APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2001; **15**: 499–510
DOI: 10.1002/aoc.186

Production of dimethyl telluride and elemental tellurium by bacteria amended with tellurite or tellurate

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The purpose of this study was to determine whether a facultative anaerobe, *Pseudomonas fluorescens* K27, would produce dimethyl telluride when anaerobic cultures were amended with differing concentrations of sodium tellurate and/or sodium tellurite and how that volatile organotellurium production varied over time. Batch bacterial bioreactor experiments were undertaken in order to observe the changes in the headspace of a growth medium solution inoculated with *P. fluorescens* and amended with tellurium salts. Gas samples were taken from the bioreactor every hour and were analyzed by capillary gas chromatography using fluorine-induced chemiluminescence detection to determine compounds in the headspace. Liquid samples were analyzed by spectrophotometer to determine optical densities, which were used as an indicator of cell growth. Verification of the identity of the dimethyl telluride produced in the bacterial headspace above a tellurate-amended culture was achieved by comparison with the chromatographic retention time of an authentic $(CH_3)_2Te$ standard and by gas chromatography/mass spectrometry. The time course production of dimethyl telluride varied with amendment salts' tellurium oxidation states and concentrations. Increasing tellurate concentrations caused slower bacterial growth, but those cultures reached the stationary phase sooner than cultures amended with tellurite concentrations 10 or 100 times less. Black elemental tellurium (Te^0) was produced by live cultures amended with tellurium salts but not by sterile controls. The amount of tellurium in the solid phase (as Te^0 and in/or on cells) harvested from replicate, anaerobic cultures of *P. fluorescens* sampled after 92 h of incubation was approximately 34%. Mixed tellurite/tellurate amendment experiments exhibited a synergistic toxic effect and yielded less final biomass and very little dimethyl telluride production compared with cultures amended with either tellurate or tellurite alone.

Keywords: tellurium; dimethyl telluride; reduction; bacteria; elemental; toxicity; bioreactor; hydride generation

Received 25 September 2000; accepted 13 December 2000

INTRODUCTION

The first notice of the methylation of inorganic compounds of tellurium occurred early in the 19th century. The odor exhaled by animals who had been fed inorganic tellurium compounds was first reported by Gmelin[1] in 1824, as discussed by Challenger.[2,3] Later, Hensen detected a garlic odor in the breath of dogs or men a few minutes after administration of potassium tellurite.[4] During his studies on the derivatives of tellurium at the University of Leeds, Challenger saw that this odor could easily be detected around those involved in working with inorganic tellurium compounds, although they had never come into contact with organic compounds of tellurium. Bird and Challenger analyzed the gases evolved from test-tube cultures of *S. brevicaulis* on bread containing potassium tellurite and concluded that it was

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Contract/grant sponsor: Cottrell College Science Award of Research Corporation.
Contract/grant sponsor: Texas Research Institute for Environmental Studies.
Contract/grant sponsor: Sam Houston State University.
Contract/grant sponsor: Robert A. Welch Foundation.

dimethyl telluride (DMTe).[5] In the 1950s the cases of organomecury-based Minamata Disease in Japan[6] led to further investigations of organometal(loid)s. Environmental applications utilizing alkylmetals have contributed to the development of this subject as well.

In 1945, Challenger suggested a biomethylation mechanism for metalloids involving methionine.[2] He suggested that the biomethylation of metalloids involved an activated methionine intermediate, which has been shown to be S-adenosylmethionine.[7–9] Challenger's proposed mechanism for selenium biomethylation involved four steps, in which a selenium oxyanion is methylated and reduced to form volatile dimethyl selenide, and he suggested that production of DMTe occurred analogously.[2] One compound missing in this proposed mechanism was dimethyl diselenide, which was later observed by workers in the headspace above selenium-resistant bacteria.[10–14]

Reamer and Zoller[10] broadened Challenger's proposed mechanism for the biomethylation of selenium based on their identification of dimethyl diselenide in purged sewage samples amended with selenium salts. They proposed a pathway for the intermediate CH_3SeO_2 to form dimethyl diselenide by reduction. Later, Doran[15] suggested another mechanism, in which selenite is first reduced to elemental selenium and then further reduced to selenide, which is methylated to dimethyl selenide. Evidence for this mechanism comes from the commonly observed elemental selenium in bacterial cultures with added selenium salts.[16–21]

Finally, based on the observation that dimethyl selenenyl sulfide, CH_3SeSCH_3, was produced in some microbial cultures, it has been suggested that either chemical exchange or disproportionation could lead to the formation of CH_3SeSCH_3 when dimethyl diselenide, methanethiol and dimethyl disulfide are present in either cells or bacterial cultures.[12] Since these organosulfur compounds are also biogenically produced in many microbial cultures, the entire process is inherently biological whether or not organoselenium is produced inside cells or by the organosulfur exudates released into microbial culture.

It is thought that biological transformations for tellurium probably follow similar pathways to selenium, which include methylation and reduction by metalloid-resistant microbes.[2] Fungi can also produce dimethyl telluride from tellurium salts, as reported by Bird and Challenger[5] and Chasteen *et al.*[11] In monocultures of some phototrophic bacteria amended with tellurate, and, most surprisingly, some amended with elemental tellurium (i.e. the powdered metal), DMTe was detected in bacterial cultures after 7 days growth.[18] In earlier work, *Candida humicola*, grown into the stationary phase on a complex growth medium, produced detectable headspace amounts of DMTe,[11] though no time course experiments were carried out. In other work by Fleming and Alexander,[22] a strain of *Penicillium* amended with tellurium oxyanions produced DMTe. Although it can be supposed that the mechanism for methylating tellurium follows the same metabolic pathway as those for selenium and arsenic, DMTe was produced and detected only in the presence of selenium in that report; therefore, transmethylation could not be ruled out. The yield of DMTe was proportional to the input of inorganic selenium, so that no methylated tellurium compound was found until the input quotient of selenium to tellurium was about 10:1.[22]

Very little is known of the biogeochemical cycle of tellurium. Alkylated forms of this element apparently form less readily under biogenic conditions than those of selenium. Although available data suggest that organotellurium compounds play a marginal role in the element's natural cycle, recently, volatile compounds, including DMTe, have been reported in gases emitted by municipal waste deposits and landfill gas,[23,24] in geological settings[25,26] and either volatile or particulate tellurium-containing species have been detected in atmospheric air, though no speciation was performed.[27] The natural abundance of tellurium in the Earth's crust is small (2 ppb). Therefore, tellurium movement as reduced volatile forms is probably small. That said, recent expanded interest in the use of tellurium in synthetic and industrial applications[28–30] may increase its spread in the environment.

The biomethylation experiments reported here took place in a 3 l bioreactor using a facultative anaerobe grown in the presence of nitrate as the terminal electron acceptor. Biomethylation of tellurium salts by *Pseudomonas fluorescens* K27, a bacterial strain that was isolated from Kesterson Reservoir in the San Joaquin valley of central California,[31] was observed and bacterial growth behavior monitored. Batch cultures of K27 were amended with different concentrations of tellurium salts and were grown in the bioreactor until they reached stationary phase. Mixed tellurate and tellurite amendments to bacterial cultures were also performed for the first time that we can find reported in the literature.

MATERIALS AND METHODS

Reagents

The reagents used throughout this research were analytical-grade chemicals and were used without further purification. Tryptic soy broth (TSB) was obtained from DIFCO Laboratories (Detroit, MI, USA). Sodium tellurate (Na_2TeO_4), and sodium tellurite (Na_2TeO_3), hydrochloric and nitric acids, sodium borohydride, ammonium persulfate, and dimethyl disulfide (DMDS, CH_3SSCH_3) were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Potassium nitrate (certified A.C.S. grade) was ordered from Fisher Scientific (Houston, TX, USA). DMTe (CH_3TeCH_3) was procured from Organometallics, Inc. (East Hampstead, NH, USA) and used as received. Tellurium atomic absorption calibration standards were diluted from 1000 ppm stock solutions (Aldrich) with 50% v/v HCl in the final calibration solutions, which matched the acid matrix of the samples.

Bacterial growth media

TSN3 medium (TSB with 0.3% nitrate) was prepared by dissolving 10.0 g of TSB and 3.0 g potassium nitrate per 1.0 l of deionized water. The freshly prepared growth media were sterilized by autoclave (15 min at 121 °C).

Tellurium bacterial amendment solutions

Tellurate

A 20 mM stock solution was prepared by dissolving 1.904 g sodium tellurate in 200 ml deionized water. This solution was sterile filtered with a disposable filter unit (0.2 μm pore size; Nalgene Company Rochester, NY, USA) using a vacuum–pressure pump.

Tellurite

A 10 mM stock solution was prepared by dissolving 4.431 g sodium tellurite in 200 ml deionized water. This solution was also sterile filtered. Higher concentrations of this aqueous reagent precipitated within a few hours.

Bacterial cultures

All the experiments were carried out using *P. fluorescens* K27 grown in TSN3. This is a metalloid-resistant bacterium harvested from Keterson Reservoir in the San Joaquin Valley of California, USA, and isolated by R. Fall at University of Colorado, Boulder.[31]

Bioreactor

Culture growth

The batch bacterial experiments were carried out in a New Brunswick BioFlow III Batch/Continuous Fermentor (Edison, NJ, USA). The fermentor was disassembled, cleaned, reassembled and filled with 2.5 l TSN3 media before every experiment. It was then sterilized in a 716 l autoclave (Wisconsin Aluminum Foundry Co., Inc.; Manitowoc, WI, USA). The fermentor was connected to a personal computer to record and/or control temperature, agitation, pH, and dissolved oxygen. The temperature was maintained at 30 °C for the entire experiment. Gas samples were taken hourly through a septum-lined gas sampling port using 1 ml gas-tight syringes with push button valves (Alltech; Deerfield, IL, USA) and were analyzed using gas chromatography with fluorine-induced chemiluminescence detection. Liquid culture samples were taken hourly and optical density readings (as a measure of cell growth) at 526 nm[32–35] were measured using a spectrometer.

Precultures of *P. fluorescens* K27 were grown aerobically at 30 °C before the experiments. A K27 colony from an agar plate was used as inoculum into 50 ml sterile TSN3, grown aerobically (with shaking) for at least 24 h, and then aseptically added to 200 ml more of sterile TSN3, which was then left to grow aerobically for another day, time enough for the bacteria to reach stationary phase. This inoculation solution was then introduced into the fermentor through one of its openings in the top plate (a 10 vol.% inoculum for a final reactor liquid volume of 2.75 l). After the fermentor was amended with the appropriate amount of the sterile tellurium salts described above, it was purged with nitrogen to remove the dissolved oxygen in the system to force these facultative anaerobic bacteria to grow anaerobically. The fermentor, thermostatically maintained at 30 °C, was stirred continuously at 200 rpm. The reactor headspace gases were allowed to expand through 3.2 mm tubing that bubbled through a 6% solution of sodium hypochlorite to oxidize and trap any volatiles and prevent volatile organotellurium species from escaping into the laboratory. A sterile filter (0.2 μm) was connected inline to prevent bacterial contamination of the culture. Liquid samples were collected from the batch every hour by very slightly pressurizing the

vessel with sterile nitrogen via an attached gas line and then collecting the displaced liquid via the liquid sampling port of the reactor, which samples the liquid from the bottom third of the reactor volume. Optical density and pH data from the liquid medium were then recorded. Bacterial controls were treated identically but not amended with tellurium-containing salts.

Cells and elemental tellurium harvesting and sample preparation

In some tellurite-amended experiments, four replicate 25 ml samples were taken from the bioreactor *via* the liquid collection method described above after cultures had reached far into the stationary phase (~92 h) and tellurium content was determined. The total volume of the bioreactor solution was recorded at this time. These well-mixed suspensions were centrifuged in 25 ml polycarbonate tubes at 5 °C (12857 *g*; 30 min) and the cells, along with black tellurium metal, were separated from the clear supernatant by decanting. 18 ml of supernatant was mixed with 2 ml of concentrated nitric acid and 10 ml of deionized (DI) water and evaporated to dryness in a beaker; this oxidizes all tellurium present to tellurate.[36] 18 ml of DI water was again added, the solution stirred well, and 5 ml of that solution mixed with 5 ml of concentrated HCl and 0.2 ml of 2% ammonium persulfate.[37,38] These latter components reduced tellurate to tellurite in preparation for analysis by hydride generation. This mixture was heated in a closed test tube in a water bath for 30 min and analyzed by hydride generation atomic absorption spectroscopy (HGAAS; see below)

The solids at the bottom of the centrifuge tubes were digested by first dissolving in 1 ml concentrated HNO_3 (decolorization and dissolution was immediate). A comparison between this simple dissolution step and boiling to dryness resulted in no significant differences. An appropriate dilution of this solution (for example 1/100) was carried out using DI water and then 5 ml of that diluted solution, moved to a clean test tube, was chemically reduced as above, then analyzed by HGAAS.

Standard deviations among the four samples taken from each run are reported, as are the averages over all four bioreactor runs. Percentage recovery data were based on an assumption of a known mass of tellurium contained in 25 ml of bioreactor solution taking into account the initial amount of tellurium added and the reactor's volume when the 25 ml samples were collected (see above). The calculated percentage recovery also assumes that the added tellurium was well mixed throughout the bioreactor's contents upon amendment and then redistributed between solution, solids, and suspended cells, which were again well-mixed when sampling occurred.

Headspace analysis

1 ml gas samples were taken from the fermentor every hour using a gas-tight syringe via a septum-lined gas sampling port and analyzed by capillary gas chromatography. Used syringes were cleaned by passing air through them in an enclosed vessel maintained at approximately 50 °C. After cleaning, the syringes were checked for carryover by injecting 1 ml of laboratory air into the gas chromatograph using the shorter syringe check temperature program (see below). The cleaning process was repeated until no peaks were seen in the syringe check chromatograms. Using this method, organo-sulfur and organo-tellurium could be determined simultaneously in each headspace sample analyzed.

Instrumentation

Fluorine-induced chemiluminescence

The instrumentation used for the gas chromatographic separation and chemiluminescence detection have been described elsewhere.[18] Briefly, a capillary gas chromatograph was interfaced to a gas-phase fluorine-induced chemiluminescence detector. A very thick film chromatographic column (30 m, 0.32 mm i.d., 5 μm 5% phenyl and 95% methyl polysiloxane film) allowed us to chromatograph relatively low boiling point compounds (b.p. >50 °C) without having to use a cryogen and sub-ambient oven temperature programs. During some calibration experiments, a split ratio of 1:50 was used to prevent overloading of the column. The peak areas obtained from these split injections were therefore multiplied by 50 in order to normalize these peak areas to those of splitless injections.

The following temperature programs were used to analyze samples or to check syringes. Analysis: 30 °C initial 1 min, 20 °C min^{-1} ramp to 180 °C, 30 °C min^{-1} to the final temperature of 225 °C for 3 min. For syringe checks: 30 °C initial 1 min, 25 °C min^{-1} to 250 °C final for 2 min.

Gas chromatography/mass spectrometry (GC–MS)

The headspace above a 0.1 mM tellurate-amended culture of K27 was analyzed by GC–MS (Varian

Saturn 3; Varian Inc, Palo Alto, CA, USA) to confirm the identity of the peak in our chromatograms that appears at 7.3 min. The capillary column used in this analysis was a 30 m, 0.25 mm i.d., 0.25 μm film, DB-5MS capillary column (J&W Scientific; Folsom, CA, USA). A carrier gas of UHP helium with a head pressure of 12.5 psi and with splitless injection was used. The MS scan rate was 1 scan s^{-1}. Since no cryogen and sub-ambient temperature program were available for this instrument, the separation of low boiling point compounds was relatively poor compared with our thick-film capillary column used in the chemiluminescence method.

HGAAS

Digested, tellurium-containing samples containing 5 M HCl acid (see above) were analyzed by HGAAS.[38] A Varian FS220 AAS with a hydride generation module was operated according to the manufacturer's procedures using the 214.3 nm line, 0.2 nm slit with air–acetylene flame to heat the optical cell. The hydride generation reagents were 50% concentrated HCl and 0.35% $NaBH_4$ (stabilized with 0.5% NaOH). Sample, acid and borohydride reagent flows were examined routinely and found to be invariant at 1.2 ml min^{-1}. No background correction was used. Standard curves were determined between every fifth sample to minimize the effects of instrument drift. Blanks contained either water or sterile medium and were carried through the oxidation and then reduction steps before HGAAS analysis.

RESULTS

As with our previous work with this microbial strain, methylated organo-sulfur compounds were routinely detected in the headspace of the K27 microbe growing on this complex growth medium at 30 °C (i.e. controls). These compounds are biologically produced from sulfur-containing components in the growth medium and released into the headspace where gas concentrations vary depending on the bacterial growth phase.[13,18,39] Figure 1 shows chromatograms from four different time slices of a 0.01 mM tellurite-amended culture early in logarithmic phase through stationary phase (2, 10, 18, and 25 h after inoculation and tellurium-salt amendment). Because of the kind of chromatographic column used in this work, relatively low boiling point compounds were not resolved chromatographically. Therefore, in Figure 1a, 2 h after tellurium amendment, only DMDS (b.p. 109 °C) is well resolved and identified (R.T. = 8.7 min) based on an authentic standard and our previous experience with this microbe.[13,39,40] No DMTe was detectable in the headspace at this point. DMTe detection limit (at $S/N = 3$) is 5 ppbv (parts per billion by volume) in a 1 ml headspace sample using this method. Earlier headspace experiments with this same microbe more clearly detailed bacterial headspace production of methanethiol, dimethyl sulfide, and DMDS.[11,13,39] In these experiments, DMDS production was usually larger

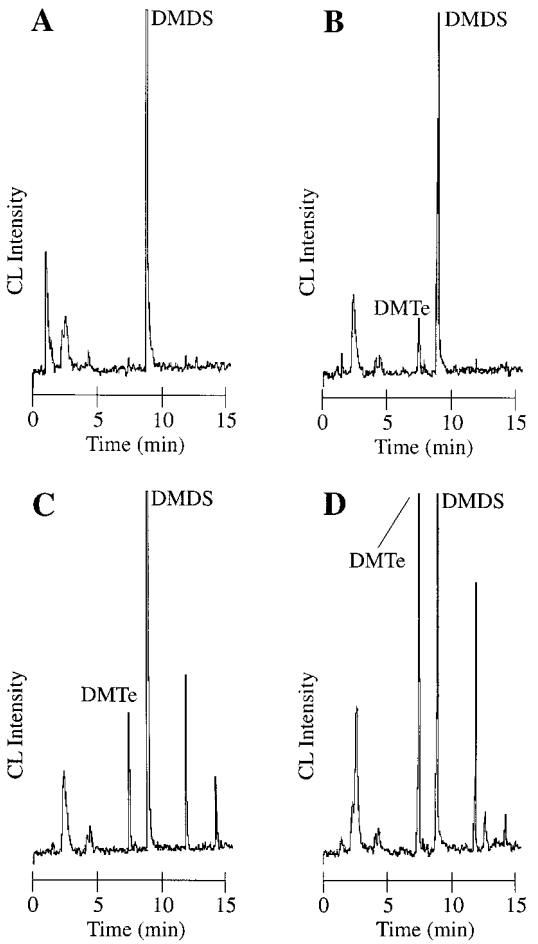

Figure 1 Chemiluminescence chromatograms from multiple samples of 1 ml of bioreactor headspace from a 0.01 mM tellurite-amended bacterial experiment. A, B, C, and D were chromatograms from samples that were taken 2 h, 10 h, 18 h, and 25 h respectively after inoculation and tellurium amendment.

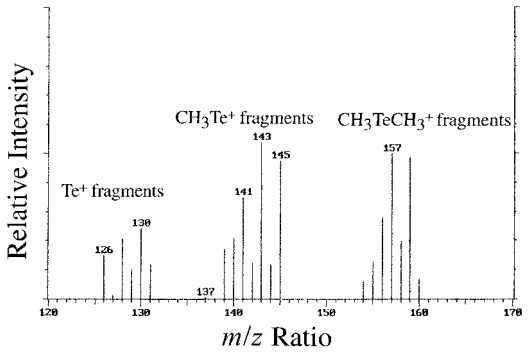

Figure 2 The difference mass spectrum (peak minus nearby background) for an unresolved DMTe peak showing only the mass range from 120 to 170 Da. The identities of the isotope groupings for DMTe are indicated.

Table 1 Overview of tellurium-amendment experiments with biological cultures of *P. fluorescens* K27

Amendments	Te amendment conc. (mM)	Max. DMTe headspace conc. observed (ppbv)
Blanks	0	0
TeO_4^{2-}	0.01	~6
	0.1	21
	1.0	19
TeO_3^{2-}	0.01	40
	0.1	15
$TeO_4^{2-} + TeO_3^{2-}$	0.1 + 0.1	10
	1.0 + 1.0	Below detection limit

than DMTe by at least a factor of 20, so, at the time slice in the culture time course seen in Fig. 1a (2 h after inoculation, still in lag phase), only DMDS is present in any significant amounts (~200 ppbv). The earlier somewhat unresolved chromatographic peaks seen in Fig. 1 are dimethyl sulfide and methanethiol—based on correlations with our previous cryogenically trapped chromatography, which better resolved the earlier eluting peaks.[18] DMTe (b.p. 94 °C) was not produced in large amounts until later in the log phase of the bacteria.

At 10 h after inoculation/amendment, DMTe was detectable in the bioreactor headspace as a small chromatographic peak at approximately 7.4 min. This retention time corresponded to that of a commercial DMTe standard. Figures 1b–d tracks the increase in DMTe production from 10 to 25 h in the bacterial culture's headspace.

In order to confirm the identity of the compound eluting at 7.4 min in our chemiluminescence chromatography initially made by comparison to the authentic commercial compound, GC–MS analyses using a different chromatographic column were carried out. Again, a 1 ml headspace sample from a tellurium-amended culture was analyzed and the difference mass spectrum (DMTe peak region minus nearby background) is shown in Fig. 2 for the mass region from 120 to 170 Da. The isotope pattern for tellurium can be seen in fragments for Te^+, CH_3Te^+, and $CH_3TeCH_3^+$.

A peak corresponding to dimethyl ditelluride ($CH_3TeTeCH_3$) was not seen in this work in the fluorine-induced chemiluminescence chromatography nor in the GC–MS data (the later eluting peak at ~12.5 min is dimethyl trisulfide). Experiments with tellurium-amended fungal cultures has shown a time-dependent production of DMTe and dimethyl ditelluride,[11] and K27 does produce the analogous compound dimethyl diselenide when amended with selenium oxyanions.[41] This then points to the probability that the more oxidized organometalloids (dimethyl ditelluride or dimethyl diselenide) detected in the bacterial headspace of this microbe are produced by bacterial metabolism instead of being the result of the headspace oxidation of the monometalloids (DMTe or dimethyl selenide), and in this case DMTe is metabolically produced by K27 and apparently dimethyl ditelluride is not.

Table 1 details the amendment experiments carried out, their concentrations, and the maximum amount of DMTe detected in the culture headspace. Figure 3 shows the time course of DMTe, DMDS, and cell population of a K27 culture amended with 0.01 mM sodium tellurite. Both of the compounds tracked in Fig. 3 show a change in concentration as the cell population increases, with DMDS decreasing and then rebounding as the culture reached stationary phase. Experiments involving tellurite amendments above 0.1 mM TeO_3^{2-} concentrations were not carried out (see tellurate experiments below) because tellurite was so poorly soluble in this aqueous growth medium that solutions made with concentrations above 0.1 mM TeO_3^{2-} precipitated immediately. Tellurite insolubility was mirrored in the aqueous stock solutions, which precipitated at concentrations above 10 mM.

Tellurate-amended bacterial cultures (0.1 mM) each showed roughly similar patterns of growth (Fig. 4, three runs) with the same overall (apparent)

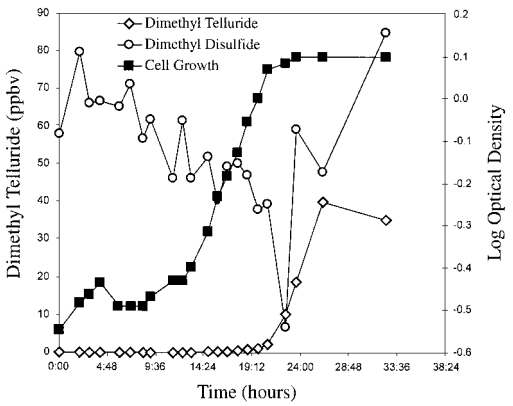

Figure 3 DMTe and DMDS headspace concentrations and cell growth versus time for a 0.01 mM TeO_3^{-2}-amended culture of *P. fluorescens* K27.

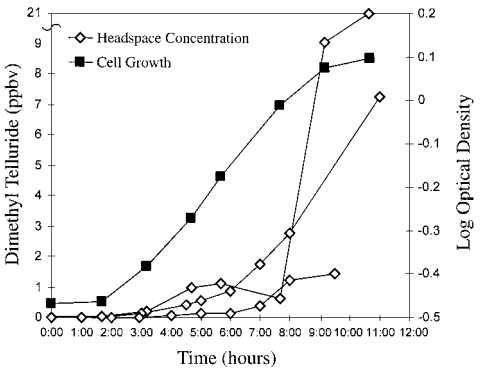

Figure 4 DMTe headspace concentration and cell growth versus time for triplicate runs of 0.1 mM tellurate-amended K27 bacterial cultures.

final biomass as determined by optical density. However, though this higher tellurium amendment concentration (compared with 0.01 mM tellurite) yielded about the same stationary phase concentration of headspace DMTe, that headspace production occurred much earlier in the time course, in the middle of the log phase. Furthermore, the specific bacterial growth rate (SGR), as estimated from the slope in the log phase of growth,[42,43] was higher in the cultures amended with tellurate (0.1 mM tellurate SGR = 0.21 h^{-1}; 1.0 mM tellurate SGR = 0.20 h^{-1}) than that of the tellurite experiments (0.01 mM tellurite SGR = 0.14 h^{-1}). Tellurate experiments with 0.01 mM TeO_4^{2-} showed very little DMTe headspace production and no significant change in growth rate compared with controls (SGR = 0.30 h^{-1}).

Proceeding to even higher added tellurium concentrations, 1.0 mM tellurate amendments yielded even less DMDS and (generally less) DMTe headspace production, and the culture log phase was less distinct (in fact, true logarithmic growth did not appear to occur at 1.0 mM tellurate), curving gently into stationary phase (Fig. 5) after 11 or 12 h; also note the differences between the distinctness of the log phases in Figures 3 and 4. Finally, a change in the amount of time necessary to reach stationary phase was also apparent between tellurite- and tellurate-amended cultures. Even the 1.0 mM tellurate experiments reached stationary phase in approximately 10 h; in the 0.01 mM tellurite-amended cultures (Fig. 3) the stationary phase was only reached after as long as 19 or 20 h.

Noting that different concentrations and different oxidation states of tellurium oxyanions had different effects on the growth and DMTe headspace production, we carried out mixed tellurium amendment experiments by adding both tellurium oxyanions, as the sodium salts, to a freshly inoculated culture of K27. A low and high mixed experiment regime was carried out: 0.1 mM TeO_3^{2-} plus 1.0 mM TeO_4^{2-} (0.2 mM total amended Te) or 1.0 mM TeO_3^{2-} plus 1.0 mM TeO_4^{2-} concentrations (2.0 mM Te total and precipitation probably occurred). In those experiments, when tellurite and tellurate were added together, as before, simultaneously with bioreactor inoculation, very little DMTe headspace production was found in any headspace analysis over the ensuing time course ($\lesssim$ 10 ppbv DMTe) and the culture yielded approximately half the final biomass of the

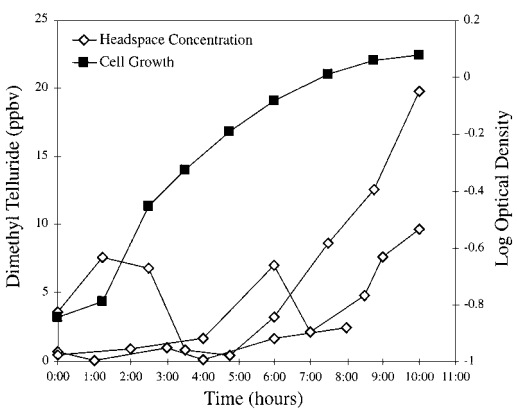

Figure 5 DMTe headspace concentration and cell growth versus time for triplicate runs of 1.0 mM tellurate-amended K27 bacterial cultures.

Table 2 Percentage distribution of tellurium among supernatant and collected solids in four duplicate bioreactor runs. Anaerobic cultures of *P. fluorescens* K27 were amended with 0.1 mM sodium tellurite, maintained at 30 °C, and then spun-down cells and solids and liquid medium were analyzed for tellurium by HGAAS as described in the text. Four samples harvested at the same time from each run were analyzed

Run	Te in solid phase (%)	Te in solution phase (%)	Standard deviation ($n = 4$ samples)	Recovery (%)
1	42	58	6.5	107
2	18	82	1.1	84
3	33	67	18.1	111
4	43	57	5.4	87
Average (%) ($n = 4$ runs)	34	66	7.8	97

individually tellurium-amended experiments (either TeO_3^{2-} or TeO_4^{2-}). This highlights the synergistic toxic effects of tellurate and tellurite on this bacterium; interestingly, this was a microbe that was isolated from a drainage system where both the oxyanions of this metalloid and many other metalloids and metals were present.[44]

Tellurite-amended cultures showed more apparent elemental tellurium production than those treated with tellurate, so four replicate bioreactor runs (all 0.1 mM tellurite amendments) were incubated continuously for 92 h and then aliquots of those bioreactor solutions were removed, spun down and analyzed for tellurium as before: four 25 ml samples were taken from each run within a few seconds of each other. The results of the total distribution of tellurium between the solution and (centrifuge-collected) solid are shown in Table 2.

DISCUSSION AND CONCLUSIONS

We propose that, as for the other chalcogens (i.e. sulfur and polonium), selenium and tellurium can be transformed from inorganic compounds to organometalloids through microbial activities (through biomethylation of polonium has not been observed yet). The resultant methylated compounds are more lipophilic and more volatile, thus changing their pattern of transport and possibly their toxicological behavior. This, of course, can be contrasted with the increased toxicity of methylated mercury compounds compared with their ionic forms.[45,46]

Owing to their present limited anthropogenic usage, environmental contamination by tellurium compounds is not a serious problem. The environmental occurrence of organo-selenium and -tellurium compounds is still not well established owing to the lack of suitable methods or the existence of analytical methods that are too laborious, and probably because of these compounds low concentrations in the environment. Although biomethylation of selenium and tellurium has been investigated, biomethylation of tellurium is not as well documented as that of selenium. In fact, very few data presently exist on the biomethylation of tellurium.

The results reported here demonstrate that a metalloid-resistant microbe, *P. fluorescens* K27, isolated from a relatively high salt and metal environment,[31] can grow in the presence of both environmentally common tellurium oxyanions, tellurate and tellurite, at concentrations up to 1 mM when grown anaerobically in this liquid medium. Previous work has shown volatile trimethylantimony [$(CH_3)_3Sb$] production from this same microbe when amended with trimethyl dibromoantimony,[40] and, when examined in analogous selenium experiments,[33] K27 has been shown to grow in a minimal medium at concentrations up to 200 mM selenate, but with very long lag phases (>4 weeks). Decreased biomass production and much longer lag phases (compared with controls) were seen with the tellurite amendments, compared with cultures with added tellurate. A report of the relative toxicity of selenate, selenite, and a proposed[2] biological intermediate, dimethyl selenone, to this bacterium has been published,[33] but those studies used a different growth medium, so no direct toxicity comparisons can be drawn here. That said, longer lag phases *were* characteristic of higher amounts of added metalloids used in that work. Therefore, based on that criterion, tellurite appears to be more toxic than tellurate to this microbe: 0.01 mM tellurate shows no change in lag phase length compared with controls, whereas 0.01 mM

tellurite showed lag phases that were as long as the entire lag + log phase in the 1.0 mM tellurate amendment experiments. Furthermore, the decrease in SGR or doubling times can also be used as a means of determining relative toxicity.[32,33] The differences between 0.01 mM tellurite experiments and both tellurate amendment ranges (0.1 and 1.0 mM) show a large difference in SGR, with tellurite again exhibiting a larger toxic effect. Reports of this order of relative toxicity for tellurite and tellurate have appeared elsewhere.[47–49]

Figures 4 and 5 show a distortion of the logarithmic phase of growth for higher tellurate amendments. Experiments with another selenium-resistant bacterium, *Pseudomonas aeruginosa*, have also reported organoselenium production when exposed to 1 mM selenate,[50] and work with a microbe of that same genus and species also demonstrated a similar distortion of the growth kinetics upon exposure to differing concentrations of a polycylic aromatic hydrocarbon, phenanthrene.[51] Recent work with bacterial cultures amended with copper has also shown these kinds of change in growth pattern.[52] Some tellurium-amended bacterial cultures produce metallic tellurium in the later phases of growth as a gray/black precipitate of elemental tellurium (Te^0).[53,54] Production of elemental metalloids by bacterial metabolization in liquid and soil samples of mono- and poly-cultures has been reported.[18,55–60] Since optical density was used in this work as a measure of cell density/population, we thought that the changes in log phase plots were, in part, influenced by the presence of elemental tellurium in the bacterial medium and/or cells. Others have reached that conclusion for elemental selenium production in bacterial cultures.[61] Our tellurium-amended cultures showed a dark green to grayish cast in the latter log phase, turning to black in the stationary growth phase, and workers have found that tellurite amendment of bacteria resulted in production of elemental tellurium and metallic tellurium deposition inside cells.[62,63] The color change seen here was more prominent in tellurite-amended cultures than those with added tellurate. Therefore, experiments were undertaken to determine how tellurium, initially added as a soluble salt, was redistributed among solution and solid phases in a culture taken far into stationary growth phase (92 h). This was accomplished by separation *via* centrifugation, acid digestion and oxidation, and then conversion of all tellurium present to tellurite via reduction with boiling HCl and analysis for tellurium by HGAAS.[36–38,64] These experiments, for four separate bioreactor runs, showed that approximately 66% of added tellurium was recovered in the liquid medium and 34% was detected in the filtered solid (cell biomass and tellurium metal). Though this last datum does not differentiate between tellurium oxidation states in or on cells and in the elemental tellurium form, we believe this is still a significant measure of the cell's ability to bioprocess soluble—and therefore more toxic—tellurium into an immobilized form.

The percentage recoveries, based on an assumed amount of tellurium in each bioreactor aliquot sampled (see above) as reported in Table 2, unsurprisingly, average approximately 100%; however, the standard deviation around that mean was large ($97 \pm 13\%$, $n = 4$ bioreactor runs). Samples of known tellurium content (either tellurate or tellurite), for comparison, were taken through all oxidation and reduction steps identically to the biological samples and these showed recovery rates of 109% with a smaller standard deviation ($109 \pm 2.9\%$, $n = 6$). Sterile blanks containing all culture medium components, also treated identically, showed an insignificant tellurium content of approximately 1 ppb. Therefore, we feel that the method of tellurium analysis we used is sound and that the larger variance among the biological samples is due to other reasons. Interferences and variances in HGAAS procedures for tellurium determination are often attributed to unstable reagents, poorly optimized reduction steps, varying sample acidity, interfering metalloids or metals, and the effect of nitric acid on the reduction and hydride generation step.[38,65–67] All of these parameters were carefully addressed in this work and a small linear working range (0 to 20 ppb) was also purposely chosen. Hydride generation reagents were made up fresh daily, calibrations were run between every five samples, and all samples and standards contained the same final HCl content. The metals in our cultures were limited to those trace elements of TSB medium, reagents and tellurium salts and when our samples were diluted into the linear range these were far below the ranges that have caused problems for others.[38] The nitric acid content in the final samples was also orders of magnitudes below that reported to cause problems. Therefore, our only explanation for poor tellurium recoveries for the biological samples is that our assumption that the bioreactor contents were well mixed is incorrect and inhomogeneous samples affected our harvesting procedures. We made an effort to increase bioreactor mixing prior to sampling the contents by doubling the mixing

speed from 200 to 400 rpm; however, foaming prevented us from using this speed throughout the entire run. This problem may indeed be the result of sampling a complex mixture of suspended solids.

The synergistic toxic effect on this organism of tellurate plus tellurite amended cultures was expected in view of other metalloid amendment work we have carried out with this bacterium.[19,68] In those experiments, 1:1, 1:2 and 2:1 selenate/selenite mixed amendments carried out with this same growth medium and organism showed a decrease in SGR (compared with unamended controls) that was greater than that for the same concentration of either selenium oxyanion alone (data not shown). This, then, is a synergistic effect that increases the depression of growth more than either oxyanion alone.

The differences in volatile headspace production for microbial exposure to different oxidation states of added metalloids has been reported before,[18] and in this work the timing of DMTe headspace production differed depending on the oxyanion to which the bacterium was exposed, at least at the tellurium concentrations we were able to evaluate. Tellurite-amended cultures showed their greatest production increase as the stationary phase was reached and growth became limiting, whereas cultures containing tellurate showed relatively significant DMTe production throughout the log phase. This may be because the enzyme expression that powers the detoxification/methylation process[69] is triggered earlier in tellurate-amended cultures and later in those with added tellurite.

The increase of organometalloidal concentration as seen in Figs 3–5 is not simply a function of continuous build-up of volatiles as the culture biomass increases. Instead, it is a function of increased bacterial production in the culture and, we believe, dynamic exchange between the bioreactor headspace and the liquid medium. Headspace concentrations change dynamically over time in these bacterial cultures as gas-phase and solution-phase components exchange, as the bacterial biomass increases, and as components in the liquid solution are consumed. As the mid-time-course drop in organosulfur in Figure 3 shows, sometimes the headspace component concentrations drop. This drop in DMDS at approximately 22 h after inoculation is a real reflection of dynamic changes in DMDS headspace amounts—not a variable injection—because the DMTe headspace content recorded on the same injection/chromatogram does not show a decrease; instead, it continues a smooth increase, begun about 4 h before, and continues on into the stationary phase. Recent work assaying reduced thiol (RSH) content in Gram-negative bacteria exposed to tellurite showed a decrease in RSH, whereas the same analysis for tellurium-resistant cells exposed to TeO_3^{2-} did not show a significant RSH decrease;[70] therefore, it is possible that the reducing ability of cells with tellurium resistance depends on additional reducing power from some other source than intracellular thiols. This might explain the temporal decrease in reduced sulfur compounds that is sometimes seen in cultures amended with tellurium oxyanions, since DMDS is clearly produced by cellular activity (see Fig. 3). Experiments under way in our laboratory now have also shown that, in bioreactor runs with continuous nitrogen purging (50 mL min^{-1}), the headspace component concentrations also vary in a manner dependent upon the growth phase (unpublished data).

One very recent result with analogous selenium-amended microbial cultures has revealed the apparent presence of selenomethionine in cells examined using X-ray absorption spectroscopy, and while elemental selenium was produced in those same cultures, the production of selenium appears to occur after small metabolic selenium requirements are met.[60]

In conclusion, *P. fluorescens* K27, when grown anaerobically in 2.75 l volume batch cultures at 30 °C and amended with either tellurite or tellurate, produced DMTe that could be detected in the gases of the bacterial headspace. The time course production of DMTe varied with tellurium amendment oxidation state and concentration. Increasing tellurate concentrations from 0.1 to 1.0 mM caused slower bacterial growth, but those cultures reached the stationary phase sooner than cultures amended with 0.01 mM tellurite. Mixed tellurite/tellurate amendment experiments exhibited a synergistic toxic effect and yielded less final biomass (as measured by optical density) and very little DMTe production compared with cultures amended with either tellurate or tellurite alone. Metallic (elemental) tellurium was, initially, detected visually in the late log phase of bacterial growth mostly in tellurite-amended cultures, and, for the conditions studied, the amount of tellurium found as elemental tellurium or as tellurium in and/or on harvested cells from cultures grown far into the stationary phase (92 h) was approximately 34%.

Acknowledgements This research was supported by a Cottrell College Science Award of Research Corporation, the Texas Research Institute for Environmental Studies, Sam Houston

State University Research Enhancement Funds, and the Robert A. Welch Foundation. Thanks also go to Dr Hakan Gürleyük for review of the manuscript and useful comments. Anonymous reviewers' comments were also appreciated.

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