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Effects of growth conditions on production of methyl selenides in cultures of *Rhodobacter sphaeroides*

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***Rhodobacter sphaeroides* 2.4.1 exposed to selenate or selenite produced volatile selenium compounds. Total amounts of dimethyl selenide, dimethyl diselenide, dimethyl sulfide and dimethyl disulfide in culture medium and headspace were determined. The highest selenate volatilization occurred in the late stationary phase of growth. However, cultures deprived of light in the stationary phase of growth produced much less of the volatile organo-selenium compounds. Lower culture pHs increased the rate of selenium volatilization. Low sulfate concentration limited biomass production and selenium volatilization; high sulfate concentrations had an enhancing effect on the release of organo-selenium compounds. Cultures of *R. sphaeroides* reacted very differently to amendments with increasing amounts of selenate and selenite. Only small amounts of selenite were volatilized; meanwhile high amounts of methylated selenides were found in selenate-poisoned cultures.**

Keywords: *Rhodobacter sphaeroides*; selenate; selenite; reduction; methylation

Introduction

The volatilization of selenium plays an important role in the global cycling of this element [9,21,31] Selenium is an essential trace element for higher organisms and some microorganisms, yet it is toxic for most organisms at concentrations only slightly above levels necessary for growth [25]. Certain bacteria and fungi are resistant to high concentrations of selenite and selenate and are capable of reducing these compounds to mostly elemental selenium and thereby detoxifying their environment. Some microorganisms reduce and methylate selenium oxyanions to volatile compounds like dimethyl selenide and dimethyl diselenide.

Resistant strains have mostly been isolated from soil, sewage or selenium-contaminated sites [2,7,10,11,13, 22,32]. Recently, it was shown that phototrophic nonsulfur bacteria are resistant to and can reduce metalloid oxyanions [19]. The high-level resistance of some members of the *Rhodospirillaceae* is regarded as a mechanism to get rid of excess electrons generated by the primary photochemical process [20]. These findings gave rise to further investigations of these organisms as bioremediating agents of metalloid-polluted sites. McCarty *et al* [18] found dimethyl selenide and dimethyl diselenide in the headspace above selenate-amended cultures of *Rhodospirillum rubrum* and *Rhodocyclus tenuis*. Thus these bacteria reduce and methylate selenium oxyanions and convert selenite and selenate into less toxic [14] volatile organoselenides.

To effectively utilize biological processes for bioremediation, it is necessary to define the effects of growth conditions on the process [12,13,16,22,32]. The well-characterized species *Rhodobacter sphaeroides* 2.4.1 was chosen in this study.

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Materials and methods

Chemicals

Na_2SeO_3, Na_2SeO_4, dimethyl selenide (CH_3-Se-CH_3), and dimethyl diselenide (CH_3-Se-Se-CH_3) were obtained from Strem Chemicals (Newburyport, MA, USA). Dimethyl sulfide (CH_3-S-CH_3), dimethyl disulfide (CH_3-S-S-CH_3) and HPLC grade acetonitrile were purchased from Aldrich Chemical Co (Milwaukee, WI, USA). All the media components used were of analytical grade.

Organism and growth conditions

Rhodobacter sphaeroides 2.4.1 (strain No. 158, Deutsche Sammlung von Mikroorganismen, Göttingen, Germany) was grown at room temperature on Sistrom minimal medium [28] with 20 mM succinate as sole carbon source at pH 6.8 except where mentioned otherwise. In the low sulfate medium, ammonium sulfate and magnesium sulfate were replaced by the corresponding chlorides (final sulfate concentration: 13 μM).

The pH was adjusted prior to autoclaving. After inoculation of liquid medium, the cultures were left in the dark overnight to deprive them of oxygen before incubation in incandescent light (100 W tungsten light bulb; 10 W m^{-2}) at room temperature. With the exception of time courses, all experiments were carried out in 16-ml Hungate test tubes sealed with Teflon-coated septa which do not adsorb volatile compounds. Since Teflon septa are not gas-tight once they have been pierced, time course experiments with single cultures were run in 100-ml Schott flasks capped with an enclosure device especially developed for repeated headspace sampling of compounds that may be adsorbed by rubber septa [29]. These flasks were slightly immersed in a 28°C water bath, gently shaken and illuminated with incandescent light of 10 W m^{-2}. For dark experiments the flasks were wrapped in two layers of aluminum foil at the end of the exponential phase of growth. Daily sampling of the headspace and the bacterial culture allowed following

growth as turbidity and the production of volatile selenium compounds over time. The optical density at 660 nm was used as a measure for biomass.

Amending with selenate or selenite

Prior to the addition of selenium, cultures of *R. sphaeroides* were put in the dark overnight and then incubated in the light for 2–3 days. Sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3) were added from sterile-filtered stock solutions with final volumes of 10 ml in Hungate tubes or 60 ml in Schott flasks. Since the cultures had to be opened for the addition of selenate and selenite, after amendment they were left in the dark overnight to re-establish anaerobic growth conditions before they could be exposed to light again. Controls consisted of sterile medium on the one hand and autoclaved bacterial cultures on the other hand, contained 1 mM selenate or selenite, and were incubated for 7 days in the light. After headspace analysis, one ml of each amended culture was used to reinoculate 9 ml of fresh medium. These subcultures were incubated in the light for up to 2 weeks and qualitatively assessed for growth.

Variation of pH

The pH of Sistrom medium (10 mM phosphate buffered) containing 20 mM succinate was adjusted with HCl or KOH to 5, 5.5, 6, 6.5, 7, 7.5, and 8 prior to autoclaving (after autoclaving, pH values were shifted less than 0.1 for pH $\leq$ 7.0, and $\leq$ 0.3 for pH $\geq$ 7.5). These pH-adjusted media were inoculated with *R. sphaeroides* and distributed into six Hungate tubes in 10-ml portions. After 2 days, three tubes at each pH were amended with 1 mM selenate; three tubes were used as controls. After 7 further days of incubation, headspace analysis for volatile, methylated selenium and sulfur compounds was carried out as described below.

Variation of carbon source

Six C-4 compounds (all at 20 mM) of different oxidation states were used as carbon sources for growth of *R. sphaeroides*: butanol, butyrate, succinate, malate, fumarate, and tartrate. After 2 days of photo-heterotrophic growth the cultures were amended with 1 mM sodium selenate using a dark sequence as described above. Then the headspace was analyzed daily for volatile selenium and sulfur compounds while the cultures were growing in the light. The electrons available from each carbon source were calculated from the number of oxygen molecules necessary to oxidize a carbon source to CO_2 and H_2O, with each O_2 molecule accepting four electrons.

Analysis of headspace samples

For analysis of volatile sulfur and selenium compounds, 1-ml gas samples were withdrawn from the headspace of the cultures by piercing the septa with a gas tight syringe (Dynatech, Baton Rouge, LA, USA). The gas samples were immediately analyzed by capillary gas chromatography coupled with fluorine-induced chemiluminescence detection [33].

Organo-selenium and -sulfur compounds were identified using the retention times of laboratory standards. All purchased standards were used as received. The identity of dimethyl selenenyl sulfide (CH_3-Se-S-CH_3) [4,5,33] and methaneselenol (CH_3-SeH) [6] were checked by GC/MS.

Determination of the concentration of headspace components

The gas phase concentrations of the volatile headspace components examined were determined by calibration with solution phase standard injections chromatographed as described above. The detection limit for these compounds was $\leq$ 15 parts per billion by volume (ppbv). Since dimethyl selenenyl sulfide is not commercially available, an average of the determined calibrations of dimethyl disulfide and dimethyl diselenide was used to estimate amounts of dimethyl selenenyl sulfide.

Solution phase concentrations of volatile components

To calculate the amount of each volatile compound that remained dissolved in the medium, the following dimensionless Henry's law constants, determined at 25°C as described by Robbins *et al* [24] were used: dimethyl sulfide 0.1441, dimethyl selenide 0.0879, dimethyl disulfide 0.0538, dimethyl diselenide 0.0879 (similar Henry's law constants for dimethyl sulfide, dimethyl selenide and dimethyl disulfide have been reported [8,15]). The stock solutions to determine the Henry's law constants were made in cell-free, sterile filtered medium from 7-day-old cultures of *R. sphaeroides*.

Results

Controls

No volatile sulfur or selenium compounds ($\leq$ 15 ppbv) were detected above sterile medium containing 1 mM selenate or selenite after 1 week of incubation. When cultures of *R. sphaeroides* were autoclaved and then amended with 1 mM selenate and incubated for 1 week, no volatile selenium compounds and only barely detectable amounts of dimethyl sulfide were found.

Selenium volatilization during growth of R. sphaeroides

Only small amounts of volatile selenium compounds were released throughout the exponential phase of growth of *R. sphaeroides* (Figure 1) and the highest amounts of dimethyl selenide and dimethyl diselenide were found after the culture grown in the light had been in stationary phase for several days. Small amounts of dimethyl selenenyl sulfide were also found (Figure 1c). Methaneselenol was not found in these studies. To test if this volatilization is a light-driven process two cultures were inoculated identically, amended with 1 mM selenate at the same time, grown until stationary phase of growth and then one of the two cultures was wrapped in aluminum foil. The dark culture produced significantly smaller amounts of volatile selenium compounds compared to the culture grown in the light throughout the stationary phase of growth (Figure 1b,c).

Effect of sulfate concentration on selenium volatilization

R. sphaeroides was grown on media at high sulfate (6.2 mM, as in the original Sistrom medium) or low sulfate

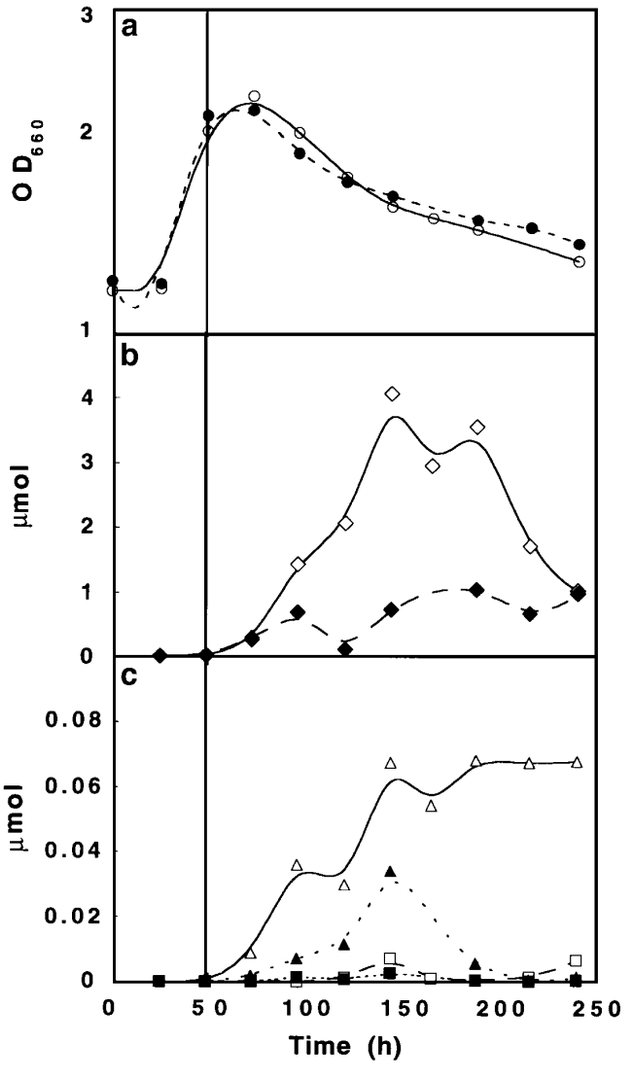

Figure 1 Growth and the release of volatile, methylated selenium compounds of *R. sphaeroides* poisoned at $t = 0$ h with 1 mM selenate at 28°C. One culture stayed in the light (open symbols) for the whole time whereas the other culture (solid symbols) was incubated in the light for 49 h (vertical line) and then kept in the dark for the rest of the experiment. (a) biomass (as turbidity); (b) dimethyl selenide; (c) dimethyl diselenide (triangles) and dimethyl selenenyl sulfide (squares). The unit μmol means the amount of a compound released into the headspace by a 1-L culture (independent of headspace volume).

concentrations ($13\ \mu M + \leq 310\ \mu M$ transferred with inoculum; Table 1). Almost twice the biomass was formed in the high sulfate Sistrom medium within 7 days as compared to the low sulfate medium. Interestingly, the higher

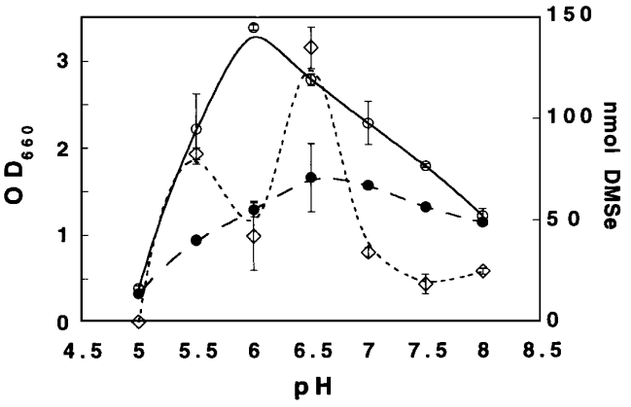

Figure 2 Effect of pH variation on biomass production (control ○; 1 mM selenate-amended ●) and evolution of dimethyl selenide (◇). Values given were obtained 7 days after selenate addition. The room temperature was 20 ± 2°C. The unit nmol means the amount of a compound released into the headspace by a 1-L culture (independent of headspace volume). The error bars represent the standard deviation of the mean of at least three samples.

sulfate concentration also enhanced the production of organo-selenium compounds.

Effect of pH on selenium volatilization

Figure 2 shows OD_{660} of Se-poisoned and unpoisoned cultures 7 days after 1 mM selenate addition. Growth of the control cultures was higher than in selenate-amended samples with the most striking effect apparent at low pH. The release of volatile selenium compounds was higher in the lower pH range. In two additional pH experiments (data not shown), results similar to the ones described above were obtained.

Effect of the oxidation state of the carbon source on selenium volatilization

R. sphaeroides was grown on six different C-4 carbon sources and monitored over the course of time (data not shown). Growth rates were rather similar on different carbon sources and so were the biomass yields, except for cultures grown on tartrate (data not shown). Figure 3 shows organo-selenium release 3 days after the addition of 1 mM selenate. The highest amounts of dimethyl selenide, much lower levels of dimethyl diselenide and traces of dimethyl selenenyl sulfide were detected above cultures grown on malate and tartrate. To estimate the yield of reduced selenium per total number of electrons available in 20 mM of each carbon source, the total amount (volatile and dissolved) of reduced selenium was calculated, based on

Table 1 Volatile selenium compounds released by *R. sphaeroides*

Sulfate	OD_{660}	DMSe (nmol)	DMSeS (nmol)	DMDSe (nmol)	nmol Se^{2-} per OD_{660}
Low	2.48 ± 0.04	25.7 ± 1.8	0	0	10.4 ± 0.5
High	4.26 ± 0.23	80.2 ± 19.9	0.05 ± 0.02	0.24 ± 0.09	18.9 ± 3.6

The cultures were amended with 1 mM selenate and grown for 7 days on high (6.2 mM) or low (13 μM) sulfate medium. The listed nmol numbers are amounts of a volatile compound released into the headspace by a 1-L culture. The room temperature was 25 ± 2°C, $n = 2$. DMSe = dimethyl selenide, DMSeS = dimethyl selenenyl sulfide, DMDSe = dimethyl diselenide.

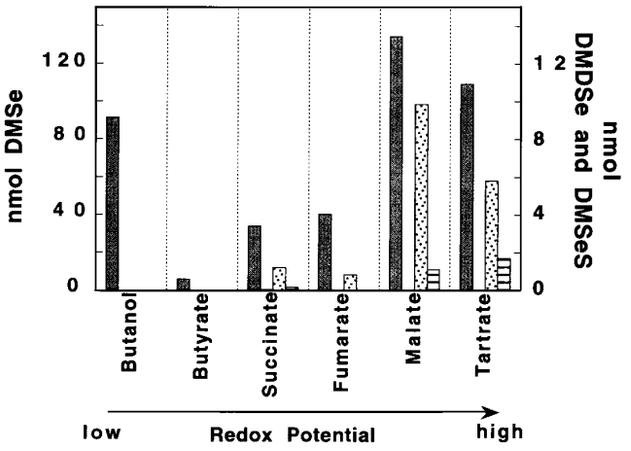

Figure 3 Effect of growth on C-4 carbon sources with different oxidation states (fumarate and malate have the same oxidation state) on the production of dimethyl selenide (▩), dimethyl diselenide (▨), and dimethyl selenenyl sulfide (▤). Values given were obtained 3 days after the addition of 1 mM selenate. In all cases the carbon source concentration was initially 20 mM. The unit nmol refers to the amount of a compound released into the headspace by a 1-L culture (independent of headspace volume). The room temperature was 25 ± 2°C.

amounts determined in the headspace above each culture (Table 2). The highest efficiencies of selenium reduction to dimethyl selenide and dimethyl diselenide were observed in the cultures grown on malate and tartrate, two of the most oxidized carbon sources used in this experiment. The highest reduced selenium yields per mmol added selenate (but still less than 0.2%) were also found with malate and tartrate. Time course experiments with the six carbon sources showed similar trends as described above. Additional organo-selenium was contained in the headspace above malate and tartrate on incubation longer than 3 days (data not shown).

Effect of increasing Se oxyanion concentrations on selenium volatilization

After 2 days of phototrophic growth, cultures of *R. sphaeroides* were supplemented with varied concentrations of sodium selenate or sodium selenite. Headspace analysis and biomass determinations were carried out 7 days later (Figure 4). Selenate at levels of 6 mM and more led to a decrease in biomass production in poisoned cultures (Figure 4a). The highest amounts of dimethyl selenide were found in the headspace above cultures amended with 6–10 mM selenate; the highest yield of 0.16% (selenium contained in dimethyl selenide and dimethyl diselenide) occurred when the cultures were amended with 6 mM selenate.

With increasing amounts of SeO_3^{2-}, optical densities at 660 nm decreased (Figure 4b). Some of this light scattering at 660 nm was from elemental selenium since in selenite-poisoned cultures the color changed from a typical brownish red to brick-red. The formation of elemental selenium in phototrophic cultures has not been investigated; however in cultures of *Pseudomonas fluorescens* we have observed a correlation between increase in optical density and the formation of red elemental selenium (unpublished results). The concentration of selenite that inhibited reinoculated cells of the phototroph from growing (30 mM SeO_3^{2-}) was considerably higher than the selenate concentration (8 mM SeO_4^{2-}. However, only very small amounts of volatile selenium were found in all selenite-amended cultures. The highest volatile selenium yield of 16.7 nmol per mmol selenite (at 2 mM) was two orders of magnitude lower than the highest yield in selenate-poisoned cultures. No dimethyl diselenide or dimethyl selenenyl sulfide was found in selenite-amended cultures.

About one third of dimethyl selenide found at 2 mM selenate was detected in cultures amended with 0.05 mM sel-

Table 2 Electrons (e^-) used to reduce SeO_4^{2-} to Se^{2-} contained in dimethyl selenide and dimethyl diselenide as compared to electrons available from C-4 carbon sources of different oxidation state and yield of reduced selenium per mmol selenate

C-4 carbon source	Yield μmol Se^{2-} per mmol SeO^{2-}_4	Available e^- per mol carbon source (mol)	e^- in reduced Se per available e^- (μmol mol^{-1})
Butanol	1.04	24	17.3
Butyrate	0.06	20	1.3
Succinate	0.41	14	11.8
Fumarate	0.48	12	15.8
Malate	1.77	12	58.4
Tartrate	1.40	10	55.0

Total amounts of dimethyl selenide and dimethyl diselenide (headspace and medium) were obtained by using the determined Henry's law constants. Eight e^- are used for the reduction of SeO_4^{2-} to Se^{2-}. The calculated data are based on the results shown in Figure 3.

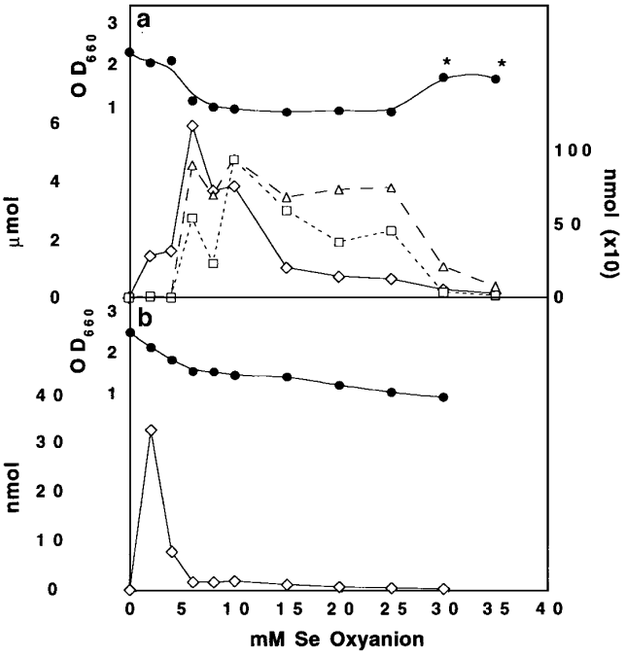

Figure 4 Effect of increasing selenate (a) and selenite (b) concentrations on biomass (●) and the release of volatile selenium compounds, given as nmol or μmol of a compound released into the headspace by a 1-L culture (independent of headspace volume): dimethyl selenide (◇: (a) μmol L^{-1}; (b) nmol L^{-1}); dimethyl diselenide (△: nmol L^{-1}); dimethyl selenenyl sulfide (□: 10 × nmol L^{-1}). ●* represents data where Se^0 is likely to contribute to OD_{660}. The room temperature was 20 ± 2°C.

enate. At 0.013 mM selenate, no organo-selenium was found whereas at 0.013 mM selenite, 4.5 ± 1.7 nmol dimethyl selenide was detected in the headspace of a 1-L culture.

Discussion

The finding that the largest release of organo-selenium compounds by cultures of *R. sphaeroides* 2.4.1 took place several days after the cells had reached the stationary phase of growth (Figure 1) means that the reduction and/or methylation are independent of growth. Dissimilatory oxyanion reductions are, in general, electron transport chain-linked processes [1,17].

Sulfur and selenium are elements of group VIB and form analogous oxyanions. They can be competitive substrates and electron acceptors as proposed for *Chlorella* and *Clostridium pasteurianum* [1,26,27]. However, this work shows that sulfate stimulates the production of volatile, methylated selenium compounds as well as the production of biomass in *R. sphaeroides* (Table 1). Similar effects have been reported for a *Penicillium* species [11].

In phototrophs the reduction potential of the carbon source regulates metabolic pathways, eg pigment synthesis [30]. In the case of tellurite, another oxyanion of a group VIB element, it has been reported for TeO_3^{2-}-amended cultures of *R. sphaeroides* that the lower the redox potential of a carbon source, the more tellurite could be detoxified by reduction [19,20]. The rate of selenium volatilization reported here was also dependent on the carbon source, but could not be correlated with the redox potentials. However, tellurite-poisoned cultures of *R. sphaeroides* turn coal black (from elemental tellurium formation). With respect to their link to biochemical reductive processes in this organism, selenium oxyanion reduction/methylation and tellurite reduction are not as similar as one might expect. It is not clear why much less selenate was volatilized upon growth on fumarate even though malate and fumarate have the same redox state. Using volatile selenium production per liter of culture and μmol electrons used to reduce selenium per mol of available electrons, cultures of *R. sphaeroides* grown on malate and tartrate showed higher volatilization/detoxification activities as compared to cultures grown on other C-4 compounds (Figure 3, Table 2). Yet, the number of electrons used to reduce selenate to Se^{2-} was in all cases only a small fraction of the available electrons.

With the phototroph examined in this work, pH, sulfate concentration, carbon source, selenate and selenite concentrations all affected the formation of dimethyl selenide, dimethyl diselenide, as well as dimethyl selenenyl sulfide. Certain physiological conditions may favor the reaction sequence for the formation of dimethyl diselenide as proposed by Reamer and Zoller [23], based on the Challenger mechanism [3]. It is unclear whether dimethyl selenenyl sulfide is formed by biological activity or by the spontaneous reaction between volatile sulfur and selenium compounds, such as methanethiol, methaneselenol (which so far has never been found above *R. sphaeroides*), dimethyl disulfide and dimethyl diselenide. Since in all experiments dimethyl selenenyl sulfide was only found together with dimethyl diselenide, it is likely that it is formed along the same pathway [23].

Various purple nonsulfur bacteria are resistant to both selenate and selenite, unlike many other bacterial species [2]. A lower minimal inhibitory concentration was found for *R. sphaeroides* with selenate (0.81 mM) than with selenite (4.6 mM) [19]. In this work, upon reinoculation of poisoned cells, growth was completely inhibited at significantly lower selenate than selenite concentrations. On the other hand a much higher yield of volatile compounds was found in selenate-poisoned cultures. It seems that even though the ability of the cells to divide is inhibited at concentrations $\geq$ 8 mM selenate, the selenate reduction/methylation mechanism is still working.

Cultures amended with lower Se oxyanion concentrations (in the range of agricultural drainage water in the San Joaquin Valley, for instance) showed lower volatilization activities. At 0.013 mM selenium oxyanion concentration *R. sphaeroides* only volatilized selenite, but not selenate, the predominant Se compound in agricultural drainage waters.

With regard to a possible future application of purple nonsulfur bacteria in bioremediation, selenium volatilization activities with a naturally occurring and thus environmentally safe organism have been observed here. In order to obtain better yields of volatile selenium, further combinations and variations of carbon source, pH, selenium oxyanion concentration, and additional growth conditions have to be investigated. Selenium reduction/methylation in the presence of nitrate which can be reduced by phototrophic bacteria [20] would be of interest with regard to the bioremediation of agricultural drainage water.

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