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Bacterial Dissimilatory Reduction of Arsenic(V) to Arsenic(III) in Anoxic Sediments

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Incubation of anoxic salt marsh sediment slurries with 10 mM As(V) resulted in the disappearance over time of the As(V) in conjunction with its recovery as As(III). No As(V) reduction to As(III) occurred in heat-sterilized or formalin-killed controls or in live sediments incubated in air. The rate of As(V) reduction in slurries was enhanced by addition of the electron donor lactate, H_2, or glucose, whereas the respiratory inhibitor/uncoupler dinitrophenol, rotenone, or 2-heptyl-4-hydroxyquinoline *N*-oxide blocked As(V) reduction. As(V) reduction was also inhibited by tungstate but not by molybdate, sulfate, or phosphate. Nitrate inhibited As(V) reduction by its action as a preferred respiratory electron acceptor rather than as a structural analog of As(V). Nitrate-respiring sediments could reduce As(V) to As(III) once all the nitrate was removed. Chloramphenicol blocked the reduction of As(V) to As(III) in nitrate-respiring sediments, suggesting that nitrate and arsenate were reduced by separate enzyme systems. Oxidation of [2-^{14}C]acetate to $^{14}CO_2$ by salt marsh and freshwater sediments was coupled to As(V). Collectively, these results show that reduction of As(V) in sediments proceeds by a dissimilatory process. Bacterial sulfate reduction was completely inhibited by As(V) as well as by As(III).

Anthropogenic sources of arsenic in the environment derive from mining and smelting activities, pesticide usage, and the combustion of fossil fuels such as coal (4). In addition, arsenic is a relatively abundant element in the Earth's crust and occurs in soils and aquatic environments as a consequence of the natural dissolution and weathering of its parent minerals. In certain types of aquatic environments, such as the hypersaline Mono Lake, Calif., dissolved arsenic concentrations are extremely high (~0.3 mM), owing to the concentration effects of hydrologic and climatic factors and an abundance of hydrothermally based sources (2, 14). Arsenic occurs in nature in three oxidation states: As(V) (arsenate), As(III) (arsenite), and As(−III) (arsine). Although the dynamics between these states can be achieved by purely chemical means, microorganisms can also mediate a diversity of reactions including reduction, oxidation, and methylation (8). These reactions were observed in culture, and their overall significance to the cycling of arsenic in the environment is not known.

Within anoxic soils, sediments, and waters, arsenic occurs primarily as As(III) (14, 15, 25, 31). A number of bacteria reduce As(V) to As(III) as a detoxification mechanism based on the enhanced outward mobility from the cell of As(III) (5). This phenomenon, however, does not extend itself logically as an explanation for the abundance of As(III) in reduced environments. Recently, two novel strains of bacteria were reported to be capable of respiratory growth by coupling the reduction of As(V) to As(III) with the oxidation of lactate (1, 12). Thermodynamic calculations showed that this reduction was sufficiently exergonic to sustain growth (12). In addition, it was hypothesized that reduction of As(V) to As(III) in anoxic sediments was carried out by similar types of bacteria in putative reactions analogous to those observed for the dissimilatory reduction of Se(VI) to Se(IV) (12, 19). We now report that the reduction of As(V) to As(III) in anoxic sediments is carried out by bacterial dissimilatory arsenic reduction (DAsR).

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MATERIALS AND METHODS

Preparation of sediment slurries. Sediments were taken from a San Francisco Bay salt marsh located in Palo Alto, Calif. (19), and from Lahontan Reservoir, a man-made freshwater lake located in eastern Nevada (22). The Lahontan Reservoir sediments were used only for the experiments with [2-^{14}C]acetate (see below). Sediments were homogenized with an equal volume of artificial bay water, unamended or supplemented with 20 mM sulfate (23), or, for the Lahontan sediments, with an equal volume of lake water. The resulting homogenates (60 ml) were dispensed into serum bottles (160 or 100 ml) which contained additional artificial bay water or lake water (final sediment-in-water dilution, 1:4). All preparations and manipulations were done under a flow of O_2-free N_2 (19). The serum bottles were crimp-sealed and flushed for 10 min with O_2-free N_2. Aerobic controls were capped with a permeable foam rubber seal and were thus open to the atmosphere for the duration of the incubations. Heat-killed controls were autoclaved twice on successive days for 1 h at 121°C and 250 kPa. Formalin-killed controls received 4% (vol/vol) formaldehyde. The resulting slurries were preincubated in the dark for 16 to 24 h at 20°C with constant rotary shaking (150 rpm). Electron acceptors, electron donors, and inhibitors were added by syringe injection from anaerobic stock solutions and are given at their the final concentrations (millimolar): Na_2HAsO_4, 10; NaH_2AsO_3, 10; sodium lactate, 10; sodium acetate, 10; sodium succinate, 10; glucose, 10; $NaNO_3$, 10; Na_2MoO_4, 20; Na_2WO_4, 20; rotenone, 0.25; 2,4-dinitrophenol, 0.25; 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), 0.1; and NaCN, 1.0. Additions of Fe(III) and Mn(IV) at 10 mmol/liter (final concentrations) were made as a chelate of Fe(III)-nitrilotriacetic acid and as MnO_2, respectively. Slurries incubated with H_2 as an electron donor were flushed with this gas for 10 min to displace the N_2 headspace. All experiments were conducted with triplicate sets of sediment slurries. Slurries were incubated as stated above, and the liquid phase was periodically subsampled (1.0 ml) by syringe. Subsamples were microcentrifuged for 10 min, filtered (pore size, 0.2 μm), and stored at −60°C until analyzed. To determine if anoxic sediments adapted to nitrate respiration were able to achieve As(V) reduction without de novo enzyme synthesis, slurries were preincubated for 48 h with nitrate plus lactate (20 mM each), at which time it was determined that neither substrate remained in the slurries. All slurries received an additional 10 mM lactate supplement and were then incubated with: 10 mM nitrate, 10 mM nitrate plus 0.4 mg of chloramphenicol per ml, 10 mM As(V), or 10 mM As(V) plus 0.4 mg of chloramphenicol per ml.

Radioisotope experiment. Sediment slurries were prepared as outlined above but modified by using reduced volumes (30 ml) incubated in smaller serum bottles (57 ml). Acetate (final concentration, 5 mM) was provided as the electron donor, with or without As(V) as the electron acceptor (final concentrations, 0.0, 0.5, 1.0, 2.5, 5.0, and 10.0 mM). Salt marsh slurries also contained 10 mM sulfate, while Lahontan Reservoir slurries had ambient lake water sulfate levels (~1

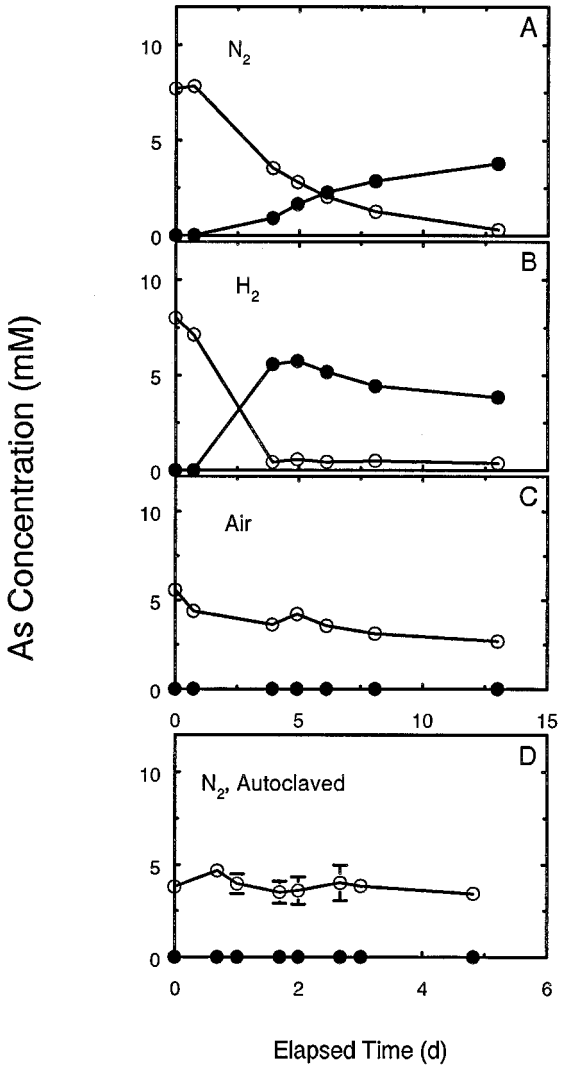

FIG. 1. Reduction of As(V) (○) to As(III) (●) in salt marsh sediments incubated in an N_2 atmosphere plus 10 mM As(V) (A), an H_2 atmosphere plus 10 mM As(V) (B), and an air atmosphere plus 10 mM As(V) (C) and autoclaved under an N_2 atmosphere with lactate plus 5 mM As(V) (D). Symbols represent the means of three slurries, and bars indicate ±1 standard deviation. The absence of bars indicates that the error was smaller than the symbols.

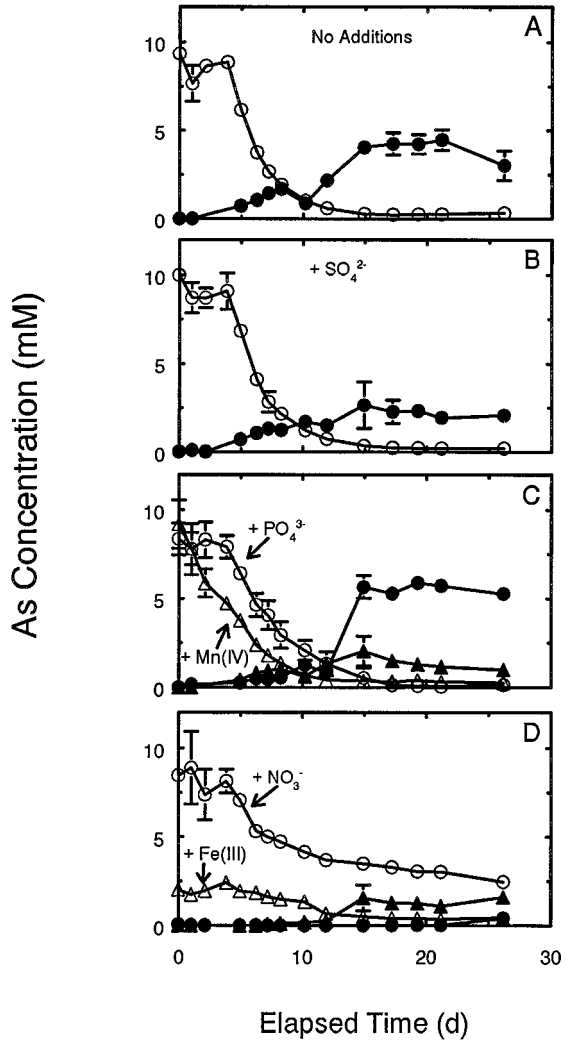

FIG. 2. Effect of electron acceptors on reduction of As(V) to As(III) in anaerobic salt marsh sediment. Open symbols, As(V); solid symbols, As(III). (A) No additions; (B) plus sulfate; (C) plus phosphate (circles) or Mn(IV) (triangles); (D) plus nitrate (circles) or Fe(III) (triangles). Symbols represent the means of three slurries, and bars indicate ±1 standard deviation. The absence of bars indicates that the error was smaller than the symbols.

mM). All slurries received 2.42 μCi of sodium [2-^{14}C]acetate (specific activity, 57 mCi/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) and were incubated under anaerobic conditions as described above. After 3 weeks (salt marsh) or 4 weeks (Lahontan), the experiments were terminated by injection of 3 ml of 12 N HCl. The slurries were shaken for an additional 20 h before the headspaces were analyzed for ^{14}C-labeled gases.

Analysis. Dissolved As(V) and As(III) were analyzed by high-performance liquid chromatography (12). Sulfate and nitrate were determined by suppressed-ion chromatography (18). Gas chromatography in conjunction with gas proportional counting was used for the determination of $^{14}CO_2$ and $^{14}CH_4$ (7).

RESULTS

Sediment slurries. Sediment slurries demonstrated an ability to reduce As(V) to As(III) (Fig. 1). Slurries incubated under N_2 completely removed As(V) from solution, with ~50% recovered as As(III) by 13 days (Fig. 1A). Slurries incubated under H_2 reduced As(V) about three times faster than those incubated under N_2 (Fig. 1B). No reduction of As(V) to As(III) occurred in live slurries incubated under air (Fig. 1C), and autoclaved controls incubated under N_2 with lactate did not reduce As(V) to As(III) (Fig. 1D). Anoxic slurries incubated with formalin demonstrated a slow loss of 10 mM As(V) with time, but 2.5 mM As(V) was still present after 13 days of incubation and As(III) production was not observed (data not shown). Presumably, much of the As(V) added in the aerobic and formalin-killed controls became sorbed to Fe(III) sites, which would remain oxidized under these incubation conditions. In a second experiment, the effect of competing electron acceptors or structural analogs of arsenic upon As(V) reduction was examined (Fig. 2). The rates of reduction in the unamended samples (Fig. 2A) were similar to sulfate (Fig. 2B) and phosphate (Fig. 2C) rates; however, nitrate exerted a strong inhibitory effect (Fig. 2D). Slurries incubated with 10 mmol of MnO_2 per liter demonstrated complete removal of As(V) by 11 days of incubation, at which time only a small quantity of As(III) (~2 mM) was detected in solution (Fig. 2C). Incubation of slurries with 10 mmol of Fe (III)-nitrilotriacetic acid per liter resulted in the reduction of As(V) to

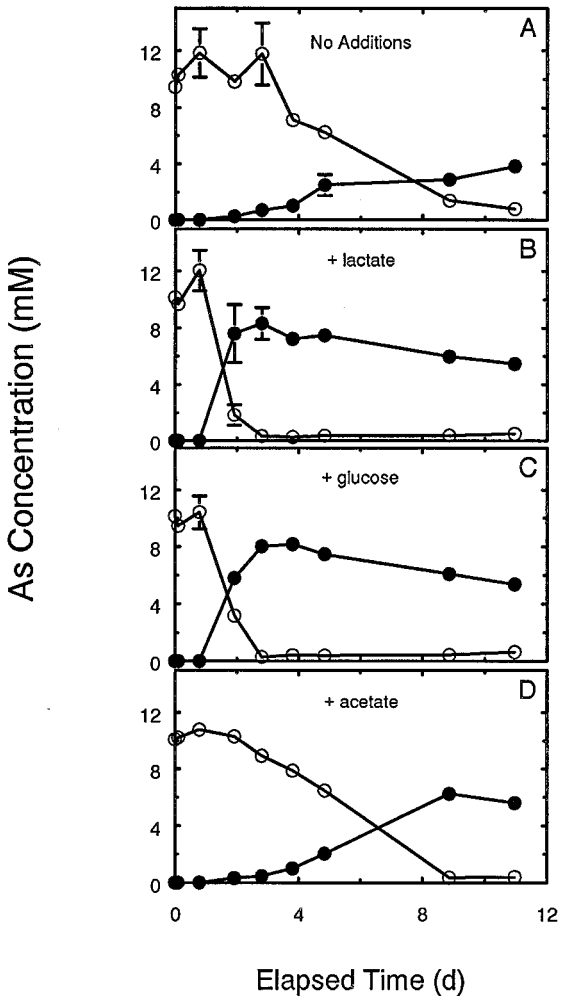

FIG. 3. Effect of electron donors on reduction of As(V) (○) to As(III) (●) in anoxic salt marsh sediments. (A) No additions; (B) plus lactate; (C) plus glucose; (D) plus acetate. Symbols represent the means of three slurries, and bars indicate ±1 standard deviation. The absence of bars indicates that the error was smaller than the symbols.

As(III); however, the arsenic species were so strongly bound that arsenic in solution never exceed 2 mM during the incubation (Fig. 2D).

The effect of soluble electron donors upon As(V) reduction is shown in Fig. 3. Reduction of As(V) to As(III) proceeded slowly in unamended samples (Fig. 3A) it was much faster in slurries incubated with lactate (Fig. 3B) or glucose (Fig. 3C) but only marginally faster with acetate (Fig. 3D). Slurries containing succinate gave results similar to the ones with acetate (results not shown). Recovery of As(III) was always higher in the substrate-amended slurries.

To reduce the necessary incubation times, we conducted all subsequent experiments with lactate-amended slurries. The respiratory inhibitors/uncouplers rotenone, dinitrophenol, HQNO, and cyanide all prevented the reduction of As(V) to As(III) for the first 42 h of incubation (Table 1). However, after this initial lag, As(V) reduction commenced but at about half the rate of that in the uninhibited samples (results not shown). In addition, whereas controls completely reduced As(V) after 4 days of incubation, inhibited slurries still contained 1 to 2 mM As(V) after 9 days of incubation (results not shown). The effect of tungstate and molybdate on As(V) reduction are shown in Fig. 4. Tungstate greatly decreased the rate of As(V) reduction, whereas molybdate had no noticeable effect.

TABLE 1. Effects of inhibitors on bacterial reduction of 10 mM As(V) to As(III) in anoxic salt marsh sediment slurries[a]

Addition	As(V) removal (% inhibition)	As(III) formation (% inhibition)
None[b]	0.0	0.0
Rotenone	68.4	83.5
Dinitrophenol	26.3	93.6
HQNO	65.7	87.2
Cyanide	ND[c]	86.2

[a] Samples were incubated for 42 h; results represent the mean of three samples.
[b] Uninhibited samples reduced 5 mM As(V) with a recovery of 4 mM As(III).
[c] ND, not detected because of coelution of As(V) with cyanide.

The effect of chloramphenicol on the capacity of sediment slurries adapted to nitrate respiration to reduce As(V) or further reduce nitrate is shown in Fig. 5. Readdition of nitrate to slurries resulted in its complete removal within a few hours, and chloramphenicol reduced the rate of nitrate removal (Fig. 5A). Nitrate-adapted slurries were able to completely reduce As(V) to As(III) over an incubation interval of a few days, and chloramphenicol totally inhibited this capacity (Fig. 5B).

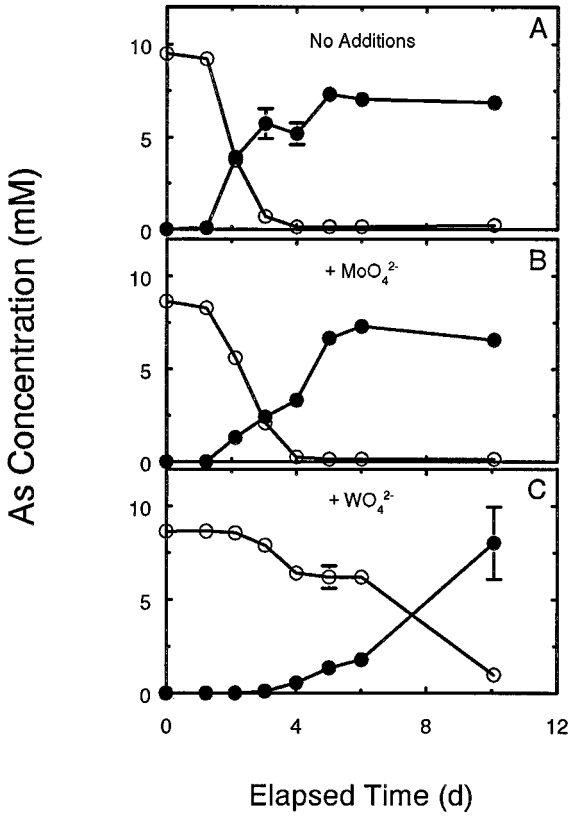

FIG. 4. Effect of molybdate and tungstate on reduction of As(V) (○) to As(III) (●) in anoxic salt marsh sediments incubated with lactate. (A) No additions; (B) plus molybdate; (C) plus tungstate. Symbols represent the means of three slurries, and bars indicate ±1 standard deviation. The absence of bars indicates that the error was smaller than the symbols.

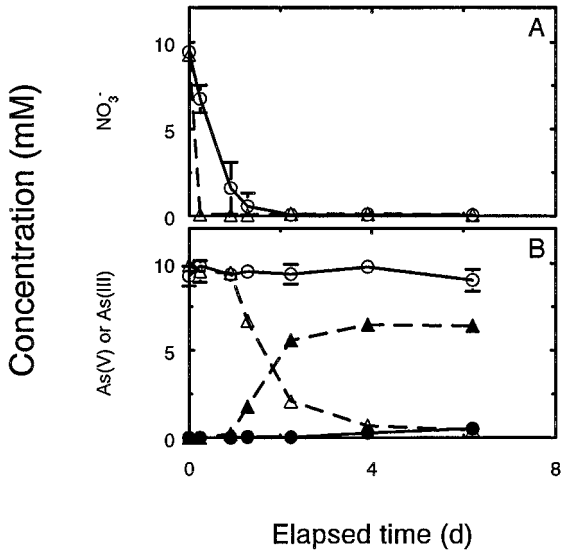

FIG. 5. Effect of chloramphenicol on reduction of nitrate and As(V) in nitrate reduction-adapted, anoxic salt marsh sediments incubated with lactate. (A) Nitrate levels in slurries without (△) and with (○) chloramphenicol. (B) As(V) (open symbols) and As(III) (solid symbols) in slurries without (triangles) and with (circles) chloramphenicol. The results are the means for three samples, and error bars indicate ±1 standard deviation. The absence of bars indicates that the error was smaller than the symbol.

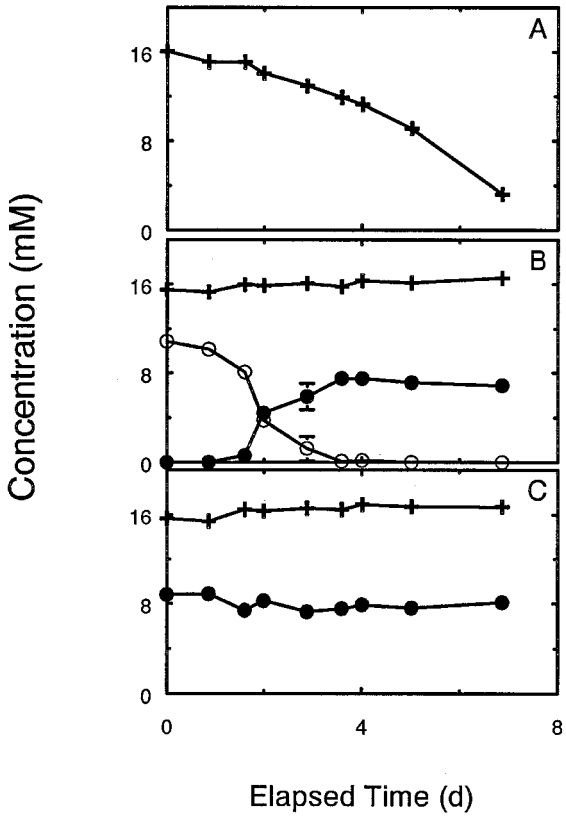

FIG. 6. Effect of As(V) and As(III) on reduction of sulfate in anoxic salt marsh sediments incubated with lactate. Symbols: +, sulfate; ○, As(V); ●, As(III). (A) Without As(V) or As(III); (B) with As(V); (C) with As(III). Results represent the means of three samples, and bars indicate ±1 standard deviation. The absence of bars indicates that the error was smaller than the symbols.

Slurries incubated with sulfate as the sole electron acceptor consumed nearly all of this anion by 7 days of incubation (Fig. 6A). However, no loss of sulfate was noted in sediments incubated with ongoing reduction of As(V) to As(III) (Fig. 6B) or in sediments incubated with only As(III) present (Fig. 6C).

Experiments with [2-^{14}C]acetate. Salt marsh sediments incubated with sulfate and [2-^{14}C]acetate metabolized ~63% of the acetate over the incubation period (Fig. 7A). About 90% of this acetate was oxidized to $^{14}CO_2$, while about 10% was reduced to $^{14}CH_4$ (Fig. 7A). Additions of 0.5 and 1.0 mM As(V) did not affect the extent of acetate degradation or the proportion of $^{14}CH_4$ and $^{14}CO_2$ formed. However, 2.5 mM As(V) totally inhibited $^{14}CH_4$ formation and caused a 90% inhibition of $^{14}CO_2$ formation. Higher levels of As(V) sustained the inhibition of $^{14}CH_4$ formation but elevated the amount of $^{14}CO_2$ formed. In slurries incubated with 10 mM As(V), 36% of the amount of acetate was oxidized to CO_2 compared with that in the controls without As(V). The production of unlabeled CO_2 and CH_4 followed the same trends as that of their radiolabeled counterparts. Freshwater sediments gave very similar results (Fig. 7B), with 112% of the acetate recovered as gaseous products in the As-free controls. There was much more methanogenic activity in the controls without As(V) than in the salt marsh sediments, and methanogenesis displayed greater sensitivity to 1 mM As(V). Exposure to 1 mM As(V) decreased $^{14}CO_2$ production by 77%, but higher As(V) levels resulted in greater oxidation of the acetate, with 10 mM As(V) samples having 85% of the oxidation of controls incubated without As(V). Production of CO_2 and CH_4 was similar to that of their radioactive counterparts.

DISCUSSION

Although much emphasis has been given to the chemical reactivity of arsenic, biological factors can also control the mobility and speciation of arsenic in nature (8). For example, the pH changes occurring in streams over a diel photosynthetic cycle affect the sorptive properties of As(V) on streambed sediments (11). The reduction of As(V) to As(III) was noted during incubation of Lake Ohakuri sediment in bacterial media; however, the biochemical mechanism(s) responsible for the reduction was unclear (10). Thus, the reports of respiratory growth of anaerobes with As(V) as an electron acceptor (1, 12) needed to be placed in the context of the significance of this phenomenon in natural systems. The reduction of As(V) to As(III) we observed in our sediment slurry incubations was caused primarily by bacterial DAsR. We will now review the evidence to justify this statement.

The biological nature of the As(V) reduction was exhibited by its elimination by autoclaving (Fig. 1D), its sensitivity to poisons like formaldehyde, and its response to respiratory inhibitors (Table 1). The absence of As(V) reduction under aerobic conditions (Fig. 1C) underscored its requirement for anoxia. Further evidence for biological involvement comes from the electron donor experiments in which H_2 (Fig. 1B), as well as lactate and glucose but not acetate or succinate, speeded As(V) reduction (Fig. 3). This suggests that a degree of substrate specificity exists within the bacterial flora of these sediments, a situation which also occurred during dissimilatory reduction of Se(VI) (19). The recovery of As(III) improved with addition of electron donors (Fig. 2). This suggests that sufficient substrate was available for reduction of ferric hydroxides, thereby eliminating this sorptive site for As(V) and making it available for bacterial reduction (12, 13). Finally, the coupling of acetate oxidation to the abundance of As(V) (Fig.

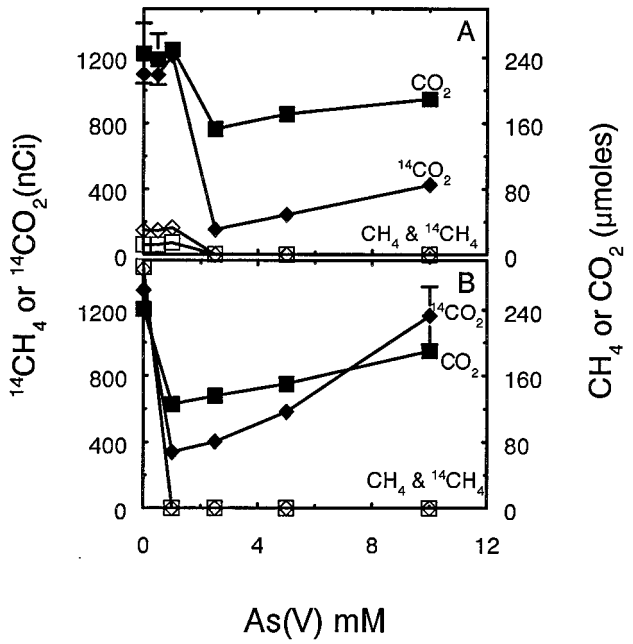

FIG. 7. Effect of As(V) on the metabolism of [2-^{14}C]acetate in anoxic salt marsh sediments incubated for 2 weeks (A) and freshwater sediments incubated for 3 weeks (B). Symbols: ■, CO_2; ◆, $^{14}CO_2$; □, CH_4; ◇, $^{14}CH_4$. Symbols represent the means of three slurries, and bars indicate ±1 standard deviation. The absence of bars indicates that the error was smaller than the symbols.

7) proves that As(V) can act as a suitable electron acceptor for the bacterial mineralization of organic matter. This phenomenon was also shown for Se(VI) reduction (19). The inability of acetate to stimulate As(V) reduction (Fig. 3) while clearly showing respiratory coupling to As(V) (Fig. 7) is not inconsistent. The energy yield of acetate oxidation by As(V) is less than that for lactate; hence, bacteria can use acetate to reduce As(V), but acetate will not speed reduction over endogenous substrates as does lactate. The fact that we have now demonstrated this to occur in marine (Fig. 7A) and freshwater (Fig. 7B) systems makes a case that DAsR is a widespread phenomenon in nature.

Addition of other potential electron acceptors or of competing group V analogs (e.g., phosphate and nitrate) should result in an inhibitory effect upon DAsR if these anions are preferentially reduced or taken up by bacteria over As(V). The chemical similarities between phosphate and arsenate result in their competition for sorptive sites in sediments (26, 29) and for uptake by bacteria (9). However, neither phosphate nor sulfate additions influenced the rate of DAsR (Fig. 2A to C), indicating that sulfate is not a competing electron acceptor and that phosphate does not serve as an analog of arsenate in this system. Nitrate, however, did have a pronounced inhibitory effect (Fig. 2D).

Complete removal of nitrate from the slurries was required before As(V) reduction could proceed, and chloramphenicol effectively blocked DAsR in nitrate-adapted sediments (Fig. 5). Therefore, nitrate reduction was carried out by a different enzyme system from that for DAsR, and the enzymes for DAsR required a de novo synthesis. This observation is somewhat at odds with the finding that DAsR activity was constitutive in nitrate-grown and selenate-grown cells of strain SES-3 (12). Nonetheless, because all slurry incubations exhibited lag periods before DAsR was noticeable (e.g., Fig. 1 and 5), an induction period for As(V) reductase synthesis was required. Nitrate acted as a preferred electron acceptor for dissimilatory reduction over As(V) rather than as a structural group V analog. This can be argued simply on the basis of standard potentials, because the E_0' of the nitrate/nitrite couple is +430 mV (32) whereas that for As(V)/As(III) is only +246 mV (30). Thermodynamic calculations and the observation of better molar growth yields when strain SES-3 uses nitrate as opposed to arsenate as an electron acceptor support this conclusion (12, 24).

Tungstate caused a prolonged period of inhibition of DAsR, whereas molybdate had no effect (Fig. 4). Although both of these Group VI A oxyanions inhibit sulfate reduction (17), the ineffectiveness of molybdate argues against the involvement of sulfate-respiring bacteria. Similarly, tungstate but not molybdate blocks selenate reduction in these sediments (19). Tungstate blocks the molybdenum-containing dissimilatory nitrate reductases of bacteria (6, 27), and therefore the enzyme(s) responsible for DAsR in these sediments probably contains molybdenum. In the case of Fe^{3+} or Mn^{4+} as potential competing electron acceptors for DAsR, most (~80%) of the added As(V) remained sorbed to the Fe^{3+}-nitrilotriacetic acid complex or as As(III) bound to Mn^{2+} as a consequence of bacterial reduction of the Mn^{4+}. It has been suggested that the solubility of arsenic in anoxic, manganese-rich soils is controlled by the $Mn_3(AsO_4)_2$ phase (16).

In some experiments, there was a discrepancy between the amount of As(V) added to the slurries (10 mM) and the quantity detected in solution (8 mM) before the onset of DAsR (Fig. 1). In addition, the recovery of As(III) was often less than the amount of As(V) added (Fig. 1 and 2), although recovery was better in sediments amended with soluble electron donors (Fig. 2). Because these sediments can contain 1 to 2 mM free sulfide, it is likely that some chemical reduction of As(V) and the formation of an As_2S_3 precipitate accounted for these imbalances. However, bacterial sulfide production during the course of these incubations did not occur, because both As(V) and As(III) completely inhibited sulfate reduction (Fig. 7). The use of sulfate-respiring bacteria to immobilize As(III) as As_2S_3 in anoxic sediments has been proposed as a means of passive bioremediation (28). The concentration of As species appears to be a critical factor, since Rittle et al. (28) used 1.3 mM As(III) and sulfate reduction proceeded whereas we used 8 to 10 mM As(V) or As(III) and achieved full inhibition. Low concentrations of As(V) (≤1 mM) did not disrupt the metabolism of [^{14}C]acetate in the salt marsh sediments (Fig. 7A), although 1 mM As(V) strongly inhibited freshwater sediments (Fig. 7B). Presumably, the oxidation of acetate in the salt marsh system was linked mainly to sulfate reduction whereas in the freshwater sediments it was linked to sulfate reduction and methanogenesis. Sulfate reduction occurs in the anoxic water column and sediments of Mono Lake, Calif. (20, 21), an environment with ~0.3 mM As (2, 14). It appears that the inhibitory threshold of the two arsenic species on sulfate reduction probably occurs at the low millimolar level, but the precise number is likely to prove variable in differing environments. Arsenic inhibits denitrification in subsurface aquifer sediments (3), and we noted a clear inhibition of methanogenesis upon incubation of sediments with As(V) (Fig. 7). Therefore, As(V) and/or its reduction product, As(III), is potentially capable of inhibiting three ecologically important anaerobic respiratory processes: denitrification, sulfate reduction, and methanogenesis.

The results we obtained with sediments indicate that no quantitatively significant reduction of arsenic occurs beyond the As(III) species. For the purpose of bioremediation, however, the As(III) species is desirable, because it is more mobile

(although more toxic) than the As(V) state (8) and therefore contaminated soils can be "treated" and the As(III) can be removed. Our results suggest that because the reduction of As(V) to As(III) is a bacterial respiratory process, provision of a suitable electron donor to contaminated, anoxic soils should greatly speed this reduction and increase the recovery of As(III). However, the complexation of As(V) with iron hydroxides or with a manganous phase or the presence of an abundance of nitrate could pose barriers to the quantitative reduction of As(V) to As(III) in these systems. It is possible that sustained provision of an electron donor to such systems can drive them toward complete reduction of all the oxidized chemical species, thereby resulting in an enhanced release of As(III).

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