (w/v) NaCl, when both were present, no growth and no red Se(0) formation were detected. At 41°C, the strain could only produce limited amounts of red Se(0), indicative of suboptimal growth conditions induced by higher temperatures.

Figure 4b presents a TEM micrograph of *P. moraviensis* stanleyae grown aerobically for 24 h in medium amended with 10 mmol l^{-1} of Na_2SeO_3. Elemental Se nanoparticles are visible on the surface of bacterial cells and display a size lower than 100 nm.

Inoculation

As this *Pseudomonas* strain was a root endophyte isolated from a Se hyperaccumulator from the Brassicaceae family (*S. pinnata*), its capacity to affect growth and accumulation of Se and other plant nutrient was investigated in related species Indian mustard (*B. juncea*), which is an economically important crop and also a popular species for phytoremediation. Inoculation with *P. moraviensis* stanleyae enhanced root growth by 38% (NS) and shoot growth by 70% ($P < 0{\cdot}05$, Table 3). Root and shoot Se levels were not significantly affected in the inoculated plants, but showed a trend to be lower compared to the control plants (Table 3). In contrast, the chemically similar element sulphur (S) was present at higher levels in roots of inoculated plants compared to control plants (Table 3), while shoot Se levels were unaffected. The effect of inoculation on plant potassium (K) levels was very similar to those of Se: K levels were higher in root

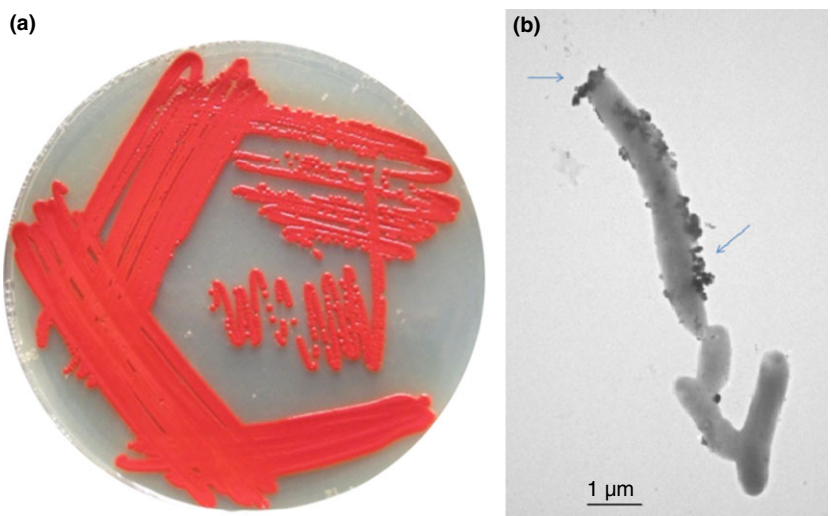

Figure 4 Biogenic Se(0) (a) Red Se(0) formation on agar plate (aerobic conditions) and (b) transmission electron micrograph of *Pseudomonas moraviensis* stanleyae producing Se(0) under aerobic conditions. Note that the arrows indicate the presence of electron-dense Se(0) nanoparticles.

Table 3 Dry weight (mg) and elemental concentration (mg kg^{-1} dry weight) for *Brassica juncea* grown without (control) and with (inoculated) *Pseudomonas moraviensis stanleyae*. Shown values are the mean and standard error of the mean ($n = 6$). All inoculated values that are significantly different from the control ($P < 0{\cdot}05$) are indicated by an asterisk (*)

	Root		Shoot	
	Control	Inoculated	Control	Inoculated
Dry weight	8 ± 1·4	11 ± 1·3	17 ± 3·4	29 ± 2·7*
Ca	29 221 ± 3040	44 513 ± 15 141*	14 580 ± 2023	9920 ± 581*
K	14 917 ± 1411	30 362 ± 5669*	61 470 ± 12 331	62 193 ± 2500
Mn	592 ± 65	1155 ± 214*	449 ± 477	735 ± 127*
S	3831 ± 291	7563 ± 1129*	17 183 ± 1997	16 396 ± 448
Se	450 ± 118	345 ± 135	1692 ± 434	554 ± 122

and unaffected in shoots of inoculated plants, as compared to control plants (Table 3). Calcium translocation from root to shoot appeared to be reduced in inoculated plants, as Ca levels were higher in the root and lower in the shoot of inoculated plants, compared to control plants (Table 3). Manganese (Mn) levels were significantly higher in both roots and shoots of inoculated plants, compared to uninoculated controls (Table 3). Other elements that were also tested but found to not be different between inoculated and control plants include Cu, Fe, Mg, Mo, Ni and Zn (results not shown).

Discussion

Phylogenetic analysis

Based on the FAMEs of strain #71, a similarity index (SIM) of 0·846 was calculated, matching with the FAME profile of *P. fluorescens*. According to the FAME protocol, SIM indices higher than 0·6 are indicative of a good species match.

The genus *Pseudomonas*, however, is very diverse and its taxonomy is still developing (Mulet *et al.* 2010). Using the multi-locus sequencing analysis, the phylogenetic position of strain #71 was further refined to *Pseudomonas koreensis*, a subgroup of *P. fluorescens,* and was finally found to be most closely related to the *P. moraviensis* type strain, with 97·3% nucleotide identity (NI) match between the concatenated sequences (Fig. 1). As 97% NI was proposed as the species boundary (Mulet *et al.* 2010), we classified strain #71 as a subspecies of *P. moraviensis* and named it *P. moraviensis* stanleyae in reference to its host plant. *Pseudomonas moraviensis* was isolated from soil by selective enrichment with nitroaromatics (Tvrzova *et al.* 2006).

Taken together, these two phylogenetic approaches show the importance of the best discriminative tool towards the proper identification of a species. The genus *Pseudomonas* has been shown to be complex and often various subspecies can have contrasting metabolic traits (Palleroni 2005). This is particularly important as different wastewaters have a complex make-up with competing oxyanions that can interfere with the target pollutants to be treated by bioremediation.

Growth

Pseudomonas moraviensis stanleyae can grow aerobically in the presence of both selenite and selenate but exhibits different growth patterns. While the presence of selenate does not appear to have a negative impact on the growth curve compared to the control treatment, the presence of selenite elicits a toxic effect. The control and the selenate-amended treatments showed a typical sigmoid growth curve and the treatments exhibited an almost similar growth pattern. In contrast, when the culture was amended with 10 mmol l^{-1} selenite, a 40% diminished cell concentration (based on CFU count) was measured during stationary phase. The comparison between growth phases exhibited by the selenite-amended culture and the control show an extended lag phase induced by selenite (6 h *vs* 4 h). Moreover, the stationary phase also starts with a 3-h delay in the selenite-amended treatment (12 h *vs* 9 h). This indicates that the cell division takes place at a slower rate when selenite is present.

Overall, the delay in growth phases and the slower growth of the selenite-amended culture indicate a toxic effect elicited by selenite. Similarly, a 40% lower cell concentration between the control and the 0·5 mmol l^{-1} SeO_3^{2-}-amended phototrophic bacterium *Rhodospirillum rubrum* cultures during stationary growth phase was reported Kessi *et al.* (1999). It has been shown that selenite reacts with glutathione forming toxic reactive oxygen species (H_2O_2 and O_2^-) that trigger the production of further oxidative stress enzymes (Kramer and Ames 1988; Bebien *et al.* 2001).

It is interesting to note that, although *Pseudomonas* is often considered an obligate aerobic genus, production of red Se(0) by *P. moraviensis* stanleyae was observed under anaerobic conditions (Fig. S1). The fact that selenite was

reduced anaerobically might indicate its possible use as a terminal electron acceptor when oxygen concentration is depleted, in analogy with *Bacillus selenitireducens* (Switzer Blum *et al.* 1998).

SeO_3^{2-} reduction

The poor depletion of selenite during the first 9 h of incubation can be linked to the longer time needed by the culture to reach the stationary phase. The high selenite reduction showed during the stationary phase points out the importance of keeping the inoculum in a steady stationary state in order to ensure the efficient and high rate conversion of selenite. Furthermore, the short period needed until the stationary phase is reached suggests that the strain under investigation could be a promising alternative to the slower anaerobic inocula. Compared with the anaerobic reduction of selenite by mixed anaerobic microbial communities (granular sludge) in a batch mode, which may take 10–14 days (Lenz and Lens 2009), *P. moraviensis* stanleyae is at least 5-fold faster under aerobic conditions. Using *Shewanella oneidensis* MR-1 grown anaerobically at 30°C, in LB media containing 0·5 mmol l^{-1} selenite and 20 mmol l^{-1} fumarate. Li *et al.* (2014) reported selenite reduction rates between 0·5 and 1·5 μmol h^{-1} for the wild type species and three mutants. As a comparison, the 0·27 mmol h^{-1} selenite reduction rate reported herein is three orders of magnitude faster. This strain therefore holds promise for the development of a more efficient aerobic treatment system of selenite-laden wastewaters.

The depletion of selenite below the highly sensitive detection limits of MCE is particularly relevant in the context of the very low (50 μg l^{-1}) permissible discharge limits imposed by the regulatory agencies (USEPA 2003).

Tolerance to selenite and selenate

The amount of selenite that can be reduced to red Se(0) is an important parameter in the design and operation of bioreactors treating Se-laden wastewaters. With increasing selenite concentration, the culture exhibited a longer delay before the onset of the reduction process and the formation of red Se(0). Similar high tolerance to selenite was reported for other bacteria (Kuroda *et al.* 2011; Lampis *et al.* 2014). A *P. stutzeri* strain collected from the drainage water of a Se refinery plant (Kuroda *et al.* 2011) was reported to aerobically reduce high concentrations of selenite (up to 94 mmol l^{-1}), although the higher levels were not completely depleted and selenite could not be reduced anaerobically. In contrast, other *Pseudomonas* strains were reported to be Se sensitive (Ike *et al.* 2000). This entails that different strains are adapted to environments with different evolutionary pressures (Rajkumar *et al.* 2012).

In contrast, within the selenate range investigated, 0·1 to 150 mmol l^{-1}, growth of *P. moraviensis* stanleyae was not inhibited. As selenate did not negatively impact the growth of the strain at any of the concentrations tested, the maximum SeO_4^{2-} that it can withstand could not be determined and it can be assumed to be higher than 150 mmol l^{-1}. Growth in the presence of SeO_4^{2-} was not accompanied by the production of red Se(0). Thus, the resistance of *P. moraviensis* stanleyae to selenate is not based on selenate reduction, but may be due to the extrusion of this oxyanion by membrane efflux systems (Bruins *et al.* 2000). Only a limited number of bacterial species have been shown to reduce SeO_4^{2-} to red Se(0) under aerobic conditions (reviewed in Kuroda *et al.* 2011).

The high Se tolerance and selenite reduction capacity exhibited by *P. moraviensis* stanleyae may be correlated with the seleniferous soils from the Pine Ridge Natural Area (Colorado) from which it originates, and the extremely high Se levels in its host plant (El Mehdawi and Pilon-Smits 2012). Selenium hyperaccumulator *S. pinnata* typically contains tissue Se levels upwards of 2000 mg kg^{-1} dry weight in all its organs (Galeas *et al.* 2007; Cappa *et al.* 2014). In *S. pinnata*, Se is stored predominantly in organic forms, which it reductively assimilates from selenate (Freeman *et al.* 2006). From an evolutionary standpoint, bacteria living in the rhizosphere or as endophytes of various metallophytes may be expected to develop tolerance to the toxic elements that are tolerated by their host plants. Apart from their intrinsic scientific merit, these plant-microbe symbioses constitute a promising source for bacteria with favourable properties for industrial applications. In our further studies with *P. moraviensis* stanleyae, we intend to explore such applications.

Production of red elemental selenium

The formation of red Se(0) was dependent on bacterial growth. The aerobic and anaerobic negative controls (no bacterial inoculum added) amended with selenite did not turn red, therefore the formation of red Se(0) was mediated by bacterial activity and not by an abiotic process (results not shown). Because Se(0) is water-insoluble and less bioavailable than Se oxyanions, it has reduced toxicity (White *et al.* 1997). This principle is used by bacteria to render Se oxyanions less harmful (Kessi *et al.* 1999) and may be similarly applied in industry. Extracellular Se particles of similar size (approx. 120 nm) were reported on the surface of *Veillonella atypica* cells grown in the presence of 5 mmol l^{-1} Na_2SeO_3 (Pearce *et al.* 2008).

The particles may be formed as a result of selenite reduction in or outside the cell (Oremland *et al.* 2004).

Bacteria that reduce Se oxyanions may be used in bioreactors to treat Se-laden wastewaters, such as the selenite-polluted waters produced by power plants (Hansen *et al.* 1998). In addition, the conversion of high levels of selenite to elemental Se(0) exhibited by the strain investigated in the current study opens the possibility to gain further insight into the production of biogenic Se nanoparticles. Further research using high-resolution Scanning Electron Microscopy is needed to assess the level of polydispersity of biogenic Se(0) nanoparticles. Having *photoconductive* properties (i.e. electrical resistance decreases with the increase in illumination) as well as *photovoltaic* properties (i.e. the direct conversion of light into electricity), elemental Se is routinely exploited in a variety of industrial applications including solar and photo cells, exposure meters, and xerography (Johnson *et al.* 1999).

Another interesting alternative would be the production of binary metal compounds like quantum dots (e.g. CdSe) (Pearce *et al.* 2008). Ayano *et al.* (2014) produced CdSe nanoparticles by incubating cadmium chloride and sodium selenite in the presence of a *Pseudomonas* isolate with high metal tolerance. However, the grand challenge of this approach is the need for a complete reduction of selenite (Se^{4+}) to selenide (Se^{2-}). If the reduction will stop at Se(0), the yield of CdSe will be decreased.

Inoculation experiment

The root endophyte *P. moraviensis* stanleyae enhanced the growth of Indian mustard, a crop relative of its natural host, *S. pinnata*. This indicates that likely *P. moraviensis* stanleyae is a mutualistic symbiont and that its host range is not limited to the Se hyperaccumulator *S. pinnata*. The finding that this bacterial species could boost plant productivity of Indian mustard by 70% is of significance as it likely can also enhance growth of other economically important Brassicaceae crop species such as canola, cabbage, broccoli, radish, turnip and others. The mechanism underlying the stimulation of plant growth by this bacterium awaits further study, but might involve the production of the growth hormone indole acetic acid. *Pseudomonas moraviensis* stanleyae enhanced plant accumulation of the plant nutrients Mn, S and K, and also affected Ca distribution in the plant. Selenium, the main element of interest, was not significantly affected, although it is noteworthy that the mean shoot Se concentration was 3-fold lower in the inoculated plants. A possible explanation is that the endophyte stimulated Se volatilization from the plant, resulting in lower plant Se levels. Many bacteria (and plants) can volatilize Se, and bacteria have been reported previously to affect plant Se volatilization (de Souza *et al.* 1999). For a fuller discussion on the microbial community structure of Se hyperaccumulators and non-hyperaccumulators the reader is referred to a related paper recently published (Sura-de Jong *et al.* 2015). In future studies, it will be interesting to further investigate the nature of these plant-microbe interactions and their ecological implications as well as their applications in agriculture and bioremediation.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Multi-locus sequence analysis parameters.

Table S2 Growth of *P. moraviensis* stanleyae based on Colony Forming Units (CFU) and absorbance at OD_{600} in the absence and presence of 10 mmol l^{-1} of Na_2SeO_3 and Na_2SeO_4.

Figure S1 Red Se(0) produced under anaerobic conditions by the reduction of (SeO_3^{2-}) = 10 mmol l^{-1}.