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Volatilization of Selenium by *Alternaria alternata*

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Seleniferous water continues to be a serious problem to wildlife in the central valley of California. Water samples collected from Kesterson Reservoir, Peck Ranch, and Lost Hills evaporation pond facilities contained between 0.005 and 5 mg of Se per liter. The objective of this study was to isolate Se-methylating organisms in evaporation pond water and to assess, through enrichment and manipulation of their optimal growth parameters, the environmental factors which govern microbial Se methylation. *Alternaria alternata* was isolated as an active Se-methylating organism. The volatile product was identified as dimethylselenide. The effects of pH, temperature, Se substrates, and methyl donors on the ability of *A. alternata* to methylate Se were investigated in liquid medium containing 100 mg of Se per liter. The optimum pH and temperature for methylation were 6.5 and 30°C, respectively. Selenate and selenite were methylated more rapidly than selenium sulfide and various organic Se compounds (6-selenoguanosine, 6-selenoinosine, seleno-DL-methionine, and 6-selenopurine). L-Methionine and methyl cobalamine (0.1 μM) stimulated dimethylselenide production. This study demonstrates that Se-methylating organisms are present in evaporation pond water and are capable of liberating substantial quantities of Se in the volatile dimethylselenide form. By determining the optimum environmental conditions which stimulate volatilization, it may be possible to design a way to remove Se from seleniferous water in situ.

Agricultural irrigation water on the western side of the San Joaquin Valley, California, removes soluble salts, including toxic trace elements, from cropland via tile drains into drainwater evaporation ponds. Selenium is of particular concern because high concentrations have been found to be toxic to wildlife inhabiting these waters. Bioaccumulation of Se in the food chain can result in reduced reproductive success, embryo deaths, and deformities of waterfowl (16, 21).

The transformation of nonvolatile Se into volatile products is an important link in the global cycling of the element and may also constitute an effective detoxification process (8). The toxicity of inorganic Se species is approximately 1,000-fold greater than that of the alkylselenide dimethylselenide (DMSe) (50% lethal dose [LD_{50}] of DMSe is 1,600 to 2,200 mg of Se per kg in the rat [15, 18, 26]). Volatilization of Se appears to be a widespread process and has been reported to be carried out by a diverse group of organisms in pure culture as well as in soils (1, 3, 5, 12, 27, 28). It was found in this laboratory and in the field (Kesterson Reservoir) that the addition of specific organic C amendments to soil greatly stimulates Se methylation (17a; W. T. Frankenberger, Jr., and U. Karlson, *Dissipation of Soil Selenium by Microbial Volatilization at Kesterson Reservoir*, U.S. Dept. of the Interior, Bureau of Reclamation, December 1988; Karlson and Frankenberger, Soil. Sci. Soc. Am. J., in press). It was therefore postulated that Se methylation might also be a means of detoxifying Se-contaminated agricultural drainage water.

Alternaria alternata is a widely distributed organism found associated with numerous kinds of organic materials in moist environments (22). It has been isolated from soil, on leaves of over 117 plant species, in leaf litter, and submerged in the sea (20, 25). *Alternaria alternata* is a saprophyte, with some strains being weak foliicolous pathogens. This is the first time that *A. alternata* has been reported to be Se resistant as well as an active methylator of Se.

MATERIALS AND METHODS

Reagents. Sodium selenite and sodium selenate (Na_2SeO_4) were obtained from Alfa Products (Danvers, Mass.), and DMSe was from Strem Chemical Co. (Newburyport, Mass.). The following chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.): selenium sulfide, 6-selenoguanosine, 6-selenoinosine, seleno-DL-methionine, 6-selenopurine, methyl cobalamine, and L-methionine. Sodium tetrahydroborate was obtained from Aldrich Chemical Co. (Milwaukee, Wis.), and ammonium persulfate was from Mallinckrodt (Paris, Ky.).

Water sampling. Water samples were collected from evaporation ponds 1, 2, 5, 6, and 7 at Kesterson Reservoir (Merced County, Calif.); from Lost Hills (Kern County, Calif.) A, B, and C evaporation ponds; and from cells 1 to 6 at the Peck Ranch (Fresno County, Calif.) (Fig. 1). The water was collected in Nalgene bottles, transported on blue ice, and stored at 5°C.

Atomic absorption spectrometry. Water samples were analyzed for Se atomic absorption spectrometry with hydride generation. The instrument used was a Varian (Mulgrave, Victoria, Australia) Spectra AA/10 with a VGA-76 vapor generation assembly. The operational conditions were as follows: acetylene, 2.4 ml/min; air, 6.3 ml/min; nitrogen, 90 ml/min; sample flow, 6.5 ml/min; acid flow, concentrated HCl, 1.2 ml/min; reagent flow, 0.6% $NaBH_4$–0.5% NaOH, 1.2 ml/min; lamp current, 10 mA; wavelength, 196.0 nm; and slit width, 1.0 nm. Samples were treated with $(NH_4)_2S_2O_8$ (final concentration, 0.2%) and concentrated HCl to give a sample acidity of 6 M HCl and boiled for 1 h immediately before analysis. Filtration of the water samples was not necessary. A quality assurance procedure for Se analysis by atomic absorption spectrometry was followed. Duplicate samples were analyzed with seven nonconsecutive measurements per day. Samples were spiked at midpoint or at the upper end of the calibration range to check for possible

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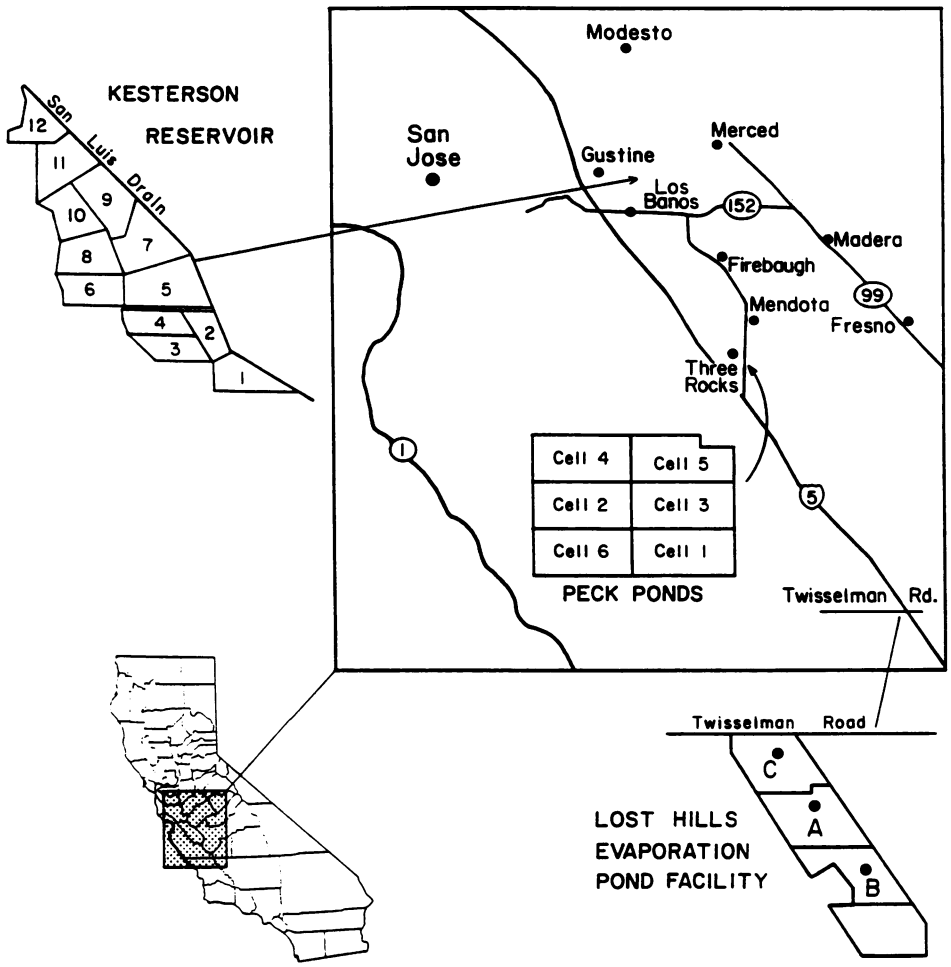

FIG. 1. Locations of evaporation ponds in the Lost Hills District, the Peck Ranch, and Kesterson Reservoir. State (circles) and interstate (shield) route numbers are shown.

interferences and note any analytical problems. Calibration and reagent blanks were analyzed at the beginning and end of each run. Acceptable data quality objectives were as follows: accuracy, 80 to 120% recovery; precision, 15% relative percent difference; detection limits, 10 μg/liter.

GC. For gas chromatography (GC), the headspace above inoculated media was analyzed for DMSe by withdrawing a 1-ml gas sample with a gas-tight series 2 Pressure-Lok gas syringe (Alltech, Deerfield, Ill.) and injecting it directly into a gas chromatograph. The GC analysis was performed on a Hewlett-Packard (Avondale, Pa.) model 5890 GC, connected to a Hewlett-Packard 3393A integrator. The operational conditions were as follows: stainless steel column (10 m by 2.2 mm inner diameter); liquid phase, 10% Carbowax 1000; solid support, Chrom W-AW; particle size, 0.18 to 0.24 mm (mesh 60/80); flame ionization detector; column temperature, 58°C rising to 80°C after 2 min at the rate of 70°C per min; injector and detector temperature, 105°C; carrier gas (He), 10 ml/min; N_2, 30 ml/min; H_2, 40 ml/min; air, 370 ml/min. Calibration of the instrument with DMSe as a standard enabled specific detection and quantification.

Enrichment cultures. The literature suggests that Se methylation is a purely biological process. Doran (11) reported that volatile Se is not produced from soils, sediments, and sewage when steam sterilized. Unamended pond water sterilized through autoclaving does not evolve DMSe (W. T.

TABLE 1. Analyses of water samples from selected evaporation ponds

Location	Date(s) collected (mo/day/yr)	Avg total Se (mg/liter)	Avg electrical conductivity (dS/m)	Avg pH	Avg dissolved oxygen (mg/liter)	Temp range (°C)
Peck cell 3	9/12/87–6/16/88	1.356	20.64	8.77	9.06[a]	15–29[a]
Peck cell 5	9/12/87	1.705	35.92[a]	8.80[a]	9.21[a]	17–29[a]
Kesterson pond 1	9/12/87	0.014	32.50	7.42	ND[b]	ND
Lost Hills A	10/02/87	0.690	144.67[c]	8.77[c]	ND	ND

[a] Average readings compiled from K. K. Tanji and M. E. Grismer, *Evaporation Ponds for Disposal of Agricultural Wastewater*, quarterly progress reports, January-April 1987, April-June 1987, and July-September 1987, WRCB no. 5-190-150-0.

[b] ND, Not determined.

[c] Reported readings from Lost Hills Water District, 1986 monitoring and reporting program. Agricultural Subsurface Drainage Evaporation Disposal Basins, Kern County. Report submitted to California Regional Water Quality Control Board, Central Valley Region. Order No. 86-240. Averaged with readings from this laboratory.

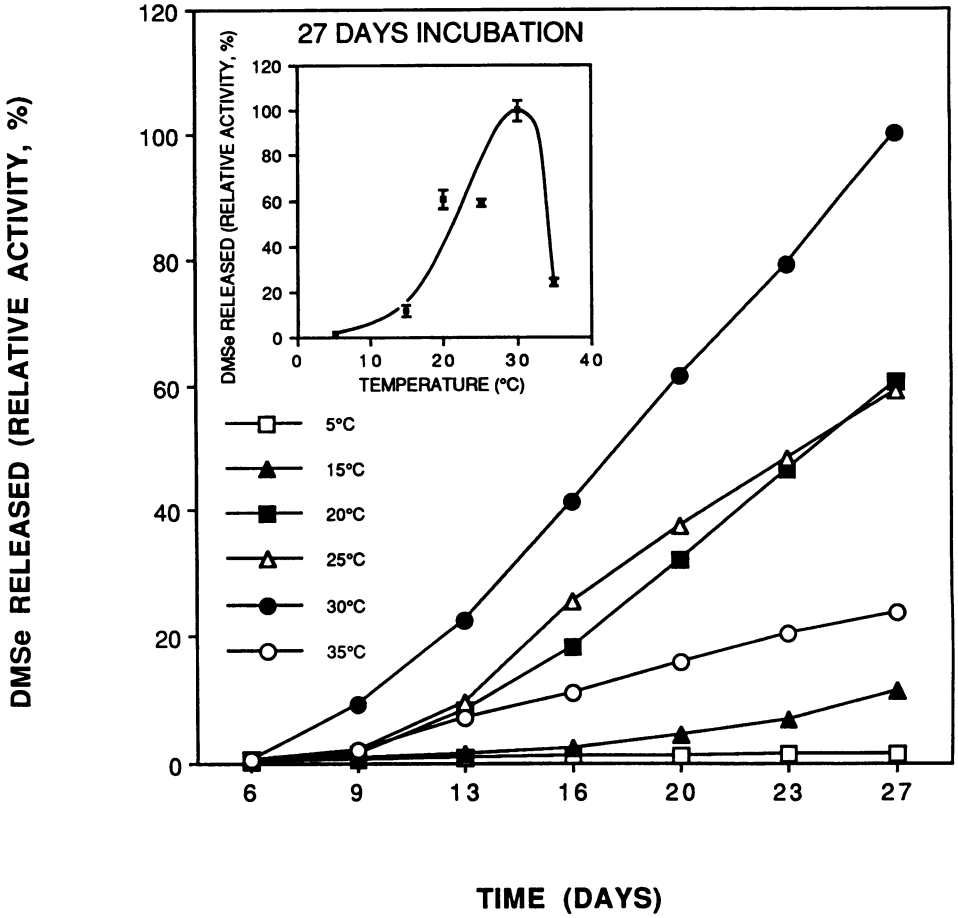

FIG. 2. Influence of temperature on DMSe production by *A. alternata*.

Frankenberger, Jr., and E. T. Thompson-Eagle, *In Situ Volatilization of Selenium. II. Evaporation Ponds*, San Joaquin Valley Drainage Program, September 1988).

Six different types of culture media were used for selective enrichment and isolation of Se-methylating organisms: (i) 0.1% (wt/vol) glucose added to 200 ml of pond water, (ii) malt extract broth at pH 7.0 (20 g of malt extract, 20 g of glucose, 1 g of peptone per liter [Difco Laboratories, Detroit, Mich.]), (iii) minimal medium at pH 8.0 (14), (iv) medium G (4), (v) Chu 10 (6), and (vi) nutrient broth (Difco) supplemented with 50 mg of cycloheximide (Sigma) and 5 g of glucose per liter. All media were autoclaved for 20 min at 121°C at 15 lb/in^2. Fifty milliliters of pond water was added to 150 ml of medium in 250-ml Erlenmeyer flasks. An autoclaved solution of Na_2SeO_4 was added to the mixture so as to achieve concentrations of 10, 100, and 200 mg of Se per liter. Ion chromatographic analysis by the method of Mehra and Frankenberger (19) confirmed that autoclaving and subsequent shelf storage in glass bottles did not affect the stability of Se(VI). The flasks were sealed with gas-tight rubber septa (Aldrich) disinfected with 70% ethanol and were placed on orbital shakers (120 rpm) at room temperature (ca. 23°C). The water samples enriched with media i and v were incubated in the light to encourage growth of algae. The headspace of each flask was sampled for DMSe by GC.

Isolation of selenium-methylating organisms. Samples (1 ml) were removed from the pond water enrichment cultures which had produced DMSe, and a series of dilutions were made in sterile water. Samples (0.1 ml) were plated onto one of the six media supplemented with agar (15 g of agar [Difco] per liter) and sterile Na_2SeO_4 (10 mg of Se per liter). The inoculated plates were incubated at room temperature (ca. 23°C) until individual colonies could be selected from the plates and further subcultured on the same medium. All isolates producing the characteristic DMSe odor were reinoculated into broth and subjected to analysis by GC. The most active methylating isolate was identified as *Alternaria alternata* (10, 13). This fungus has short, pigmented conidiophores with chains of flask-shaped, multicellular, pigmented conidia which are laterally and longitudinally septate. Purification of this fungus was achieved through serial transfers on solid medium. The fungus was examined microscopically during identification and was found to have no bacteria adhering to it.

Factors affecting DMSe production by *A. alternata*. Malt extract broth (MEB) was added in 75-ml quantities to screw-capped 125-ml Erlenmeyer flasks and sterilized by autoclaving (20 min, 121°C, 15 lb/in^2) before the addition of Se(VI) at 100 mg/liter [MEB(100)]. The fungus was inoculated aseptically into the broth in the form of mycelial plugs, 1 cm in diameter. The flasks were then capped with 70% ethanol-disinfected Mininert valves (Dynatech, Baton Rouge, La.), and except for the temperature study, flasks were placed on an orbital shaker (120 rpm) at room temperature. The headspaces were analyzed by GC every 2 to 5 days. After each sampling, the caps were removed and the

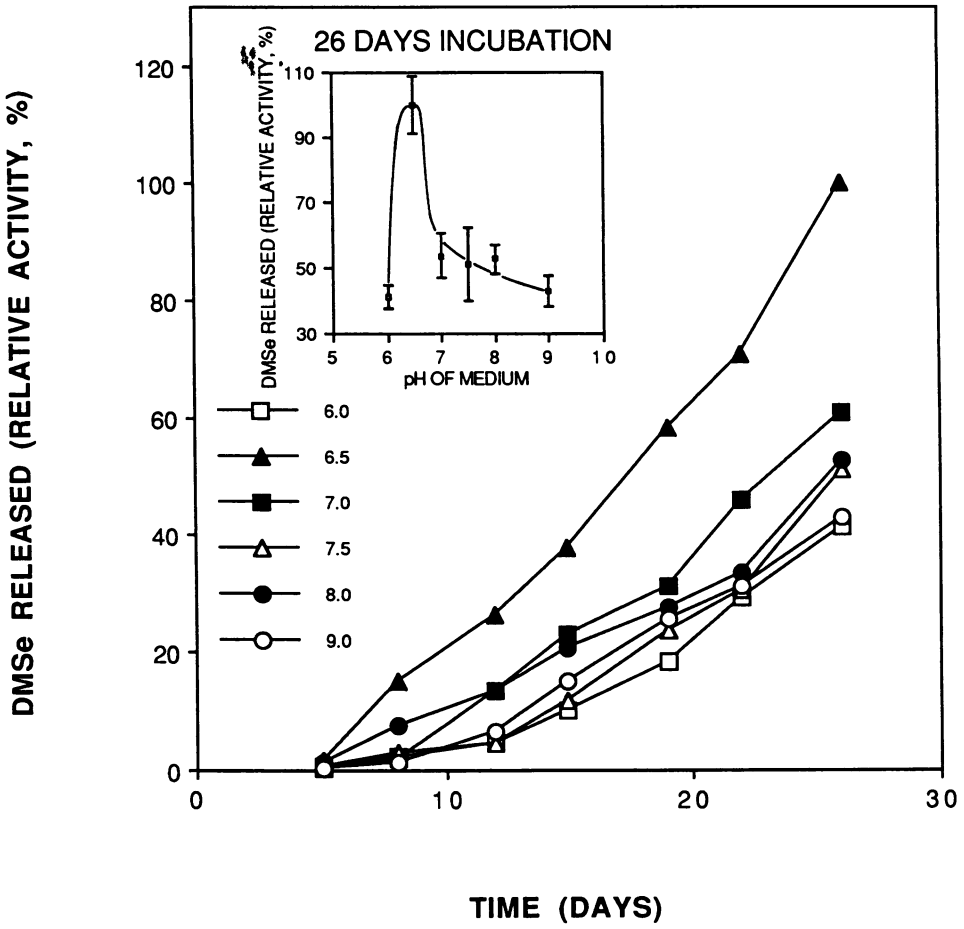

FIG. 3. Influence of pH on DMSe production by *A. alternata*.

headspace was evacuated for 0.3 h (flow rate, 8 liters/min) under aseptic conditions. Each treatment consisted of five replicates. Error bars in each figure depict the standard error of the data. Evolution of Se by fungal cultures was expressed as percent relative activity based on the cumulative maximum of DMSe released for each parameter tested.

Temperature. Cultures of *A. alternata* were incubated statically in MEB(100) and placed in incubators set at 5, 15, 20, 25, 30, and 35°C. Flasks were sampled for DMSe over a 27-day incubation period. Temperature coefficient (Q_{10}) values were calculated from the Se-methylating activity of *A. alternata* in the presence of 100 mg of Se(VI) per liter with the temperature of incubation ranging from 5 to 30°C, by the following equation:

$$Q_{10} = \left(\frac{\text{DMSe at } T_2}{\text{DMSe at } T_1} \right) \frac{10}{T_2 - T_1} \quad (1)$$

pH. Buffered MEB(100) was prepared by adding 20% modified universal buffer stock solution (23) to the medium. The pH was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0 by using HCl or NaOH. Flasks were sampled for DMSe over a 26-day incubation period.

Selenium substrates. The substrates sodium selenate, sodium selenite, 6-selenoguanosine, 6-selenoinosine, seleno-DL-methionine, and 6-selenopurine were filter sterilized (0.22-μm pore size; Millipore Corp., Bedford, Mass.) in Delrin plastic in-line holders (Fisher Scientific Co., Pittsburgh, Pa.) and added to sterile MEB so as to give a final concentration of 100 mg of Se per liter. Sodium selenate, sodium selenite, and seleno-DL-methionine were dissolved in water. The remaining substrates were dissolved as follows: 6-selenoinosine was dissolved in 1 M NaOH, and both 6-selenoguanosine and 6-selenopurine were dissolved in 1 M NH_4OH. Selenium sulfide was insoluble and thus was added in solid form. All media were pH-corrected to pH 7.0. Flasks were sampled for DMSe over a 42-day incubation period.

Methyl donors. The methyl donors L-methionine and methyl cobalamine were filter sterilized (0.22-μm pore size) before addition to MEB(100) to give final concentrations of 0, 0.1, 1, 10, 100, and 1000 μM. Flasks were sampled for DMSe during a 35-day incubation period.

RESULTS

Water sampling. Selenium concentrations as high as 0.6 mg/liter were found at Kesterson Reservoir but usually ranged from 0.005 to 0.060 mg/liter (Table 1). At the Peck Ranch and Lost Hills, Se levels ranged between 0.6 and 5 and between 0.1 and 0.8 mg/liter, respectively. The pH and electrical conductivity of the waters sampled ranged from pH 7.4 to 8.8 and from 20.6 to 144.7 dS/m, respectively. The electrical conductivity values were almost 10-fold greater in the Lost Hills evaporation ponds than at the Peck Ranch and Kesterson Reservoir. Other parameters affecting microbial

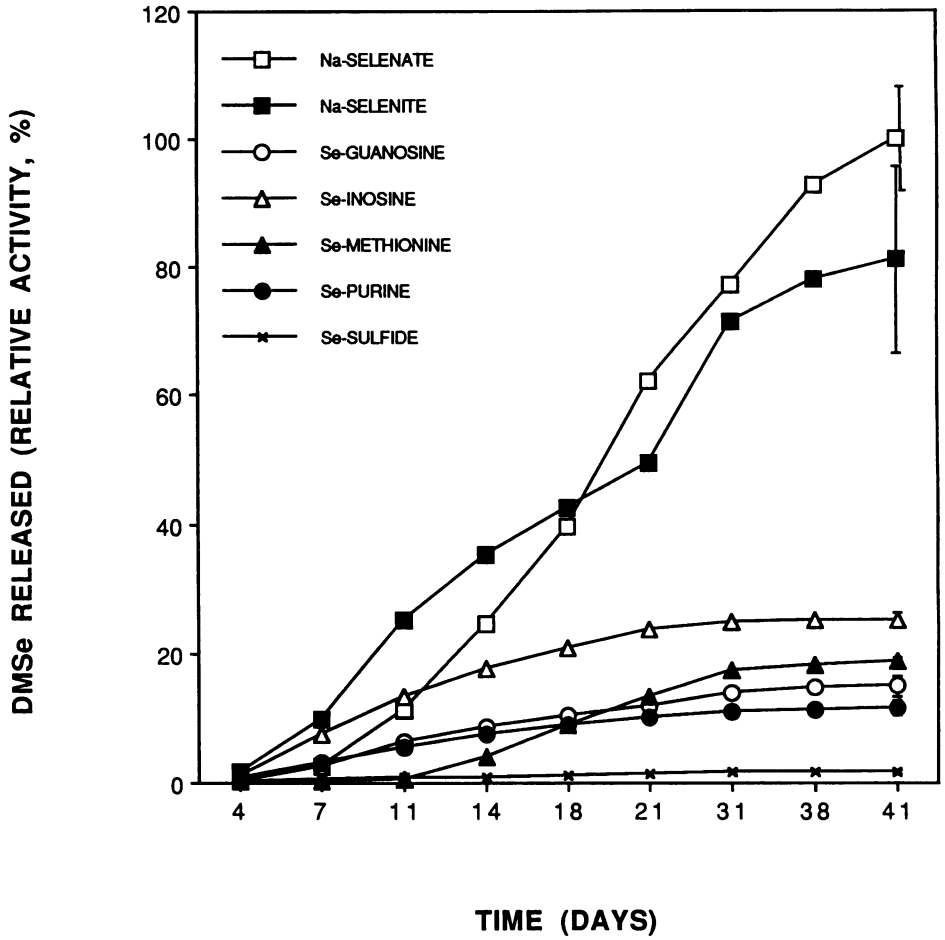

FIG. 4. Influence of Se substrates on DMSe production by *A. alternata*.

activity, such as dissolved oxygen, and the water temperatures for the Peck Ranch ponds are reported in Table 1.

Isolation of selenium-methylating organisms. Many of the microorganisms isolated from the evaporation ponds were resistant to Se concentrations of up to 100 mg/liter, but only approximately 3% of these isolates produced detectable amounts of DMSe. The most active Se-methylating isolates in these waters were found to be fungi. *Alternaria alternata* was identified as the organism with the highest production of volatile Se. The gaseous Se was identified as DMSe by GC-mass spectroscopy; no dimethyldiselenide was detected (Frankenberger and Thompson-Eagle, San Joaquin Valley Drainage Program, September 1988).

Factors affecting DMSe production by *A. alternata*. The optimum temperature for DMSe production by *A. alternata* was 30°C (Fig. 2). The activity was 1.73-fold greater at 30°C than at 25°C. DMSe was detected even when the fungus was cultured at the lowest temperature, 5°C. The Q_{10} values for Se methylation decreased progressively with increasing temperature and ranged from 5.99 to 1.70.

Modified universal buffer was selected in this study because of its buffering ability over a wide pH range. Tests showed that the pH of the reaction mixture did not deviate more than ±0.2 pH unit. DMSe production by *A. alternata* was optimal at pH 6.5 in the presence of MEB(100) and 1.64-fold greater than at pH 7.0 (Fig. 3). Sterile controls within the pH range of the experiment confirmed that the Se transformation was solely biological.

Selenium substrates. Among the substrates tested, inorganic Se(IV) and Se(VI) were more efficient substrates for methylation by *A. alternata* than the organic selenium compounds and selenium sulfide when added at equivalent amounts of 100 mg of Se per liter (Fig. 4). The most effective substrates for Se methylation after 42 days of incubation were ranked as follows: selenate > selenite ≫ selenoinosine > selenomethionine > selenoguanosine > selenopurine > selenium sulfide. Selenite(IV) was methylated at a greater rate than Se(VI) initially, while there was a lag before Se(VI) eventually produced larger quantities of DMSe. Selenium sulfide was methylated poorly, most likely because of its insolubility and thus unavailability to *A. alternata*.

Methyl donors. L-Methionine (Fig. 5) and methyl cobalamine (Fig. 6) promoted methylation of Se(VI) by *A. alternata* at 0.1 μM. L-Methionine promoted a 10-fold increase and methyl cobalamine an 8.6-fold increase in DMSe production over the controls.

DISCUSSION

Microbial isolates obtained from evaporation pond waters yielded a diverse group of organisms resistant to Se(VI) at between 10 and 100 mg of Se per liter. Selenium was added in the Se(VI) form for enrichment because it is the dominant species found in the San Joaquin River, the San Luis Drain Canal, and various evaporation ponds throughout the central valley of California, including Kesterson Reservoir (7).

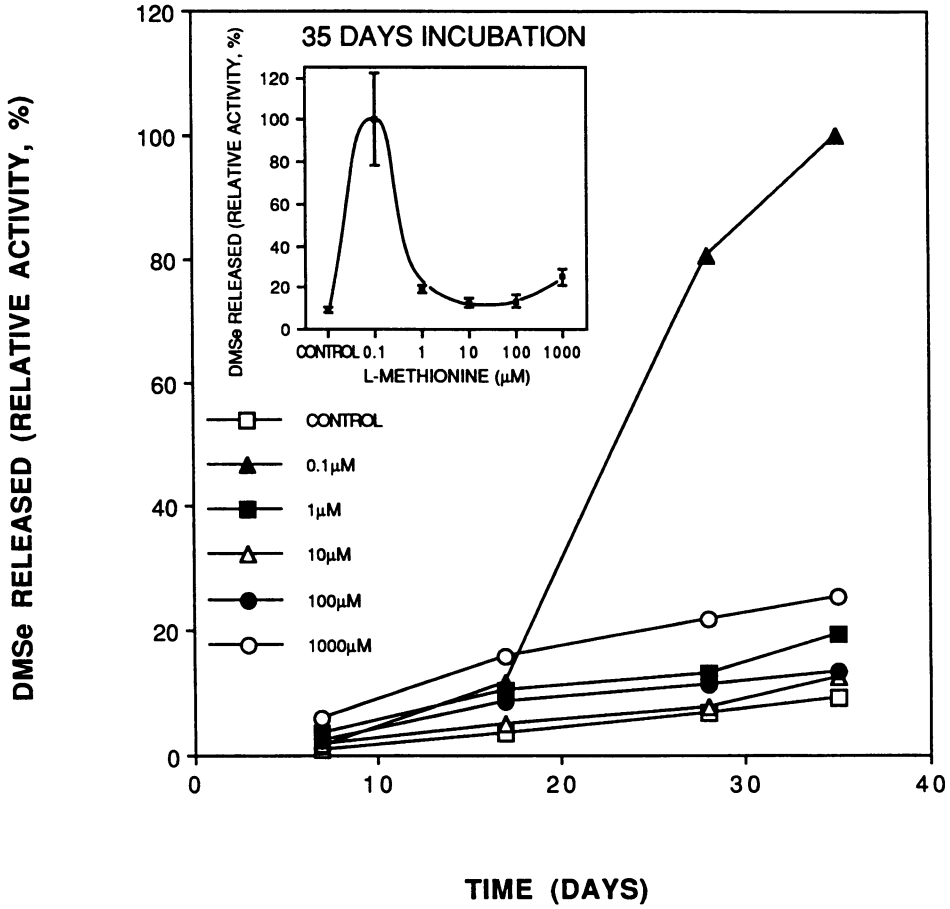

FIG. 5. Influence of L-methionine on DMSe production by *A. alternata*.

Some of these bacterial isolates were able to carry out Se reduction, an ability easily identified because of the presence of a brick red coloring [Se(0)] of the bacterial colonies.

The most active Se-methylating organism isolated was found to be *A. alternata*. Optimal conditions for Se volatilization by this fungus were determined to be 30°C and pH 6.5, with the use of Se(VI) and in the presence of a methyl donor, L-methionine or methyl cobalamine (0.1 μM).

The minimum daytime temperature recorded at the Peck Ranch was 15°C, with a maximum of 29°C (Table 1). Increasing the incubation temperature from 5 to 30°C stimulated Se methylation by *A. alternata*. The spring and summer months with warmer temperatures would be expected to promote Se volatilization directly from these evaporation ponds.

Selenium methylation by *A. alternata* in buffered MEB(100) occurred in substantial quantities throughout the pH range of 6 to 9. The average pH of evaporation pond water sampled varied between pH 7.4 (Kesterson Reservoir) and 8.8 (Peck Ranch) (Table 1). The optimum pH of Se methylation by *A. alternata* was pH 6.5. Baker et al. (2) reported that the optimal conditions for mercury methylation were pH 5.5 to 6.5. Arsenic has been shown (2) to be methylated over a much wider pH range of 3.5 to 7.5; however, the highest levels of methylated As compounds are often detected under acidic conditions.

Forms of Se other than the Se(VI) species were tested as methylation substrates because many of these compounds have been found in surface waters (7, 9). All the Se compounds tested were methylated by *A. alternata*. Approximately six times as much DMSe was evolved from Se(VI) as from the organic species, while five times more DMSe was evolved from Se(IV). In contrast, Doran and Alexander (12), using Se-spiked loam and clay soils, found that selenomethionine was methylated in greater quantities than either Se(IV) or Se(VI). They reported a 5.3-fold and 15.8-fold increase in DMSe evolution from selenomethionine compared with that from Se(IV) and Se(VI), respectively. Other Se substrates reported to be methylated in soil include trimethylselenonium chloride, selenocysteine, and Se^0 (11). The discrepancy in DMSe evolution from Se substrates reported in Doran and Alexander's work (12) and that reported here may be explained by the fact that communities of methylating soil and sediment organisms were monitored in the former study rather than a single organism grown in monoculture.

The effects of L-methionine and methylcobalamine on methylation of Se by *A. alternata* were investigated because of Challenger's hypothesis that compounds such as betaine, choline, and methionine may act as methyl donors in the Se methylation reaction. Challenger (3) discovered that if sodium selenite is heated with betaine, it yields DMSe. The stimulatory effect observed with methyl donors and *A. alternata* was interesting because of the conflicting results in the literature. Challenger et al. (4) found that methionine stimulated Se methylation by *Scopulariopsis brevicaulis*, while Fleming and Alexander (14) reported that methionine concentrations of 20, 100, and 200 mg/liter suppressed DMSe formation by *Penicillium* spp. in the presence of Se(VI).

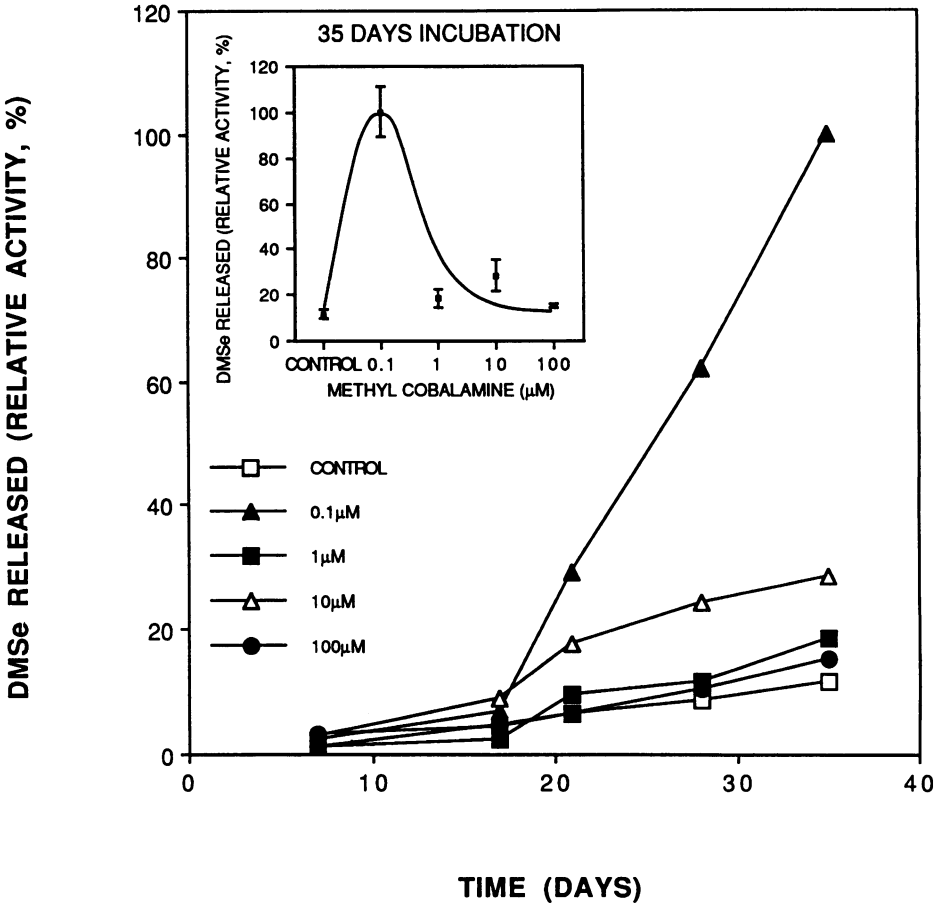

FIG. 6. Influence of methyl cobalamine on DMSe production by *A. alternata*.

Imura et al. (17) investigated methylcobalamine as a potential methyl donor and showed that when 0.1 μM methylcobalamine was added to 0.5 μM mercuric chloride in the presence of potassium phosphate buffer at 37°C in the dark, methylated mercury was formed through an abiotic route. A number of other compounds are known to donate methyl groups in various metabolic reactions, including *S*-adenosylmethionine, sodium formate, D-1,5-methyl tetrahydrofolate, glutathione, and choline chloride (24). It would be of interest to test the effect of these compounds on Se methylation.

This study demonstrates that Se-methylating organisms are present in agricultural evaporation pond water that can liberate Se in the volatile form. *Alternaria alternata* released 1,000 times more DMSe than was shown to be evolved by a lake sediment amended with sodium selenate in a previous study (5). By determining the optimum environmental conditions which stimulate volatilization, it may be possible to design a way to remove Se from seleniferous water in situ.

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