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Transformations of selenate and selenite by *Stenotrophomonas maltophilia* isolated from a seleniferous agricultural drainage pond sediment

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Summary

A Gram-negative bacterium, identified as *Stenotrophomonas maltophilia* by fatty acid analysis and 16S rRNA sequencing, was isolated from a seleniferous agricultural evaporation pond sediment collected in the Tulare Lake Drainage District, California. In cultures exposed to the atmosphere, the organism reduces selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) to red amorphous elemental selenium (Se°) only upon reaching stationary phase, when O_2 levels are less than 0.1 mg l^{-1}. In 48 h, *S. maltophilia* removed 81.2% and 99.8% of added SeO_4^{2-} and SeO_3^{2-} (initial concentration of 0.5 mM), respectively, from solution. Anaerobic growth experiments revealed that the organism was incapable of using SeO_4^{2-}, SeO_3^{2-}, SO_4^{2-} or NO_3^- as a terminal electron acceptor. Transmission electron microscopy of cultures spiked with either Se oxyanion were found to contain spherical extracellular deposits. Analysis of the deposits by energy-dispersive X-ray spectroscopy revealed that they consist of Se. Furthermore, *S. maltophilia* was active in producing volatile alkylselenides when in the presence of SeO_4^{2-} and SeO_3^{2-}. The volatile products were positively identified as dimethyl selenide (DMSe), dimethyl selenenyl sulphide (DMSeS) and dimethyl diselenide (DMDSe) by gas chromatography-mass spectrometry. Our findings suggest that this bacterium may contribute to the biogeochemical cycling of Se in seleniferous evaporation pond sediments and waters. This organism may also be potentially useful in a bioremediation scheme designed to treat seleniferous agricultural wastewater.

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Introduction

In the western side of California's San Joaquin Valley, agricultural irrigation waters leach naturally elevated levels of selenium (Se) from soil. The drainage waters are then disposed of in evaporation ponds, where Se accumulates to toxic levels in the sediment and water. Although Se is an essential micronutrient for humans and animals, at excessive concentrations in the environment, Se bioaccumulates in the food chain and is a serious threat to wildlife. Evaporation pond water containing as little as 15 μg Se l^{-1} has been associated with the bioaccumulation of Se in waterfowl eggs (Skorupa *et al*., 1990). The most notable case in California was observed in Kesterson Reservoir, where severe Se contamination was linked to embryonic deformities of aquatic birds, which ultimately led to the closure of the Reservoir in 1985 (Ohlendorf *et al*., 1986).

Over the last two decades there has been considerable interest in the microbiological transformation of Se oxyanions [i.e. selenate (SeO_4^{2-}) and selenite (SeO_3^{2-})] to volatile forms of Se such as dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe) and insoluble forms such as elemental Se (Se°) (Dungan and Frankenberger, 1999). The volatilization of Se is thought to be a protective mechanism used by microorganisms to avoid Se toxicity and it is considered to be an important process in the global cycling of Se (Haygarth, 1994). The reduction of Se oxyanions to Se°, which is less toxic and biologically unavailable because of its insoluble nature, has been reported to occur by numerous bacteria and in some cases is also thought to be a detoxification mechanism. Regardless, these biotransformation mechanisms are potentially useful for reducing *in situ* Se contamination in agricultural sediments and wastewaters. Unfortunately, little progress has been made in implementing technologies for the treatment of Se-contaminated agricultural drainage waters, largely because of the prohibitive cost associated with treating such large volumes of water and also due to the high salt content of most agricultural drainage waters.

In this study, we have isolated a strain of *Stenotrophomonas maltophilia* from Se-contaminated evaporation pond sediment in the Tulare Lake Drainage District, California. *Stenotrophomonas maltophilia* is a non-fermenting, aerobic Gram-negative rod which is currently being studied for its potential for bioremediation (Juhasz

et al., 2000; Rousseaux *et al*., 2001) and the biological control of plant pathogens (Zhang and Yuen, 2000; Yuen *et al*., 2001). This species has also emerged as a multidrug-resistant opportunistic pathogen responsible for nosocomial infections (Denton and Kerr, 1998) and is a threat to patients with cystic fibrosis (Denton, 1997; Hutchison *et al*., 2000). Overall, we were interested in studying the biotransformation of Se oxyanions by this organism. The organism is capable of reducing SeO_4^{2-} and SeO_3^{2-} to Se° and volatilizing Se. In addition, we were particularly interested in identifying the volatile Se compounds produced by this organism. Because of the bacterium's ability to rapidly transform SeO_4^{2-} and SeO_3^{2-}, *S. maltophilia* may be useful in a low-cost remediation scheme (e.g. bioreactor or *in situ* process) designed to treat seleniferous agricultural wastewater.

Results and discussion

Isolation and identification of the organism

Enrichment of evaporation pond sediment from the Tulare Lake Drainage District in California, which receives seleniferous agricultural drainage water at Se concentrations ranging from less than 1 μg l^{-1} to more than 1000 μg l^{-1} (Skorupa, 1998), yielded an organism that was capable of reducing SeO_4^{2-} and SeO_3^{2-} to Se° and producing volatile Se and S compounds when incubated under aerobic conditions. The bacterium is of rod-shape morphology, stains Gram-negative, and was positively identified as *Stenotrophomonas maltophilia* by FAA and 16S rRNA sequencing. Sequence identification (500 bp) using the BLAST database (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) also revealed that our isolate matched 100% with *Stenotrophomonas maltophilia* (accession no. AJ293468).

Electron microscopy

To identify the location of the Se deposits, whole mounts of cells grown in media containing Na_2SeO_4 or Na_2SeO_3 were viewed by transmission electron microscopy (TEM). An electron micrograph of *S. maltophilia*, grown in media containing no Se, revealed that the cells are about 2.8 μm in length and 0.6 μm in diameter and contained no deposits (Fig. 1A). Figure 1B and C show cells taken from a Na_2SeO_4 and Na_2SeO_3 culture during stationary phase respectively. The cells did not exhibit altered morphology when grown in the presence of either Se oxyanion, but deposits (≤270 nm in diameter) were observed near the periphery of the cell wall. Interestingly, cells grown in Na_2SeO_3-containing media possessed only one or two large deposits located near the ends of the cell (Fig. 1C), whereas cells grown in Na_2SeO_4-containing media developed multiple deposits at random locations on the cell surface (Fig. 1B). These deposits were also found free in the medium. Elemental analysis of deposits was performed using EDX spectroscopy (EDXS). Characteristic energy peaks for Se were found at 1.379, 11.207 and 12.492 keV (Fig. 2). This evidence, in combination with the red colour of the deposit, suggests that it is amorphous Se°, not crystalline Se°, which is grey in colour. In addition, the Se particles were also found to contain calcium and potassium; however, the significance of these elements is unknown. It should be noted that EDXS does not provide definitive proof that Se is the only element in the deposit, but it certainly does confirm the presence of this element.

Unfortunately, this information does not elucidate the mechanism the organism uses to reduce SeO_4^{2-} and SeO_3^{2-} to Se°. Several researchers have found intracellular Se deposits in Se-reducing bacteria (McCready *et al*., 1966; Silverberg *et al*., 1976; Tomei *et al*., 1992; 1995; Garbisu *et al*., 1996) and in a sulphur-reducing bacterium (Nelson *et al*., 1996); however, because we used whole cell mounts we were unable to assess whether *S. maltophilia* contained intracellular deposits of Se. Based on the observed location of the Se deposits, it may be possible that the Se oxyanions are reduced to Se° on the surface of the cell wall. Alternatively, the Se oxyanions may be reduced in the inner membrane space, followed by expulsion of Se° from the cell. Liberation of the Se° deposits by *S. maltophilia* did not appear to occur as a result of cell lysis. Losi and Frankenberger (1997) observed that *Enterobacter cloacae* SLD1a-1, a SeO_4^{2-} and SeO_3^{2-} reducing bacterium, contained Se deposits. The deposits were associated with the cell wall of the organism and found in the medium. It was speculated that the Se oxyanions were reduced via membrane-associated reductase(s) and then the Se precipitate was expelled into the medium.

Reduction of selenate and selenite

Figure 3 shows the removal of SeO_4^{2-} (Fig. 3A) and SeO_3^{2-} (Fig. 3B) [initial concentration of 0.5 mM (39.5 mg Se l^{-1})] from solution by *S. maltophilia* over a 48 h period. Full recovery of Se was achieved in the sterile controls throughout the experiment, indicating that the reduction reaction was biotically driven. Live cultures spiked with Se began to turn red upon reaching stationary phase (arrows in Fig. 3), indicating the presence of extracellular amorphous Se°, and became progressively darker red in colour over time. Overall, 81.2% of the SeO_4^{2-} and 99.8% of the SeO_3^{2-} was removed. These results indicate that *S. maltophilia* is capable of rapidly removing SeO_4^{2-} and SeO_3^{2-} from solution by reducing the oxyanions to Se°, but it appears that the reduction reaction is only taking place under microaerophilic conditions. Measure-

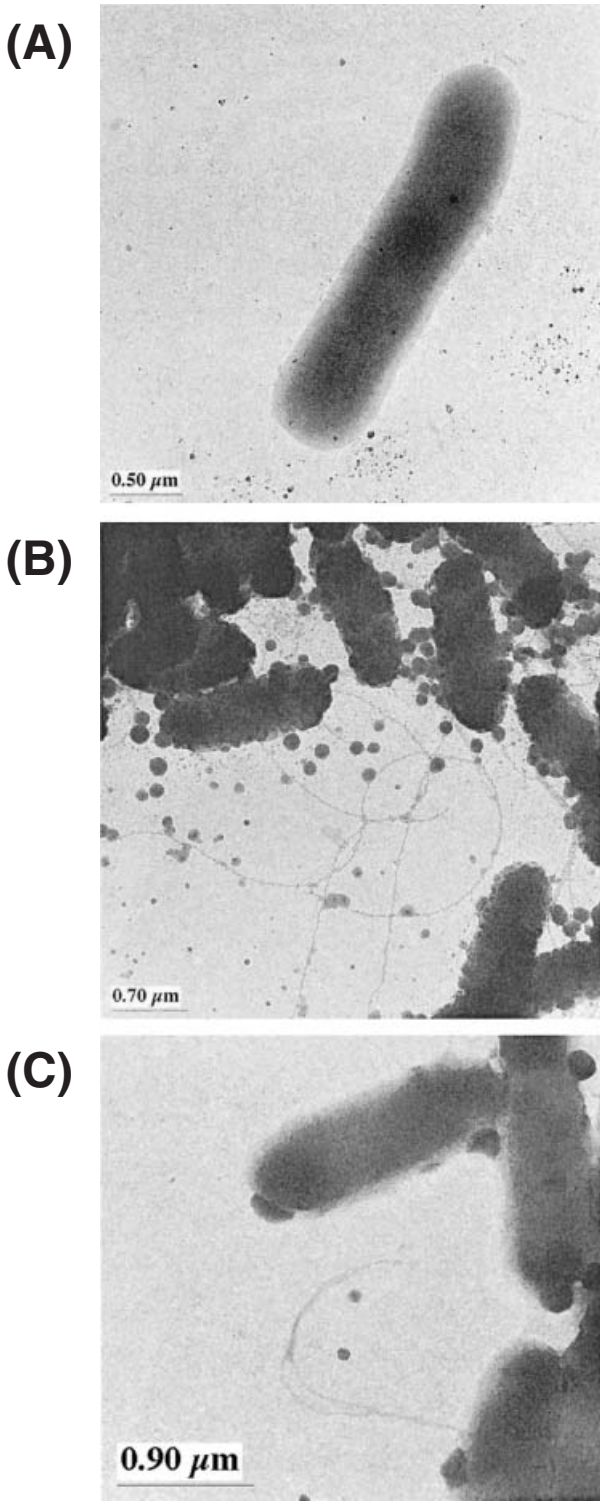

Fig. 1. Transmission electron micrographs of whole mounts of cells of *S. maltophilia* from a stationary phase culture grown in TSB medium containing (A) no Se; (B) 0.5 mM Na_2SeO_4; and (C) 0.5 mM Na_2SeO_3. B and C, deposits of Se are visible near the cell surface and free in the medium.

ments of dissolved O_2, in media containing either Se oxyanion, showed that O_2 was rapidly depleted by 15 h to concentrations as low as 0.05 mg l^{-1} (Fig. 4). Based on the data in Fig. 3, the rate of the reduction reaction was similar between the two Se oxyanions, but there was a delay in the reduction of SeO_4^{2-} compared with that of SeO_3^{2-}. This can be explained by the fact that SeO_4^{2-}, of higher redox potential, must first be reduced to SeO_3^{2-} before it can be reduced to Se°. As seen in Fig. 3A, SeO_3^{2-} is a transient intermediate during the reduction of SeO_4^{2-}. It should be noted that the reduction of SeO_4^{2-} and SeO_3^{2-} to Se° may not be the only Se removal mechanism as other reduced intermediates or end products may exist (organic and or inorganic forms), as the bacterium produces volatile alkylselenides.

Simultaneous optical density measurements at 600 nm showed that no lag period occurred during the growth of *S. maltophilia* when in the presence of either Na_2SeO_4 or Na_2SeO_3 (Fig. 4). Apparently, Se does not present a toxicity problem to *S. maltophilia* at these growth conditions and Se concentrations, which are about one to four orders of magnitude higher than in the environment from which the organism was isolated. The slight increase in the optical density observed during stationary phase in the culture containing SeO_3^{2-}, compared to the control, is due to the formation of Se°. Optical interference from Se° was not apparent in the growth curve of the SeO_4^{2-} containing culture as Se° formation is first visible at about 28 h.

Whereas the bacterial reduction of SeO_4^{2-} to SeO_3^{2-} is known to occur through dissimilatory pathways (Macy, 1994; Oremland *et al.*, 1994; Losi and Frankenberger, 1997), the reduction of SeO_3^{2-} to Se°, which is a common feature of many diverse microorganisms, is not very well understood. It has been reported that SeO_3^{2-} reduction may be catalysed by a periplasmic nitrite reductase (DeMoll-Decker and Macy, 1993), hydrogenase I (Yanke *et al.*, 1995) or through non-enzymatic reactions (Tomei *et al.*, 1992). Because *S. maltophilia* is incapable of using either SeO_4^{2-} or SeO_3^{2-} as a terminal electron acceptor during anaerobic growth (data not shown), the reduction of both Se oxyanions may be a mechanism of resistance. Lortie *et al.* (1992) reported that the aerobic reduction of SeO_4^{2-} and SeO_3^{2-} to Se° by a *Pseudomonas stutzeri* isolate functioned as a detoxification mechanism as there was no evidence of dissimilatory Se reduction. In *S. maltophilia* a detoxification mechanism may be responsible, but it appears unlikely as the formation of Se° occurs during stationary phase and not during exponential growth. Clearly, additional work is needed to elucidate the enzymology involved in the reduction processes.

Regardless of the reduction mechanism, the fact that *S. maltophilia* reduces SeO_4^{2-} independent of dissimilatory pathways is a potential advantage if the organism is to be used in a remediation scheme to treat

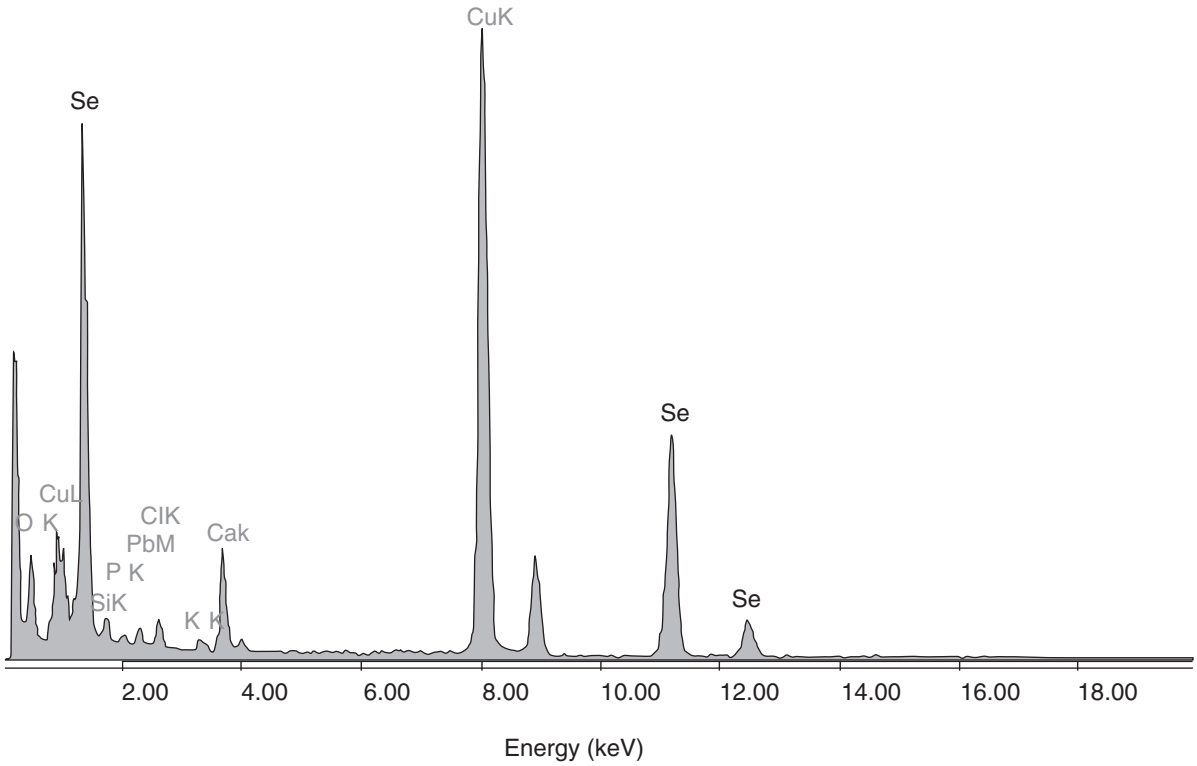

Fig. 2. A representative EDXS spectrum of deposits found in cultures containing 0.5 mM Na_2SeO_4 or Na_2SeO_3. The large Cu peak is from the copper electron microscopy grid used to support the cells. The energy range is from 0 to 20 keV. The dwelling time for the EDX analysis was about 1 min and the applied spot size ranged from 5 to 200 nm. The largest peak is 2459 counts.

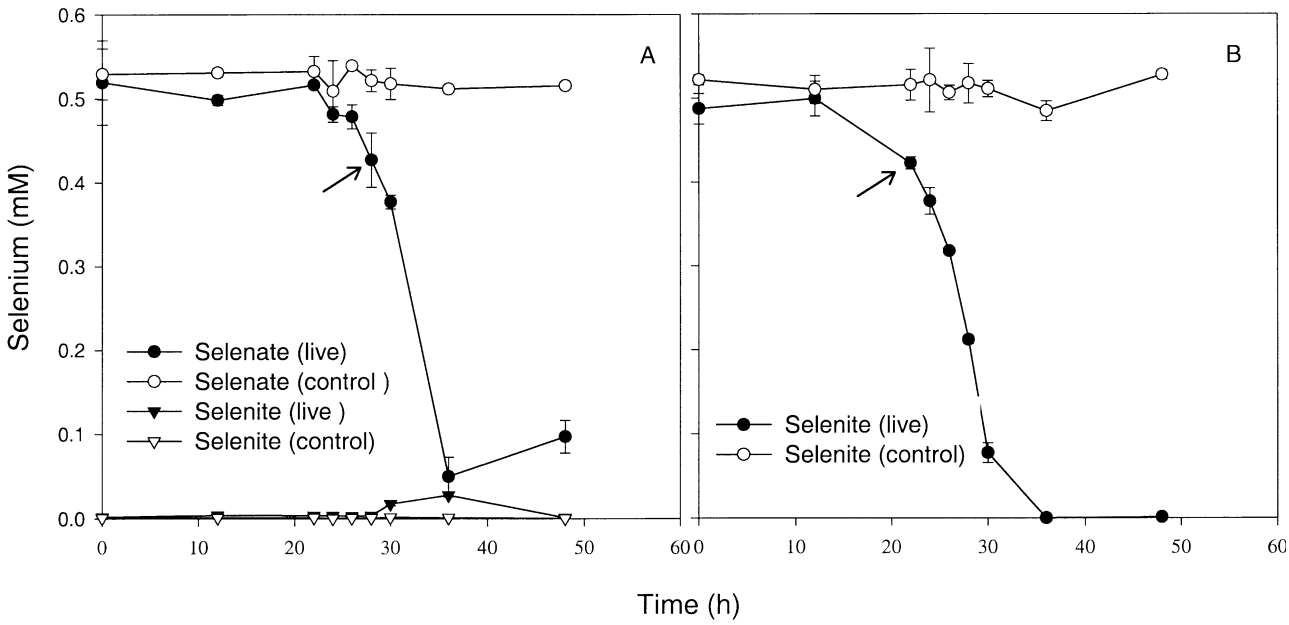

Fig. 3. Removal of (A) SeO_4^{2-} and (B) SeO_3^{2-} by *S. maltophilia* growing in cultures exposed to the atmosphere in TSB at room temperature with shaking (150 rev min^{-1}). Arrows indicate the approximate time the medium began to turn red. Error bars represent the standard deviation of three replicate samples.

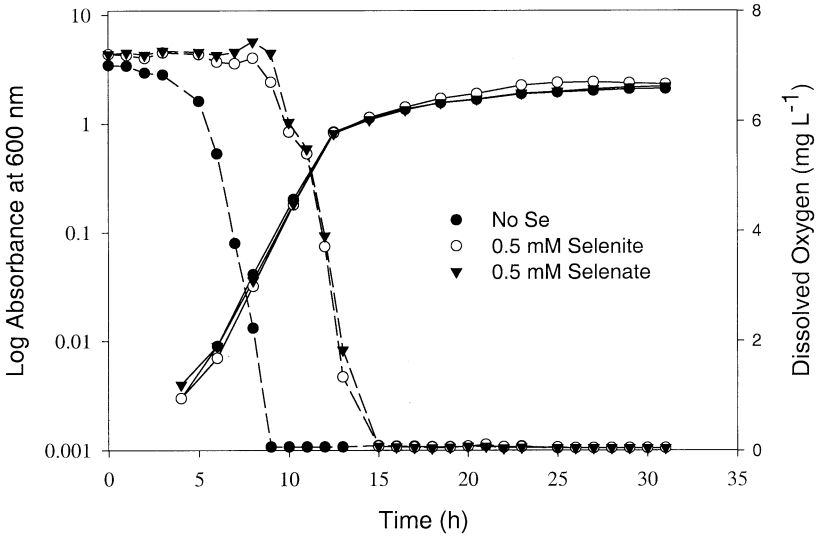

Fig. 4. Simultaneous measurement of the growth of *S. maltophilia*, expressed as absorbance at 600 nm (solid line), and removal of oxygen (dashed line). The bacterium was grown in TSB containing no Se or Na_2SeO_4 or Na_2SeO_3 at 0.5 mM. Cultures were exposed to the atmosphere at room temperature with shaking (150 rev min^{-1}).

Se-contaminated wastewater, which often contains high levels of NO_3^-. Although SeO_4^{2-}-respiring bacteria are ubiquitous in nature, functioning even in highly saline sediments and waters, if NO_3^- is present SeO_4^{2-} reduction does not occur (Steinberg *et al*., 1992). This is because many SeO_4^{2-}-respiring bacteria reduce SeO_4^{2-} via a NO_3^- reductase. Therefore, NO_3^- must first be removed from seleniferous wastewater before dissimilatory SeO_4^{2-} reduction can occur (Gerhardt *et al*., 1991). In addition, NO_3^- is a thermodynamically preferred terminal electron acceptor and Se will not reduced until most (or all) of the NO_3^- is removed. In the case of *S. maltophilia*, NO_3^- should not interfere with the reduction of either Se oxyanion since the organism cannot respire NO_3^- (data not shown), but additional studies are scheduled to determine if the organism displays a negative physiological response to high NO_3^- concentrations. Of additional importance, *S. maltophilia* can reduce SeO_4^{2-} and SeO_3^{2-} under microaerophilic conditions; in contrast, the majority of work on Se-reducing bacteria has focused on organisms that require strict anaerobicity (Gerhardt *et al*., 1991; Tomei *et al*., 1992; Macy, 1994; Oremland *et al*., 1994; 1995; Rege *et al*., 1999). Because bioreactor influent will contain O_2, the ability of *S. maltophilia* to grow in the presence of O_2 and rapidly reduce Se oxyanions in the presence of low levels of O_2 could be extremely advantageous.

Identification of volatile selenium

To determine the forms of volatile Se produced by *S. maltophilia*, gas samples were collected from above cultures grown on TSA plates containing Na_2SeO_4 or Na_2SeO_3, and analysed by GC-μECD and GC-MS. Colonies growing on the plates turned red, demonstrating the ability of the organism to reduce these Se oxyanions to amorphous Se°. The appearance of Se° was found to coincide with the presence of volatile Se compounds. As shown in Fig. 5, three alkylselenides were detected: DMSe, DMSeS and DMDSe. The GC retention time for these compounds was 3.16, 7.49 and 10.06 min respectively. All three Se gases were emitted from TSA plates containing either Na_2SeO_4 or Na_2SeO_3. Analysis of the headspace by GC-MS further verified the existence of these alkylselenides (Table 1). In addition, DMS was detected above plates containing Na_2SeO_3, whereas DMDS was detected above plates containing Na_2SeO_4 or Na_2SeO_3. Each of the volatile Se and S products were identified by peak retention time, molecular ions and mass fragmentation patterns as compared to authentic standards, except in the case of DMSeS, which was synthesized as described in the *Experimental procedures*. Among all of the volatile compounds identified, DMSe and DMDSe were the most abundant at concentrations ranging from 10 to 50 μg l^{-1} (Table 1). The production of all three alkylselenides (i.e. DMSe, DMSeS and DMDSe) from Se oxyanions has been reported to occur by bacteria (Chasteen *et al*., 1990; Rael and Frankenberger, 1996)

Table 1. Volatile selenium and sulphur compounds detected by GC-MS above cultures of *S. maltophilia* growing on TSA plates.

	Compound				
Se substrate (0.5 mM)	Selenium DMSe	DMDSe	DMSeS[a]	Sulphur DMS	DMDS
Na_2SeO_4	++	++	+	+	+
Na_2SeO_3	++	++	+	–	+

a. Detected but no standard is available for quantification.
DMSe, Dimethylselenide; DMDSe, Dimethyldiselenide; DMSeS, Dimethylselenylsulphide; DMS, Dimethylsulphide; DMDS, Dimethylsulphide. (–) not detected; (+) <10 p.p.b.; (++) 10–50 p.p.b.

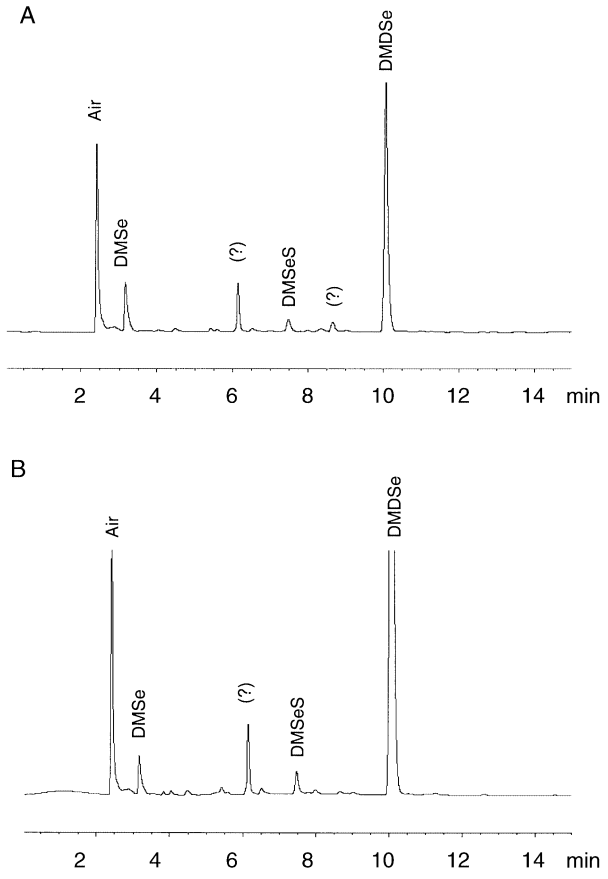

Fig. 5. Chromatograms of headspace samples taken from above *S. maltophilia* grown in the presence of 0.5 mM (A) Na_2SeO_4 and (B) Na_2SeO_3. A 0.5 ml headspace sample was analysed by GC-μECD as described in the *Experimental procedures*. Peaks with questions marks above were unidentifiable.

and algae (Fan *et al*., 1997). A number of fungi have also been reported to volatilize Se, but mainly as DMSe only. The formation of volatile Se first requires assimilation of the extracellular Se oxyanions, followed by their reduction to organo-Se compound(s) and methylation. However, the biochemistry of these reduction and methylation reactions has not been elucidated, and the Se precursor molecules have not been identified to date. Selenium biomethylation and volatilization are of particular interest because this process permanently removes Se from contaminated sediment and water (Frankenberger and Karlson, 1994).

In conclusion, *S. maltophilia* is capable of rapidly reducing SeO_4^{2-} and SeO_3^{2-}, the predominant Se species found in agricultural drainage waters, to amorphous Se°. Reduction of both Se oxyanions, which does not occur through dissimilatory pathways, may be a detoxification mechanism. Additional research is needed to elucidate the reduction mechanism. The ability of the organism to reduce both Se oxyanions to Se° represents a potentially useful mechanism for the remediation of Se in seleniferous waters. Se°, which is insoluble, is largely unavailable for biological uptake and is amenable to removal using filtration techniques. In addition, the bacterium is able to convert these Se oxyanions to volatile forms of Se including DMSe, DMSeS and DMDSe. This evidence suggests that *S. maltophilia* may be an active participant in the biological transformation and natural removal of Se that occurs in the Tulare Lake Drainage District evaporation ponds. Future work with *S. maltophilia* will be geared towards determining the optimal environmental conditions under which the organism reduces SeO_4^{2-} and $SeO3^{2-}$ and volatilizes Se. Important factors for consideration should be aeration status, utilization of various electron donor sources, salinity level, Se concentration, NO_3^- interference, temperature, and pH.

Experimental procedures

Chemicals

Tryptic soy broth (TSB) and tryptic soy agar (TSA) were obtained from Difco (Detroit, MI). Sodium selenate (Na_2SeO_4), sodium selenite (Na_2SeO_3), dimethyl diselenide (DMDSe), dimethyl disulphide (DMDS) and dimethyl sulphide (DMS) were obtained from Aldrich Chemical Company (Milwaukee, WI). Dimethyl selenide (DMSe) was obtained from Strem Chemical Company (Newburyport, MA). Dimethyl selenenyl sulphide (DMSeS) was synthesized according to the method of Chasteen *et al*., 1990).

Isolation and identification

Sediment (about 1 g) from evaporation pond 10 in the Tulare Lake Drainage District, Tulare, CA, was placed into a 250 ml Erlenmeyer flask containing 200 ml of minimal salts medium containing the following: Na_2SeO_4, 1.9 g l^{-1}; K_2HPO_4, 0.225 g l^{-1}; KH_2PO_4, 0.225 g l^{-1}; NaCl, 0.460 g l^{-1}; $(NH_4)_2SO_4$, 0.225 g l^{-1}; $MgSO_4$–$7H_2O$, 0.117 g l^{-1}; $NaHCO_3$, 0.05 g l^{-1}; $FeCl_3$, 0.005 g l^{-1}; yeast extract, 0.05 g l^{-1}; casamino acids, 0.1 g l^{-1}; acetate, 1.0 g l^{-1}; and trace element solution (Focht, 1994), 1 ml l^{-1}. The pH of the medium was adjusted to 7.2 with NaOH. The enrichment culture was incubated at room temperature on an orbital shaker (150 rev min^{-1}). After one week, the enrichment began to turn red, which is an indication that the SeO_4^{2-} was being reduced to amorphous Se°. After two weeks of incubation, the enrichment was streaked onto TSA plates containing 0.5 mM Na_2SeO_4 or Na_2SeO_3. The plates were then incubated at 25°C and the next day abundant red colonies began to appear. In addition, the plates began to emit a strong garlic-like odour characteristic of DMDSe. Red colonies were then isolated and streaked onto fresh TSA plates.

Once a pure culture was obtained, the bacterium was identified by fatty acid analysis (FAA) and 16S rRNA sequencing. Fatty acid extraction and analysis were conducted using the methods of the Sherlock Microbial Identification System (1999). The fatty acid profile of the isolate was determined by gas chromatography (HP 6890; Hewlett-

Packard, Wilmington, DE) with flame-ionization detection, and statistical pattern-recognition software was used to compare the profile to those in the database. The determination of the 16S rRNA gene sequence for species identification was performed by MIDI Laboratories (Newark, DE). The 16S rRNA gene from the bacterium was PCR amplified from genomic DNA using primers that correspond to *E. coli* positions 005 and 1540 and 005 and 531. Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators, and the samples were electrophoresed on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). Sequence analysis for bacterial identification was performed using MicroSeq microbial analysis software and database (Perkin-Elmer/Applied Biosystems).

Transmission electron microscopy and energy-dispersive X-ray spectrometry

Whole mounts of cells were observed with a Philips Electron Optics, B.V. (Eindhoven, the Netherlands), model CM300 transmission electron microscope (TEM) equipped with an EDAX International (Mahwah, NJ) energy-dispersive X-ray (EDX) spectrometer. The cells were collected, by centrifugation (5000 *g* for 2 min), from a stationary phase culture grown in TSB containing 0.5 mM Na_2SeO_4 or Na_2SeO_3. The cell pellet was washed three times in sterile tap water and 4 μl of the suspension was viewed on copper TEM grids (400 mesh with an ultrathin carbon film) after drying. The dwelling time for the EDX analysis was about 1 min and the applied spot size ranged from 5 to 200 nm.

Reduction of selenium oxyanions

To determine the ability of *S. maltophilia* to reduce SeO_4^{2-} and SeO_3^{2-} during its growth, a growth experiment was conducted in TSB (pH 7.3). To initiate the experiment, 100 ml aliquots of TSB were added into 250 ml Erlenmeyer flasks, aseptically spiked with Na_2SeO_4 or Na_2SeO_3 to a final concentration of 0.5 mM, and capped with foam plugs. The inoculum (100 μl) consisted of a 20 h culture grown in TSB and stored in 50% glycerol at −74°C. The negative controls contained no inoculum and were treated the same as the live growth cultures. The flasks were incubated aerobically at room temperature and shaken on an orbital shaker (150 rev min^{-1}). One millilitre samples were collected at 0, 12, 22, 24, 26, 28, 30, 36 and 48 h. The reduction reaction in the samples was stopped by centrifugation (16 000 *g* for 2 min) and subsequent freezing at −74°C. A simultaneous experiment was conducted under the same conditions to assess the uptake of O_2 during growth. Optical density measurements were taken at a λ of 600 nm (OD_{600}).

The Se concentrations were determined by hydride-generation atomic absorption spectrophotometry (HGAAS; Varian SpectraAA-10, Mulgrave, Victoria, Australia). The operational conditions were as follows: acetylene, 2.4 ml min^{-1}; air, 6.3 ml min^{-1}; nitrogen, 90 ml min^{-1}; sample flow, 6.5 ml min^{-1}; acid flow, 10 M HCl, 1.2 ml min^{-1}; reagent flow, 0.35% $NaBH_4$-0.2% NaOH, 1.2 ml min^{-1}; lamp current, 10 mA; wavelength, 196.0 nm; and slit width, 1.0 nm. As the instrument directly detects Se^{4+} (i.e. SeO_3^{2-}), Se^{6+} (i.e. SeO_4^{2-}) was measured by reducing Se^{6+} to Se^{4+}. The reduction reaction was accomplished by preparing the sample in 6 M HCl (adding 200 μl of 5% ammonium persulphate per 12 ml sample) and then heating the sample at 90°C for 20 min. To analyse for Se^{4+}, the samples were prepared in 6 M HCl and directly subject to HGAAS. Recovery of Se from the TSB medium was ≥97%.

Anaerobic growth experiment

To determine the ability of *S. maltophilia* to use SeO_4^{2-}, SeO_3^{2-}, SO_4^{2-} or NO_3^- as a terminal electron acceptor during respiratory growth, agar plates were made with the minimal salts medium as described in the isolation and identification section. The concentration of each of the oxyanions was 10 mM. Plates were prepared in duplicate, streaked with fresh inoculum and incubated anaerobically (in a GasPack container, Becton Dickinson, Franklin Lakes, NJ) at 25°C for two weeks. To confirm the ability of *S. maltophilia* to respire O_2 using the minimal salts medium, a separate set of plates containing no SeO_4^{2-}, SeO_3^{2-}, SO_4^{2-} or NO_3^- was incubated aerobically.

Gas chromatography

To identify volatile Se gases produced by the *S. maltophilia*, the organism was streaked onto TSA plates containing 0.5 mM Na_2SeO_4 or Na_2SeO_3 and allowed to grow overnight at 25°C. The plates were then sealed with Parafilm (American National Can, Chicago, IL) and a small hole in the cover of the Petri dish served as a gas sampling port. A 0.5 ml sample was withdrawn using a gas-tight syringe and manually injected into a Hewlett-Packard 6890 gas chromatograph (GC) equipped with a microelectron capture detector (μECD). The operating conditions were as follows: column, RTX-624 capillary column (30 m, 0.25 mm × 1.4 μm, Restek Corp., Bellefonte, PA, USA); carrier gas, He, 1.0 ml min^{-1}; inlet temperature, 230°C; detector temperature, 280°C; column temperature, isothermal at 110°C for 15 min. Identification of compounds was achieved by peak retention time by injecting the headspace from above chemical standards.

To further verify identification of the volatile Se compounds, a 0.5 ml gas sample was also analysed by GC-mass spectrometry (MS). The GC (Hewlett-Packard model 5890), equipped with an RTX-624 capillary column, was connected to a Hewlett-Packard model 5971 Mass Selective Detector. The operating conditions were as follows: carrier gas, He, 0.7 ml min^{-1}; injector temperature, 230°C; column temperature, 40°C rising to 130°C at 7°C min^{-1} after 1 min. For quantitative analysis, selective ion monitoring was utilized. The following ions were monitored: *m*/*z* 61 and 62 for DMS; *m*/*z* 79, 94, and 96 for DMDS; *m*/*z* 95 and 110 for DMSe; *m*/*z* 127, 140, and 142 for DMSeS; and *m*/*z* 175, 188, and 190 for DMDSe. Calibration standards for DMSe, DMDSe, DMS, and DMDS were prepared by pipetting 2–5 μl of authentic liquid standard into a 164 ml headspace vial. After the liquid had completely volatilized, secondary standards were prepared by serial dilution into 124 ml headspace vials. The correlation coefficients of the standard curves were ≥0.98.

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