Microbial Respiration and Precipitation of Arsenic

by

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Abstract

This dissertation explores the biogeochemistry, physiology, molecular biology, and environmental impact of dissimilatory arsenate reducing bacteria. A new species, *Desulfotomaculum auripigmentum* strain OREX-4, was found to precipitate amorphous arsenic trisulfide (As_2S_3) both intra- and extracellularly. Intracellular precipitation of As_2S_3 nucleates precipitation in the bulk milieu, and results from the reduction of arsenate and sulfate during respiration. Strain OREX-4 differs from the previously described arsenate respiring isolates strains MIT-13 (*Geospirillum arsenophilus*) and SES-3 (*Geospirillum barnesii*): strain OREX-4 grows on different substrates, and falls within the gram-positive group of the Bacteria whereas MIT-13 and SES-3 fall together in the epsilon subdivision of the Proteobacteria. Strain OREX-4 prefers arsenate to sulfate, and can grow on the arsenate-bearing mineral scorodite. Dissimilatory arsenate reductase, the enzyme responsible for growth on arsenate, was characterized and purified from strain SES-3. This enzyme is membrane-bound, unlike the arsenate reductases of *Escherichia coli* and *Staphylococcus aureus*, which are cytoplasmic and used for reductive detoxification of arsenate but not for growth. Enzyme activity in washed cell suspensions exhibited a V_{max} for arsenate reduction of approximately 0.1 μmol/min/mg and an apparent K_m of 200 μM arsenate. Evidence for the presence of a *b*-type cytochrome in cells grown on arsenate was found. NADH can serve as an electron donor for the dissimilatory arsenate reductase, and Fe-S clusters appear to be prosthetic groups in the enzyme. These studies suggest a novel mechanism of arsenate reduction. The ability of bacteria indiginous to arsenic-polluted soil to reduce arsenate was also examined. Microbial arsenate reduction was stimulated in the presence of organic carbon and microaerophilic conditions. Nitrate did not inhibit arsenate reduction, yet the formation of $Fe(OH)_3$ significantly retarded arsenate reduction as long as iron(III) remained oxidized. These results contrast with the behavior of arsenate-reducing consortia in previous studies. It is now apparent that dissimilatory arsenate reduction is widespread and readily performed by diverse bacteria from site to site. The ability of microbes to moblize arsenic through reduction is cause for concern.

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Acknowledgments

Creating this dissertation has been something akin to reliving the Odyssey in a modern (and far less picturesque) laboratory setting. Although I was fortunate to find my way somewhat faster than Odysseus, in my time, I have experienced my share of divine intervention, Scyllas and Charybdises, Circean-like distractions, and the kindness of people as generous as the Phaeacians, which together, make the analogy seem appropriate.

Many people have influenced me during this journey, but none more so than my primary advisor, François Morel, and it is to him that I am most grateful. Over the years, François, like Athena, has been my best advocate and mentor: there when I needed him, yet wise enough to maintain the distance necessary for my professional development; arranging opportunities for me from afar; teaching me about what it means to be a rigorous scientist, a clear and creative thinker, and a good writer; reminding me that patience is a necessary component of scientific maturity; encouraging me to pursue my interests even if they increasingly took me outside his area of expertise; and setting an example of generosity: both intellectual and personal. Without François' belief in me and my ability to learn, I might still be at sea, or at least, trying vainly to study the hydrodynamics thereof.

My gratitude also extends to Terry Beveridge, who, from the start, has been entirely supportive of my efforts and ambitions. Thanks to his guidance (not to mention his tolerance of my endless emails) I have become a better microbiologist. Terry has been a role model for me in many ways: he, like François, has been a true mentor, and has inspired me by his scientific passion, insightfulness, and humility.

Several others have helped me develop professionally: Dianne Ahmann got me started by handing me a yellow bottle and teaching me how to grow things in it; John Coates became my most reliable microbiology consultant, teaching me all I know about bacterial characterization, and opening his lab to me or answering a question over the phone at a moment's notice; Debbie Ellis and Stephen Tay shared their expertise in phylogeny and photography; Derek Lovley, Harry Hemond and Phil Gschwend asked tough questions which forced me to think more deeply about my experiments; Ron Oremland facilitated my venture into the world of biochemistry by introducing me to John Stolz, in whose laboratory I pursued it; Todd Lane patiently taught me the fundamentals of molecular biology; and Erin Kennedy and Josh Sharp helped me realize that one of the best ways to learn something is to teach it.

I would not have been able to successfully complete (much less begin) this dissertation without the emotional support I have received from my friends and family. At various times and in various places, these people came through for me in critical moments, shared the mundane and exciting details of my day, made me laugh, cooked me meals, loaned me their computers, opened up their homes, paid me visits, asked me to visit them, helped me keep my perspective and sense of humor, danced with me in the lab or outside of it, helped me pack, helped me move, called to check in on me, sent me encouraging messages, tolerated my mood swings, kept me company on runs, encouraged me to try my best and not to be afraid to fail, maintained their faith in me even when mine was low, and most of all, gave me the confidence that no matter what I did professionally, they would love me all the same. Out of a desire to be inclusive, and in the interests of space, I will not list these people by name. I trust that those I am referring to will know I speak of them, and accept my most sincere gratitude.

Two people, however, deserve explicit mention here, and they are my mother and father. It is to them that I dedicate this thesis, for their gifts are ones I shall carry with me throughout my life, and are ultimately responsible for who I have been, am, and will become.

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Chapter 1: Introduction

This thesis explores the subject of dissimilatory arsenate reduction by bacteria from the perspectives of biogeochemistry, physiology, molecular biology, and environmental engineering. Chapter 2 serves as a brief review of microbial arsenate reduction, where highlights of the thesis are presented in the context of previous work by other authors. The principal findings are explored in detail in the subsequent chapters: Chapter 3 focuses on the ability of a gram positive bacterium, *Desulfotomaculum auripigmentum* strain OREX-4, to precipitate amorphous arsenic trisulfide (As_2S_3); Chapter 4 explores how arsenate reduction and the consequent precipitation of As_2S_3 are linked to respiration; Chapter 5 considers how such respiration is accomplished at the molecular level in the gram negative arsenate-reducing bacterium, strain SES-3; and Chapter 6 discusses the relevance of arsenate reduction by bacterial consortia in arsenic-contaminated sites.

The geomicrobiology chapter (Chapter 3) describes how a bacterium isolated from the Upper Mystic Lake in Winchester, MA, precipitates amorphous As_2S_3. This chapter has been published as "Precipitation of Arsenic Trisulfide by *Desulfotomaculum auripigmentum*" in the journal *Applied and Environmental Microbiology* (vol. 63(5):2022-2028, May 1997). Precipitation of As_2S_3 by *Desulfotomaculum auripigmentum* strain OREX-4 results from its reduction of As(V) to As(III) and S(VI) to S(-II). At the As(III) concentration range of interest (0.1 - 1 mM), the stability of As_2S_3 is highly sensitive to pH and [S(-II)]. Thus, the relative rates at which *D. auripigmentum* reduces As(V) and S(VI) are critical to its formation of As_2S_3. Other As(V) or S(VI)-reducing bacteria are unable to precipitate As_2S_3 either due to their inability to reduce both As(V) and S(VI) or because they reduce S(VI) too rapidly. Electron microscopy of thin sections showed that the precipitate forms both intra- and extracellularly. Microbial As_2S_3 formation nucleates precipitation of the mineral in the bulk milieu whereas heat-killed cells alone do not serve as templates for its formation. Precipitation of As_2S_3 by *D. auripigmentum* suggests that As_2S_3 formation may be important in the biogeochemical cycle of arsenic.

The physiological basis of As_2S_3 precipitation is examined in Chapter 4, which will appear as "Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov." in *Archives of Microbiology*

(volume 168(5):380-388, November 1997). Strain OREX-4, a gram-positive bacterium with a hexagonal S-layer on its cell wall, differs from the previously described arsenate reducing isolates strains MIT-13 and SES-3 which are both gram negative. MIT-13 and SES-3 grow on nitrate but not on sulfate, whereas strain OREX-4 does not respire nitrate but grows on lactate with either arsenate or sulfate serving as the electron acceptor, and even prefers arsenate. Strain OREX-4 metabolizes compounds commonly used by sulfate reducers, and both arsenate and sulfate reduction are inhibited by molybdate. Scorodite [$(FeAsO_4)◊2H_2O$], an arsenate-containing mineral, provides micromolar concentrations of arsenate which support cell growth. Physiologically and phylogenetically, strain OREX-4 is far removed from strains MIT-13 and SES-3: OREX-4 grows on different electron donors and electron acceptors, and falls within the gram-positive group of the Bacteria whereas MIT-13 and SES-3 fall together in the epsilon subdivision of the Proteobacteria. Together, these results suggest that organisms spread among diverse bacterial phyla can use arsenate as a terminal electron acceptor, and that dissimilatory arsenate reduction might occur in the sulfidogenic zone at arsenate concentrations of environmental interest. 16S rRNA sequence analysis indicates that strain OREX-4 is a new species of the genus *Desulfotomaculum*, and accordingly, the name *Desulfotomaculum auripigmentum* is proposed.

The biochemistry of dissimilatory arsenate reduction is the subject of Chapter 5. It is intended to be submitted for publication in the *Proceedings of the National Academy of Sciences* as "Characterization and purification of dissimilatory arsenate reductase from strain SES-3." In this study, dissimilatory arsenate reductase, the enzyme responsible for growth on arsenate, has been characterized and purified from strain SES-3. An activity assay was devised based on the coupling of arsenate reduction to the oxidation of methyl-viologen (MV). Dissimilatory arsenate reductase was determined to be membrane-bound, unlike the arsenate reductases of *Escherichia coli* and *Staphylococcus aureus* (ArsC enzymes), which are cytoplasmic and used for reductive detoxification of arsenate but not for growth. Dissimilatory arsenate reductase comprises three highly hydrophobic polypeptides with molecular weights of 65, 31, and 22 kDa, in equimolar proportion. Enzyme activity in washed cell suspensions exhibited a V_{max} for arsenate reduction of approximately 0.1 μmol/min/mg at 25 °C and pH 7.2

and an apparent K_m of 200 μM arsenate. Arsenate-coupled MV oxidation was inhibited by arsenite, phosphate, molybdate and nitrate. Fumarate exhibited strong MV oxidizing activity in arsenate-grown cells, yet other alternative electron acceptors used by strain SES-3 showed only weak activity. Evidence for the presence of a *b*-type cytochrome in cells grown on arsenate was found. No significant cross-reaction with the antibody to *E. coli*'s ArsC was observed. NADH can serve as an electron donor for the arsenate reductase, and Fe-S clusters appear to be prosthetic groups in the enzyme. This suggests a novel mechanism of arsenate reduction.

The relevance of microbial arsenate reduction in arsenic-polluted sites is considered in Chapter 6. The work described in this chapter is intended to be submitted as a short note to *Environmental Science and Technology* as "The ability of bacterial consortia from arsenic-contaminated soils to mobilize arsenate." Microbial respiration of arsenate is now known to occur in a variety of environments by diverse populations of bacteria. Furthermore, dissimilatory arsenate reduction has been shown to catalyze rapid dissolution of arsenic from iron arsenate, a solid-phase surrogate for sedimentary arsenic. Here, the capability of an indigenous population of bacteria to reduce and mobilize arsenate from arsenic-contaminated soil is examined. Soil bacteria were grown in mineral medium supplemented with organic substrates and a high concentration of sodium arsenate. Arsenate-reduction in the resulting enrichment culture was measured with varying carbon sources and O_2 concentrations. Glucose and microaerophilic conditions appeared to stimulate arsenate-reduction. Potential alternative electron acceptors were examined for their ability to inhibit arsenate reduction. Nitrate did not affect arsenate reduction, sulfate and MnO_2 slightly retarded it, whereas ferric iron significantly delayed arsenate reduction. The ability of the enrichment culture to remove arsenic from contaminated soil was also examined. These results suggest indigenous bacteria have the ability to mobilize arsenate under a variety of conditions, which may be particularly important in sites that are both contaminated with arsenic and rich in organic matter.

As will be seen, many more questions have been raised than have been answered by this thesis. Among the more intriguing are: how have microbes evolved the ability to respire arsenate? How old is this process? Could the enzymatic machinery of arsenate respiration have sprung from that associated with the respiration of other electron acceptors, such as fumarate?

What is the evolutionary relationship between genes for dissimilatory arsenate-reduction in environmental isolates and genes for reductive detoxification in clinical isolates? How are these genes spread from one population to another? What are the selective pressures? How is arsenate respiration and resistance related to that of other toxic chemicals? Are arsenate reducing bacteria present and active in arsenic-contaminated sites or in environments with naturally high concentrations of arsenic, such as hot springs? Do they dominate these sites? Could probing the environment for molecular signatures of arsenate-reduction allow us to determine how important microbial activity is for arsenic's geochemical cycle? Might the isotopic signature of biogenic As_2S_3--or any other arsenic containing mineral, for that matter--enable us to identify mineral deposits where microbes have been active? And finally, how should we apply our understanding of microbial arsenate reduction to remediate arsenic-contaminated sites?

It is my hope that this thesis will inspire others to pursue these questions and ask different ones, and provide guidance to help them find the answers.

Chapter 2: A brief review of dissimilatory arsenate reduction

to be submitted to the *Geomicrobiology Journal*
by

Dianne K. Newman, Dianne Ahmann, and François M.M. Morel

Abstract

In this review, we summarize some of the important recent findings relating to dissimilatory arsenate reduction by bacteria. A brief discussion of freshwater arsenic cycling is provided, with attention placed on the microbial contributions to this cycle. The basic evidence for dissimilatory arsenate reduction is presented for studies with both consortia and isolates, followed by a summary of the physiology and phylogeny of four arsenate-reducing bacteria: *Chrysiogenes arsenatis* strain BAL-1^T, *Desulfotomaculum auripigmentum* strain OREX-4, strain MIT-13, and strain SES-3. Drawing on enzymatic studies of the dissimilatory arsenate reductase from strain SES-3, a preliminary biochemical model for growth on arsenate is proposed. We conclude with a discussion of the importance of dissimilatory arsenate reduction in the environment.

Background

Arsenic is ubiquitous, found at trace levels in the earth's atmosphere, waters, soils, and organisms. In the earth's crust, arsenic is present at levels from 0.1 to several hundred ppm, and is highest in marine shale materials, magmatic sulfides, and iron ores where it occurs as arsenopyrite (FeAsS), realgar (AsS), and orpiment (As_2S_3) (Bhumbla & Keefer, 1994). Arsenic is naturally introduced to aquatic systems by weathering of arsenic-containing minerals and enters the atmosphere through volcanic emissions, wind erosion, sea spray, forest fires, and biological formation of volatile arsenicals (Cullen & Reimer, 1989). Since the industrial revolution, arsenic has been distributed over the earth through mining activities, dispersed into the air during ore smelting, discharged into waterways as an unwanted byproduct of sulfuric acid manufacturing, and sprayed directly onto soils as a pesticide (Bhumbla & Keefer, 1994). Anthropogenic emissions of arsenic during the past century have been estimated as high as 28,000 metric tons per year, nearly four times the 7800 metric tons emitted each year from natural sources (Nriagu & Pacyna, 1988). Industrial emissions have concentrated arsenic to dangerous levels (thousands of ppm) near many centers of human activity (Bhumbla & Keefer, 1994). As arsenic inputs into the biosphere accelerate, biological interactions with arsenic are assuming increasing importance.

Arsenic is a group V element in the periodic table, isoelectronic with nitrogen and phosphorus. The stable oxidation states of arsenic are +5, +3, 0, and -3, corresponding to the inorganic compounds arsenate, arsenite, arsenic metal, and arsine gas, respectively. The main chemical forms of these compounds in aqueous solution at pH 7.0 are $HAsO_4^{2-}$ and $H_2AsO_4^-$, H_3AsO_3, As, and AsH_3, respectively. Under oxidizing conditions (0.2-0.5 V), both anionic arsenate species occur in appreciable proportions, whereas the uncharged arsenite species dominates in reducing environments (0-0.1V) (Mok & Wai, 1994). Arsenic metal occurs rarely, and arsine is stable only under highly reducing conditions. Methylated forms such as monomethylarsonic acid [MMAA(V)], dimethylarsinic acid [DMAA(V)], monomethylarsonous acid [MMAA(III)] and dimethylarsinous acid [DMAA(III)] can be synthesized by bacteria and phytoplankton (Cullen & Reimer, 1989), although their importance varies from one environment to another (Anderson & Bruland, 1991; Kuhn & Sigg, 1993; Sohrin et al. 1997); arsenobetaine, arsenosugars, and other complex organic compounds are

formed by lobsters, fish, and phytoplankton, but contribute little to arsenic speciation in natural waters (Cullen & Reimer, 1989).

Freshwater arsenic cycling

The arsenic cycle in marine waters has been examined by Andreae (Andreae, 1979). Here, we restrict our discussion to freshwaters. For a fuller examination of this topic, we refer the reader to reviews by Ferguson and Gavis (1972) and Mok and Wai (1994). Once arsenic enters an aquatic body, it may enter the sediments or cycle in surface waters (Figure 1). At near-neutral pH, arsenate adsorbs onto hydrous iron, manganese, and aluminum oxides, although adsorption onto iron oxides (FeO_x) predominates (Mok & Wai, 1994). Coprecipitation with FeO_x may also occur, leading to the formation of the mineral scorodite ($FeAsO_4 \cdot 2H_2O$) (Mok & Wai, 1994). Arsenic removal by ferric iron is optimal when the pH is below 7.2 to 7.5 (Sorg & Logsdon, 1978). It is thought that such adsorption and co-precipitation bind aqueous arsenate to colloids and particles that sink from the water column to the sediments, consistent with observations that arsenic concentrations in sediments are usually much higher than in the overlying water (Cullen & Reimer, 1989). Arsenite also adsorbs onto metal oxides under laboratory conditions (Manning & Goldberg, 1997) but is observed to be much more mobile than arsenate under field conditions (Gulens & Champ, 1979). Synthetic birnessite (∂-MnO_2) has been shown to oxidize arsenite directly with the release of arsenate and Mn(II), with maximal rates of adsorption and release occuring at pH 4 (Scott & Morgan, 1995). Recently, clays such as kaolinite and illite have been shown to adsorb and enhance the oxidation of arsenite to arsenate, with arsenite adsorption maximized between pH 7.5 and pH 9.5 (Manning & Goldberg, 1997). Humic acids can also retain arsenic in acidic environments (Mok & Wai, 1994).

Arsenic remains adsorbed to FeO_x as long as the sedimentary environment is sufficiently oxidized. High inputs of organic carbon to the sediments, however, usually result in oxygen depletion and lowering of redox potentials, with the lowest potentials present in the deepest layers (Zehnder & Stumm, 1988). Reduction of sedimentary $Fe(III)O_x$ solubilizes Fe(II) and releases adsorbed arsenic into porewaters (Aggett & O'Brien, 1985). Dissimilatory Fe(III)-reducing microorganisms might contribute to this release, as they are thought to be major catalysts of $Fe(III)O_x$ reduction in

anoxic sediments (Lovley, 1993). Porewater arsenic that diffuses upward into oxidizing zones may reassociate with FeO_x and reprecipitate (Belzile & Tessier, 1990), and internal arsenic cycling in sediments with sufficiently oxidized surface zones frequently results in the accumulation of arsenic just below the surface sediment layers (Cullen & Reimer, 1989). Desorbed arsenate that diffuses downward into sulfate-reducing zones may be reduced to arsenite and, under acidic conditions, precipitate as the arsenic sulfides As_2S_3 or AsS (Moore et al., 1988). Diagenetic sulfides control the distribution of arsenic in reduced, sulfidic sediments, but if physical disturbance moves the sediments to an oxidizing environment, the arsenic will be re-released into the water column. Chemolithotrophic sulfur-oxidizing bacteria may also assist this process and enhance the mobilization of arsenic (Moore et al., 1988).

Microbial interactions with arsenic

Apart from these chemical and biological factors that indirectly affect arsenic's mobility in natural waters, microorganisms can transform arsenic directly. As early as 1918, bacteria from cattle-dipping tanks were reported to both oxidize and reduce arsenic (Green, 1918). Biochemical work on an arsenite-oxidizing soil strain of *Alcaligenes faecalis* suggested that arsenite oxidation may be coupled to energy generation (Osborn & Ehrlich, 1976), and *Pseudomonas arsenitoxidans* was shown to grow on arsenite by using it as the sole source of energy in chemoautotrophic metabolism (Ilyaletdinov & Abdrashitova, 1981). In addition to aqueous arsenite, *P. arsenitoxidans* can attack the arsenic in arsenopyrite, and bacteria such as *Thiobacillus ferrooxidans* can oxidize arsenopyrite, orpiment, and enargite (Cu_5AsS_4) (Ilyaletdinov & Abdrashitova, 1981; Ehrlich, 1963; Ehrlich, 1964). The reverse process, microbial reduction of arsenate, has been described for a variety of organisms. Cell extracts of *Micrococcus lactilyticus* and whole cells of *M. aerogenes* are known to reduce arsenate to arsenite with H_2 as a reductant (Woolfok & Whiteley, 1962), various *Pseudomonas* sp. and *Alcaligenes* sp. reduce arsenate and arsenite to arsine (Cheng & Focht, 1979), and whole cells and cell extracts of *Methanobacterium* strain M.O.H. can produce dimethylarsine from arsenate (McBride & Wolfe, 1971). The capacity of these bacteria to reduce arsenate, however, has not been linked to growth.

If biologically available phosphate is scarce, or present in concentrations comparable to that of arsenate, arsenate will interfere with

microbial phosphate uptake and metabolism (Willsky & Malamy, 1980). Bacteria can alleviate this competition in two ways: by inducing a more specific phosphate uptake system (Pst system), or by inducing a system for arsenate detoxification (Rosen et al., 1994). The only known means of microbial arsenate detoxification involves reduction of arsenate to arsenite, followed by arsenite expulsion (Rosen et al., 1994). At this time, the minimum arsenate and/or phosphate concentrations necessary for induction have not been established, and so the quantitative significance of detoxifying reduction to arsenic cycling cannot be estimated. Nevertheless, a recent study of arsenic speciation in the Upper Mystic Lake in Winchester, MA showed that bacterial arsenate reduction may contribute as much, or more, than phytoplankton activity to the production of epilimnetic arsenite. Size fractionation of arsenate-reducing lake water showed that significant reducing activity resided in particles less than 1.0 μm in size; in addition, treatment of lake water with antibiotics diminished reduction capacity by approximately 60%, while the photosynthetic inhibitor DCMU had little effect (Spliethoff, 1995). A freshwater bacterial assemblage associated with *Anabaena oscillaroides* from the Waikato River in New Zealand demonstrated similar arsenate-reducing activity (Cullen & Reimer, 1989).

In addition to reductive detoxification which may occur in both aerobic and anaerobic zones, dissimilatory arsenate reduction (i.e., coupled to energy generation) may also contribute to arsenate reduction in anaerobic sediments (Dowdle et al, 1996). As oxic sediments become anoxic, microbial communities exploiting energy generation from particular substrates rise and fall in a predictable sequence, with the most energetically favorable metabolisms preceding the less favorable ones (Zehnder & Stumm, 1988). Thermodynamic considerations dictate that aerobic respiration will dominate as long as molecular oxygen is available, to be succeeded by denitrification, dissimilatory manganese reduction, dissimilatory nitrate reduction to ammonia, ferric iron reduction, sulfate reduction, and methanogenesis. If we assume molecular hydrogen is used as the electron donor, the free energy generated by arsenate respiration is expected to fall below that available by nitrate, Mn(IV), and Fe(III) respirations, indicating that dissimilatory arsenate reduction may occur only after these stronger oxidants have been depleted (Table 1). The remainder of this review explores our current understanding of this process.

The Basic Evidence for Dissimilatory Arsenate Reduction

Efforts to isolate dissimilatory arsenate-reducing organisms have been made from both pristine and arsenic-contaminated environments in the United States and Australia (Ahmann et al., 1994; Laverman et al., 1995; Macy et al. 1996; Newman et al., 1997). These bacteria have been successfully enriched in anaerobic minimal medium supplemented with arsenate and organic carbon sources. Generally, once primary enrichments reach the exponential stage of growth, they can rapidly reduce several millimoles per liter of arsenate in about a day if they are provided with a strong reductant such as glucose, but take significantly longer if acetate serves as the reductant (Fig. 2) (Dowdle et al., 1997; Newman et al., *submitted*). Nevertheless, the ability of these cultures to grow and completely reduce arsenate to arsenite in the presence of acetate (a non-fermentable substrate) indicates that such arsenate reduction is dissimilatory. Electron micrographs taken of bacteria from these cultures suggest they have a tendency to group together in a biofilm matrix, which in some cases may contain arsenic-bearing solids such as amorphous arsenic trisulfide (Fig. 3). While strict anaerobic conditions are normally used to isolate dissimilatory arsenate reducers, recent evidence suggests these organisms can tolerate small amounts of oxygen, and at least in consortia, even appear to be stimulated by microaerophilic conditions (Newman et al. *submitted*).

Bacteria from pristine freshwater and saline environments have also been shown to reduce arsenate to arsenite in sediment slurries (Dowdle et al., 1996). The arsenate reduction occurring in these slurries was demonstrated to be dissimilatory, as the respiratory inhibitors/uncouplers dinitrophenol, rotenone, or 2-heptyl-4-hydroxyquinoline *N*-oxide blocked arsenate reduction. In addition, acetate was observed to be oxidized to CO_2 with the concomitant reduction of arsenate to arsenite (Dowdle et al., 1996). Other evidence for dissimilatory arsenate reduction has been provided from studies with isolates that show growth is proportional to the concentration of arsenate in the medium (Ahmann et al., 1994; Newman et al. 1997). While this appears to be generally true up to arsenate concentrations of 5 mM, as reduction proceeds and arsenite concentrations rise, a toxic effect begins to slow growth in some cases (Laverman et al., 1995). Finally, the demonstration that arsenate reduction stoichiometrically follows the

oxidation of lactate in pure culture, provides further evidence that arsenate reduction by these bacteria is dissimilatory (Laverman et al., 1995; Newman et al., 1997). Rates of arsenic reduction by arsenate-respiring bacteria have been measured at 2×10^{-7} to 5×10^{-7} μmol As/cell/day in both strain MIT-13 (Ahmann et al., 1994) and in strain SES-3 (Laverman et al., 1995).

Physiology and Phylogeny of Four Arsenate-Reducers

Four dissimilatory arsenate-reducing organisms have now been isolated and characterized: strain MIT-13 from arsenic-contaminated sediments near the Industri-Plex Site, a Superfund site in Woburn, MA (Ahmann et al., 1994), strain SES-3 from the Massie Slough marsh in western Nevada (Laverman et al., 1995), *Desulfotomaculum auripigmentum* strain OREX-4 from the surface sediments of the Upper Mystic Lake in Winchester, MA (Newman et al., 1997), and *Chrysiogenes arsenatis* strain BAL-1^T from a reed bed at the Ballarat Goldfields in Australia (Macy et al, 1996). Although these organisms share the capacity to grow on arsenate, they are physiologically (Table 2) and phylogenetically very different from one another. For example, *D. auripigmentum* belongs to the gram positive group of bacteria, whereas strains MIT-13 and SES-3 belong to the epsilon subdivision of the Proteobacteria (Newman et al., 1997). Notably, the only other electron acceptor which the organisms appear to have in common is fumarate. Studies have shown that strain MIT-13 and strain SES-3 respire nitrate and arsenate but not sulfate (Ahmann, 1996; Laverman et al., 1995), whereas *D. auripigmentum* cannot grow on nitrate, yet prefers arsenate to sulfate (Newman et al., 1997). *D. auripigmentum* is the only isolate capable of precipitating As_2S_3 when grown on arsenate and sulfate (Fig. 4) (Newman et al., 1997), although strain SES-3 can effect similar precipitation when grown on arsenate and thiosulfate (unpublished results). *Wolinella succinogenes*, a close relative of strain MIT-13 and strain SES-3, is also known to use arsenate as a terminal electron acceptor (Woodward & Lovley, unpublished results). Recently, two gram-positive alkaliphilic moderate halophiles from Mono Lake in eastern CA, MLS-10 and E1-H, have been found to grow on arsenate and are currently being characterized (Oremland, *pers. comm.*). Preliminary results indicate that E1-H also grows by the reduction of selenate to selenite, and MLS-10 by the reduction of selenite to elemental selenium (Oremland, *pers. comm.*).

It is now clear that dissimilatory arsenate reduction is widespread and performed by diverse bacteria (Newman et al., *submitted*). The next question then becomes: how important is their activity in the environment? As 16S rRNA data for the current group of arsenate-reducing isolates seem to indicate, there is no signature oligonucleotide sequence that defines this group (i.e. dissimilatory arsenate-reducing organisms, like the Fe(III)-reducing organisms (Lonergan et al., 1996), do not form a tight cluster as do the nitrifying bacteria (Ward, 1996)). Accordingly, it seems that molecular probes designed against the genes that encode the enzymes that are involved in arsenate reduction will be the most useful for molecular ecology studies. While much remains to be done before we can design effective probes, a first step in this direction has been taken by the recent characterization and purification of the dissimilatory arsenate reductase from strain SES-3 (Newman et al, *submitted*).

A Biochemical Model of Dissimilatory Arsenate Reduction

The dissimilatory arsenate reductase of strain SES-3 appears to be fundamentally different from the enzymes responsible for the reductive detoxification of arsenate in *E. coli* and *S. aureus* (known as the ArsC enzymes). An activity assayed based upon the oxidation of methyl viologen coupled to the reduction of arsenate to arsenite was used to identify the reductase (Newman et al., *submitted*). The dissimilatory arsenate reductase is a multimeric integral membrane protein, with the molecular weight of the native complex exceeding 100 kDa (Newman et al., *submitted*)). The ArsC enzymes are cytoplasmic monomers with molecular weights close to 15 kDa (Chen et al, 1986; Ji et al., 1994). Although it remains to be proven, the dissimilatory arsenate reductase has a presumptive chromosomal locus: the ability to respire arsenate is highly stable from one generation to the next, regardless of whether arsenate is present in the medium; the ArsC enzymes are carried on plasmids (R773 and pI258, for *E. coli* and *S. aureus*, respectively) (Gladysheva et al, 1994; Ji et al., 1994). The V_{max} for the ArsC enzymes are comparable, but the V_{max} estimated for the dissimilatory arsenate reductase is several times greater (Newman et al., *submitted*). *S. aureus* is reported to have a 2 mM K_m, (although a 1μM K_m is claimed for the same enzyme, yet with a significantly lower V_{max}) (Ji et al., 1994), *E. coli* an 8 mM K_m (Gladysheva et al., 1994), and strain SES-3 a 0.2 mM K_m (Newman et al.,

submitted) These kinetic differences may help explain why strain SES-3 can not only grow on arsenate, but can survive over a wider range of arsenate concentrations than either *E. coli* or *S. aureus*.

The mechanism by which the dissimilatory and detoxifying enzymes pass electrons on to arsenate is also quite different: reduced dithiols perform this transfer for the ArsC enzymes (Rosen et al., 1994), whereas the dissimilatory arsenate reductase appears to utilize prosthetic groups such as Fe:S clusters (Newman et al., *submitted*)). A *b*-type cytochrome is present in membranes of strain SES-3 when it is grown on arsenate, and may also be involved in electron transfer (Newman et al., *submitted*)). Based on these results, we propose the following model for growth on lactate and arsenate for strain SES-3 (Fig. 5). This model is only one of several possibilities, however, and must be verified by future studies. The main feature of this model is that the reductase spans the cytoplasmic membrane, with the active site for the reduction of arsenate to arsenite facing the cytoplasm. Such an orientation would allow a proton motive force to be generated from the flow of electrons from a cytoplasmically-oriented lactate dehydrogenase to the arsenate reductase, either through a proton-pumping intermediary such as menaquinone (as shown in the model) or through diffusion of H_2 (formed by a cytoplasmic hydrogenase) through the membrane to the outside (where it would be oxidized by a periplasmic hydrogenase), allowing electrons to flow back to the arsenate reductase through membrane-bound electron carriers. A similar model has been proposed for the generation of a proton motive force during dissimilatory sulfate-reduction (White, 1995). We locate the reductase to the cytoplasmic membrane not only because this is the convention for reductases involved in respiratory chains (Ingledew & Poole, 1984), but also because transmission electron micrographs have shown that arsenic-trisulfide particles form in this location during arsenate respiration in another arsenate-reducing bacterium (Newman et al., 1997). Recent work with *Shewanella putrefaciens* strain MR-1, however, has suggested that key components of the electron transport chain involved in Fe(III) and Mn(IV) respiration are active in the outer membrane (Myers & Myers, 1997), and we could thus imagine that the arsenate reductase might alternatively reside in this location.

Environmental Importance

Although molecular probes may be necessary to assess the importance of dissimilatory arsenate reduction rigorously, evidence already exists which suggests that arsenate-reducing organisms may be active in the environment. Work on deposits of orpiment and realgar from an active geothermal site in Kamchatka, Russia, indicate that bacteria may be involved in the formation of these deposits (Benning, *pers. comm.*). Furthermore, studies with isolates, enrichment cultures, and natural consortia all show that dissimilatory arsenate-reducing organisms can reduce arsenate to arsenite at environmentally relevant levels. In the case of *D. auripigmentum* strain OREX-4, scorodite was found to support cell growth in the presence of H_2 and acetate (Newman et al., 1997), which suggests that bacteria may be able to generate sufficient energy for growth by arsenate reduction in environments close to naturally occurring arsenate minerals. Similarly, strain MIT-13 was shown to grow on micromolar concentrations of arsenate provided either by arsenic-contaminated sediments or by synthetic iron arsenate, and enrichment cultures native to these sediments were also shown to mobilize arsenic from synthetic iron arsenate (Ahmann et al., 1997). Bacterial consortia from arsenic-contaminated soils from a Superfund site in Pennsylvania were found to reduce the arsenate present in these soils as well (Newman et al., *submitted*). It thus seems likely that bacteria in arsenic-polluted sites may contribute to the mobilization of arsenic through dissimilatory arsenate reduction.

Indirect evidence for the activity of arsenate-reducing bacteria can also be found in field data. Several studies have now shown arsenic speciation in stratified lakes to deviate from thermodynamic equilibrium: arsenite predominates in the oxidized epilimnion, and arsenate persists in the anoxic hypolimnion (Kuhn & Sigg, 1993; Spliethoff et al., 1995). While the arsenate reducing (and methylating) activity of phytoplankton is commonly invoked to explain the abundance of arsenite in summer surface waters, bacteria are now thought to be involved in this process as well (although such reduction is most likely not dissimilatory) (Spliethoff, 1995). The appearance of arsenite in anoxic sediments, however, is likely to be mediated by dissimilatory arsenate-reducing microorganisms. One such example is the anoxic hypolimnion of the Upper Mystic Lake, in which significant amounts of both arsenite (>200 nM) and arsenate (<200 nM) were found to persist in sulfidic waters (25 μM) during the fall of 1994 (Spliethoff et al., 1995). It may not be

coincidental that the arsenate and sulfate-respiring isolate, *D. auripigmentum*, was retrieved from the sediments at this time. This was the only time of the year when hypolimnetic concentrations of arsenite exceeded those of arsenate (Spliethoff et al., 1995). While it is possible that abiotic reduction of arsenate by sulfide could explain this profile, both laboratory and field measurements indicate that this process is kinetically slow (Newman et al., 1997; Kuhn & Sigg, 1993). Therefore, it seems equally reasonable to suggest that dissimilatory arsenate-reducing microorganisms might play an active role in such environments.

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Figure legends

Fig 1. Freshwater arsenic cycling diagram.

Table 1. Free energies of various electron acceptors coupled to H_2 oxidation.

Fig 2. Arsenate reduction by consortia in the presence of glucose & acetate. Errors bars represent the range of duplicate samples.

Fig 3. SEM of arsenate-reducing consortia in a biofilm matrix.

Table 2. Physiological profiles of 4 arsenate-reducing isolates, taken from the literature.

Fig 4. TEM of an arsenic trisulfide coated whole cell of *D. auripigmentum*. Length of the bacterium is approximately 2-3 micrometers.

Fig 5. Biochemical model of dissimilatory arsenate reduction in strain SES-3.

Figure 1

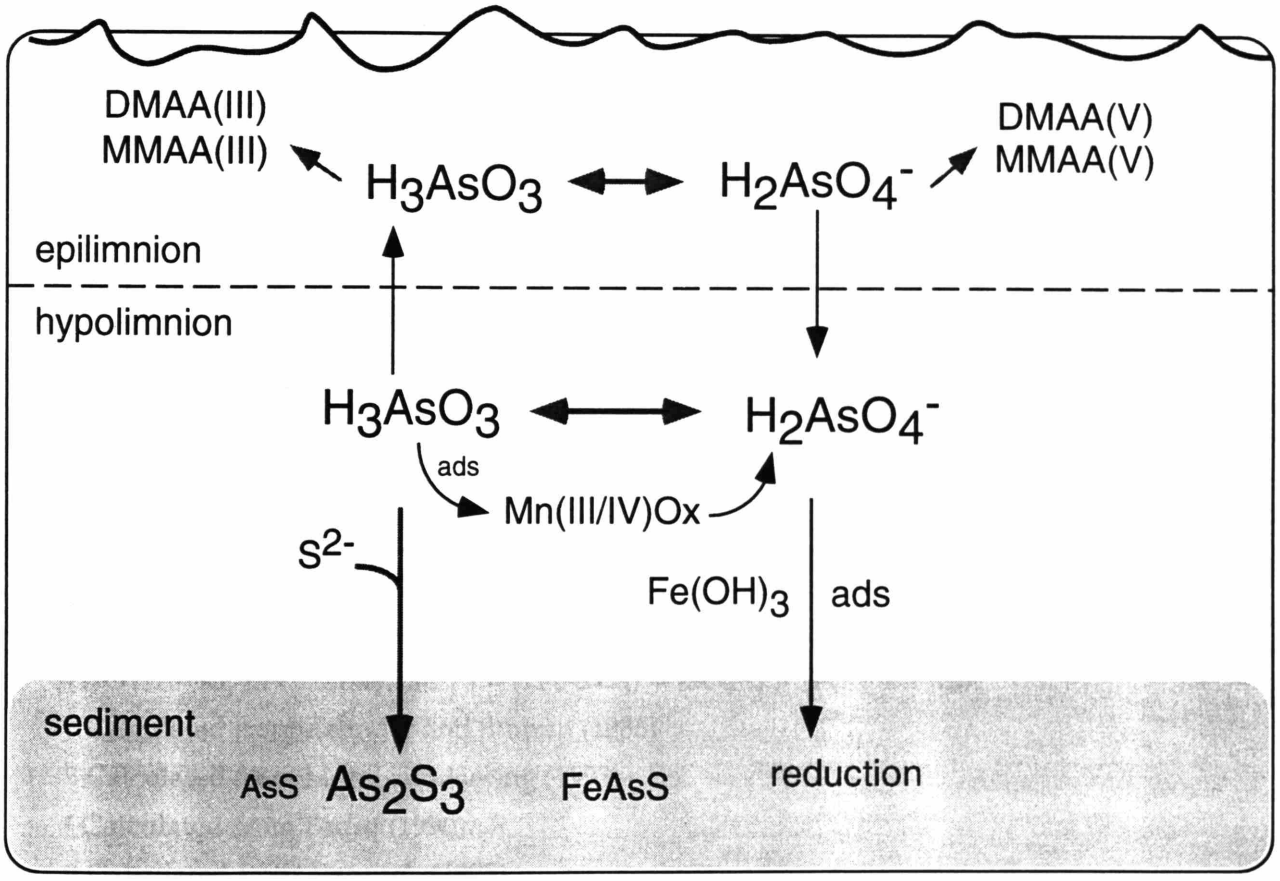

DMAA(III)
MMAA(III)
H_3AsO_3
$H_2AsO_4^-$
DMAA(V)
MMAA(V)
epilimnion
hypolimnion
H_3AsO_3
$H_2AsO_4^-$
ads
Mn(III/IV)Ox
S^{2-}
$Fe(OH)_3$
ads
sediment
AsS
As_2S_3
FeAsS
reduction

Table 1. Free energies of electron acceptor reduction coupled to H_2 oxidation

Reaction	delta G' kcal/mol e-
$1/4\ O_2(g) + 1/2\ H_2 \rightarrow 1/2\ H_2O$	-23.55[a]
$1/5\ NO_3^- + 1/5\ H^+ + 1/2\ H_2 \rightarrow 1/10\ N_2(g) + 3/5\ H_2O$	-20.66[a]
$1/2\ MnO_2(s) + H^+ + 1/2\ H_2 \rightarrow 1/2\ Mn^{2+} + H_2O$	-22.48[b]
$1/2\ SeO_4^{2-} + 1/2\ H^+ + 1/2\ H_2 \rightarrow 1/2\ HSeO_3^- + 1/2\ H_2O$	-15.53[b]
$1/8\ NO_3^- + 1/4\ H^+ + 1/2\ H_2 \rightarrow 1/8\ NH_4^+ + 3/8\ H_2O$	-13.42[a]
$1/3\ CrO_4^{2-} + 5/3\ H^+ + 1/2\ H_2 \rightarrow 1/3\ Cr^{3+} + 4/3\ H_2O$	-10.76[b]
$Fe(OH)_3(am) + 2\ H^+ + 1/2\ H_2 \rightarrow Fe^{2+} + 3\ H_2O$	-10.4[b]
$1/4\ HSeO_3^- + 1/4\ H^+ + 1/2\ H_2 \rightarrow 1/4\ Se^0 + 3/4\ H_2O$	-8.93[b]
$1/2\ H_2AsO_4^- + 1/2\ H^+ + 1/2\ H_2 \rightarrow 1/2\ H_3AsO_3 + 1/2\ H_2O$	-5.51[c]
$1/3\ H_3AsO_3 + 1/2\ H_2 \rightarrow 1/3\ As^0 + H_2O$	-2.58[d]
$1/8\ SO_4^{2-} + 1/8\ H^+ + 1/2\ H_2 \rightarrow 1/8\ HS^- + 1/2\ H_2O$	-0.10[a]

$P_{H2} = 10^{-6.6}$ atm = 10 nM P_{N2} = 0.78 atm P_{O2} = 0.21 atm pH = 7.0

$(NO_3^-) = (NH_4^+) = (Mn^{2+}) = (Fe^{2+}) = (SeO_4^{2-}) = (HSeO_3^-) = (CrO_4^{2-}) = (Cr^{3+}) = (H_2AsO_4^-) = (H_3AsO_3) = (SO_4^{2-}) = (HS^-) = 1\ \mu M$.

[a] Calculated from Zehnder and Stumm (1988)

[b] Calculated from Morel and Hering (1993)

[c] Calculated from Sadiq (1990).

[d] Calculated from Vanysek (1995)

Figure 2

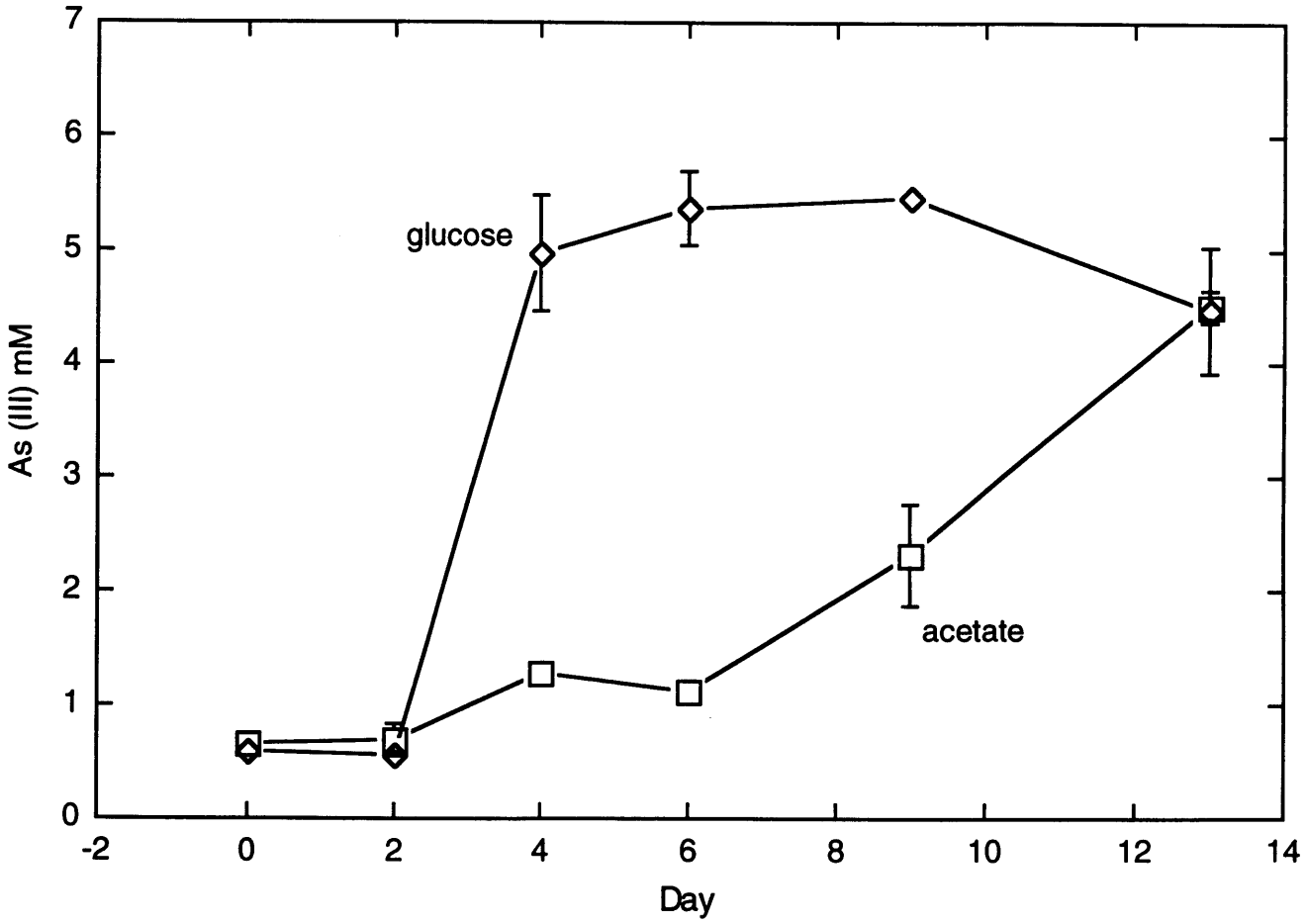
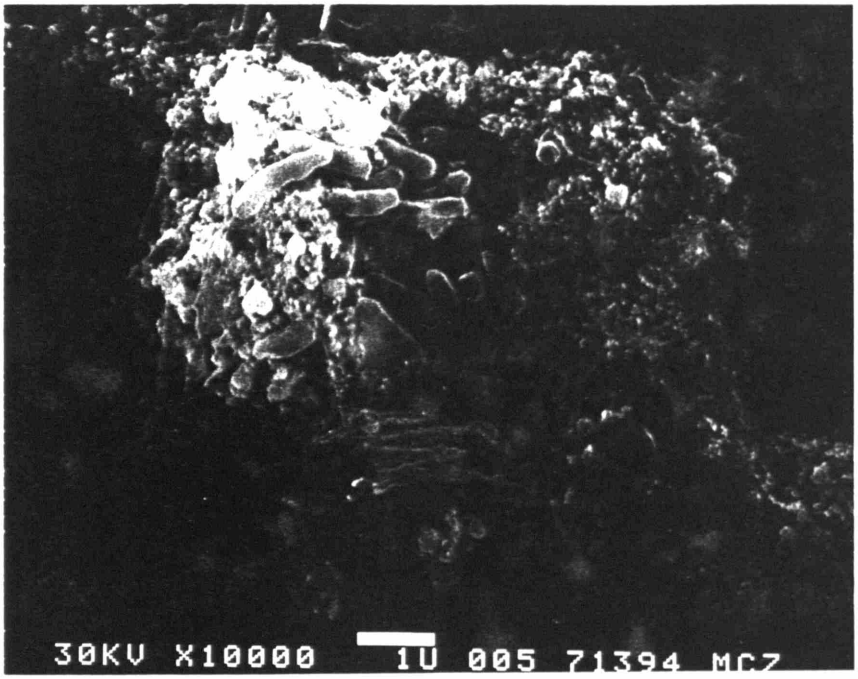


Figure 3



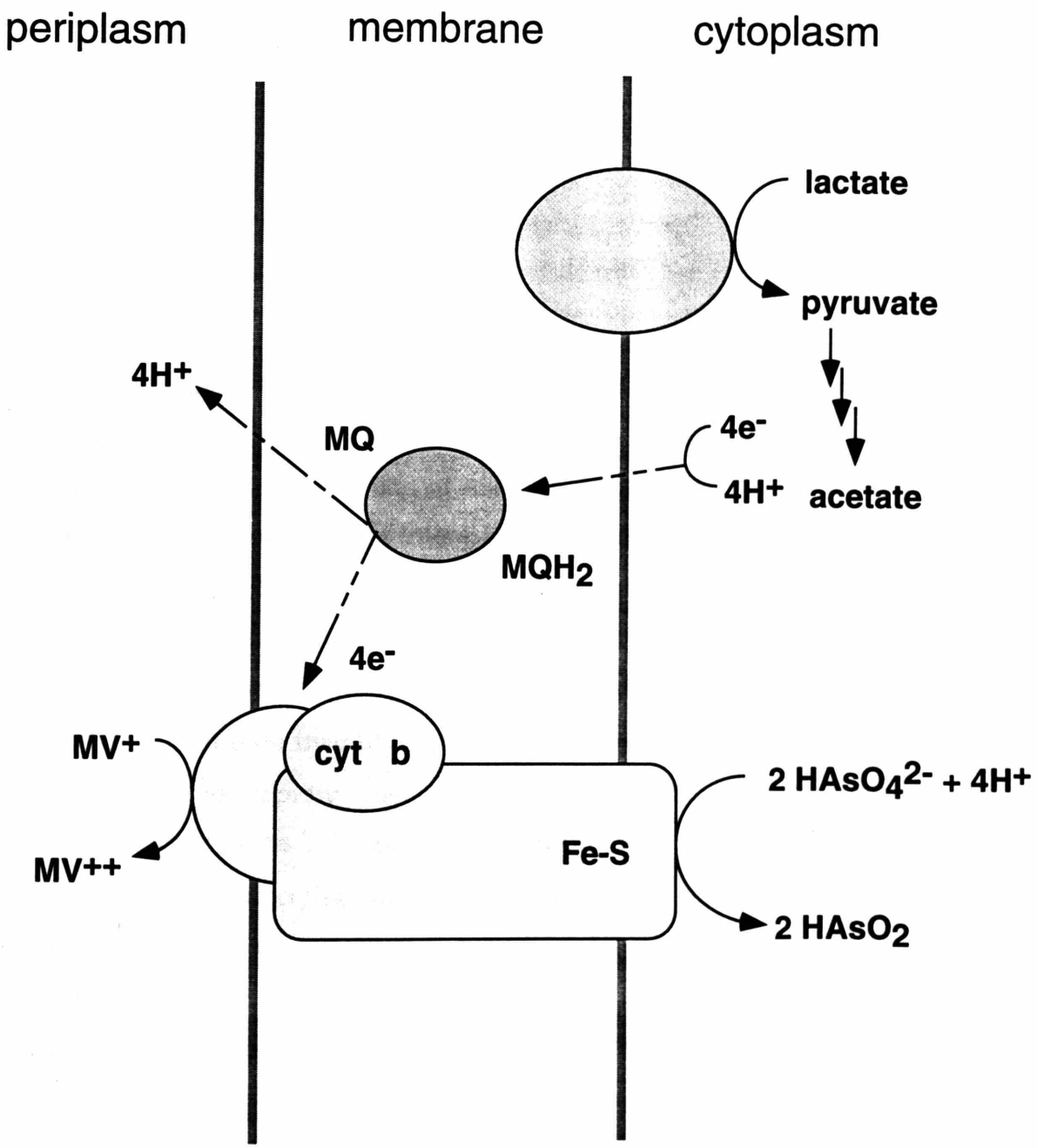
Electron donor	BAL-1^T	OREX-4	SES-3	MIT-13	Electron acceptor	BAL-1^T	OREX-4	SES-3	MIT-13
H_2	-	+	-	-	Oxygen	-	-	-	-
H_2 + acetate	+	+	+	+	Nitrate	+	-	+	+
Methane + acetate	0	-	0	0	Mn(IV)	0	-	+	-
Formate	-	-	+[a]	+[a]	Fe(III)	-	-	+	0
Acetate	+	-	-	-	Sulfate	-	+	-	-
Propionate	0	-	0	-	Sulfite	0	+	0	0
Butyrate	0	+	0	+[a]	Thiosulfate	-	+	+	0
Pyruvate	+	+	0	+	S_0	0	0	+	-
Lactate	+	+	+	+	Se(IV)	0	-	+	-
Fumarate	+	-	0	+	As(V)	+	+	+	+
Succinate	+	-	+	0	Fumarate	0	+	+	+
Citrate	-	-	+	0	Malate	0	-	0	0
Malate	+	+	0	0	DMSO	0	-	0	0
Palmatate	0	-	0	0					
Benzoate	-	-	0	-					
Glucose	-	-	0	0					
Glycerol	0	+	0	0					
Ethanol	0	+	0	0					
Methanol	-	-	0	0					
Phenol	0	-	0	0					
Casamino acids	0	-	0	0					
Yeast extract	0	-	0	0					
Octane	0	-	0	0					
Cyclohexane	0	-	0	0					
Benzene	0	-	0	0					
Toluene	0	-	0	0					

[a] Growth occurs only in the presence of acetate; 0 = not reported

Table 2

Figure 4

Figure 5


periplasm
membrane
cytoplasm
lactate
pyruvate
4H+
MQ
4e-
4H+
acetate
MQH2
4e-
MV+
cyt b
2 HAsO4 2- + 4H+
MV++
Fe-S
2 HAsO2

Chapter 3: Precipitation of As_2S_3 by *Desulfotomaculum auripigmentum*

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by

Dianne K. Newman, Terry J. Beveridge, and François M. M. Morel

Abstract

A newly discovered bacterium, *Desulfotomaculum auripigmentum*, precipitates arsenic trisulfide (As_2S_3). Precipitation of As_2S_3 by this organism results from its reduction of As(V) to As(III) and S(VI) to S(-II). At the As(III) concentration range of interest (0.1 - 1 mM), the stability of As_2S_3 is highly sensitive to pH and [S(-II)]. Thus, the relative rates at which *D. auripigmentum* reduces As(V) and S(VI) are critical to its formation of As_2S_3. Other As(V) or S(VI)-reducing bacteria are unable to precipitate As_2S_3 either due to their inability to reduce both As(V) and S(VI) or because they reduce S(VI) too rapidly. Electron microscopy of thin sections showed that the precipitate forms both intra- and extracellularly. Microbial As_2S_3 formation nucleates precipitation of the mineral in the bulk milieu whereas heat-killed cells alone do not serve as templates for its formation. Precipitation of As_2S_3 by *D. auripigmentum* suggests that As_2S_3 formation may be important in the biogeochemical cycle of arsenic.

Introduction

Over the past two decades, appreciation for the role of microbes in the precipitation of minerals and the redox tranformation of many elements has grown (5,17,18,22). Bacteria are now believed to be involved in the deposition of magnetite in marine sediments (28), the formation of placer gold (30), and the precipitation of dolomite at low temperatures (29), to name but a few examples. Our understanding of the tremendous metabolic diversity of the microbial world has expanded in recent years with the discoveries that microbes can use a variety of elements, including Fe(III), Mn(IV), Se(VI), U(VI), and As(V) as terminal electron acceptors in anaerobic respiration (17,22,2,16). In this report, we present an example of the convergence of these two microbial processes, biomineralization and dissimilatory metal reduction, in the case of As(V) reduction and precipitation of arsenic trisulfide.

Arsenic trisulfide (As_2S_3), a bright yellow solid, was once used as a golden pigment for dye and paint but today finds primary application in the semiconductor industry (11,14). The formation of As_2S_3 in nature as the mineral orpiment has previously been observed only in extreme environments such as geothermal reservoir fluids and hot springs, and has been assumed to be abiotic (7). We now provide evidence that a newly discovered As(V)-reducing bacterium, *D. auripigmentum* (23), precipitates As_2S_3 both intra- and extracellularly. Microbial precipitation of As_2S_3 is the first example of the biomineralization of a toxic compound that is intimately tied to bacterial metabolism, which, in this case, involves the respiration of As(V) and S(VI) (23). Until now, dissimilatory reduction of As(V) to As(III) has been thought to increase the element's mobility (2,16). The precipitation of As_2S_3 by *D. auripigmentum*, however, suggests that this is not necessarily the case.

Materials and Methods

Medium: *Desulfotomaculum auripigmentum* and the enrichment culture from which it derived were grown in freshwater minimal medium, supplemented with 10-20 mM sodium lactate, either 1-10 mM sodium sulfate or 1mM cysteine, and 1-10 mM sodium arsenate. The medium was buffered at a pH of 6.8 with bicarbonate, and reduced with $PdCl_2$+H_2 (300 mg/ml). Salts supplied per liter of medium were: 0.14 g KH_2PO_4, 0.25 g NH_4Cl, 0.50 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 1.0 g NaCl, and 0.62 g $MgCl_2 \cdot 6H_2O$. The medium contained per liter: 0.05 mg *p*-aminobenzoic acid, 0.02 mg biotin, 0.05 mg nicotinic acid, 0.05 mg calcium pantothenate, 0.05 mg thiamine HCl, 0.1 mg pyridoxin HCl (B_6), and 0.001 mg cyanocobalamin (B_{12}); trace metals supplied per liter were: 0.001 ml conc. HCl, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.12 mg $CoCl_2 \cdot 6H_2O$, 0.07 mg $ZnCl_2$, 0.06 mg H_3BO_3, 0.025 mg $NiCl_2 \cdot 6H_2O$, 0.015 mg $CuCl_2 \cdot 2H_2O$, 0.025 mg $Na_2MoO_4 \cdot 2H_2O$, and 1.5 mg $FeCl_2 \cdot 4H_2O$. All chemicals were purchased from Sigma Chemical Co. The isolation of *D. aruipigmentum* is described elsewhere (23).

Analyses. Total arsenic (oxidized to As(V)), As(V) and background phosphate were measured by the molybdenum blue spectrophotometric assay (12); As(III) was determined by measuring the difference between the oxidized and untreated samples. Sulfide was determined by the methylene blue method (10). Cells were DAPI stained and counted on a Zeiss Axioskop.

Comparisons to other bacteria. Experiments comparing *D. auripigmentum* to *Desulfobulbus propionicus* (31) (gift of Cindy Gilmour of The Academy of Natural Sciences) and strain MIT-13 (2) (gift of Dianne Ahmann of Duke University) were carried out in the medium described above, amended with 10 mM lactate, 14 mM sulfate, and 1 mM arsenate. As(V) and S(VI)

reduction were followed over a 2-week period, as was cell growth. *D. propionicus* was tested separately for growth on 1, 5, and 10 mM arsenate with 10mM lactate as the carbon/electron source, and S(VI) was excluded from this medium.

Scanning electron microscopy. SEM samples were prepared by filtration onto a 0.2 mm filter, followed by fixation in 2% (v/v) glutaraldehyde for 1 hour, two 15-minute rinses in Na-cacodylate (pH 6.8), and 10-minute dehydrations in an ethanol series: 50%, 70%, 85%, 95%, 100%. Samples were critical point dried using a Tousimis Samdri Model PVT3, sputter-coated with 20 nm of Au/Pd using a Technics Hummer II, and imaged with an Amray model 1000A operating at 30 kV under standard conditions.

Transmission electron microscopy and energy dispersive X-ray spectroscopy (EDS). Thin sections of *D. auripigmentum* were prepared after the cells were fixed in 2% glutaraldehyde in 50 mM HEPES buffer (pH 6.8) for 1 hour, followed by 3 rinses in HEPES buffer, 5 minutes each. Dehydration in ethanol followed: 20%, 50%, 70%, 85%, 90% for 10 minutes each, and 100% ethanol 3 times for 10 minutes each. Samples were embedded in LR White (Ted Pella Co.). After 24 hours at 55-60∞C, samples were cut on a Leica Ultracut S to 60 nm thickness. Confirmation of mineral identity in thin sections was performed with a Philips C20 TEM operating at 80 kV and equipped with an EDAX 9600 series analyser for EDS. EDS was conducted with a counting time of 200 s, live time.

Scanning transmission electron microscopy and EDS. Whole cell and thin-section specimens were mounted on carbon films supported on 200-mesh nickel EM grids (Ladd Research Industries). The relative arsenic and sulfur concentrations were determined from the average of six individual spectra acquired from thin sections of a microbial sample and compared to preparations of synthetic As_2S_3 (Aldrich) using a Link Analytical EDS system

on a VG-HB603 STEM. The area analysed for each spectrum was about 2 nm^2. By measuring spectra from areas of known but different thickness, it was determined that a self-absorption correction was not required. The counting time was 240 s, live time.

Results

Microbial precipitation. Samples from the enrichment culture were inoculated into minimal medium supplemented with 10 mM lactate, 1 mM arsenate (As(V)) and 1 mM cysteine. After one week, a bright yellow precipitate was observed to form overnight (Fig. 1A). The isolate *D. auripigmentum* (Fig. 1B) also precipitated the bright yellow substance after a week's growth when 1 mM sulfate (S(VI)) was substituted for cysteine (Fig. 1C). Cells grown on solid medium amended with As(V) and S(VI) formed bright yellow colonies, typically less than 1 mm in diameter. After several days, the agar near the colonies began to turn yellow.

Identification of Precipitate. The identity of the yellow precipitate was established using EDS in conjunction with electron microscopy. Preliminary EDS analysis of the small particles in Fig. 1C showed the precipitate to be inorganic, composed of As and S (data not shown). Finer mineral analysis with a scanning transmission electron microscope revealed the relative arsenic and sulfur compositions of a freshly precipitated sample to be 60.3 ± 0.6 % As and 39.7 ± 0.6 % S by weight (Fig. 2A). These proportions were found to be statistically indistinguishable from those of a synthetic As_2S_3 sample (60.9 % As, 39.1 % S).

X-ray maps of a single cell of *D. auripigmentum* illustrated that the arsenic and sulfur profiles corresponded to the regions of highest density, and when analyzed for percent composition, gave the expected ratio for As_2S_3 (Fig. 2B). Electron diffraction patterns gathered from particles near the poles of this cell indicated that the precipitate was amorphous. While As_2S_3 was clearly predominant in all samples analyzed regardless of age, small alteration products were observed to form over time. High resolution mineral analysis (nm scale) showed spicular structures containing a slightly higher As:S ratio (possibly corresponding to amorphous AsS) to be present in

samples that had aged for several weeks (data not shown). These structures clearly contrasted with the monodisperse spherical As_2S_3 particles.

Redox transformations underlying As_2S_3 precipitation. The importance of microbial As(V) reduction in the precipitation of As_2S_3 was first demonstrated in an experiment with an enrichment culture. In this experiment, As(V) reduction and As_2S_3 precipitation only occured in the presence of bacteria and not in abiotic controls, and after 12 days the bacteria had removed 40-50% of the dissolved arsenic in the system (Fig. 3A). Seeding of the medium with heat-killed cells also failed to nucleate precipitation.

In cultures of *D. auripigmentum* which were amended with As(V) and S(VI) in place of cysteine, the precipitation of As_2S_3 implied that this organism reduced arsenic and sulfur from As(V) to As(III) and S(VI) to S(-II). To verify and quantify these redox transformations, *D. auripigmentum* was grown on 10 mM pyruvate in the presence of 1 mM As(V) and 1 mM S(VI). Rapid As(V) reduction and concomitant accumulation of As(III) in the medium were observed (Fig. 3B). As the level of As(III) rose, trace quantities of S(-II) also appeared (Fig. 3C). Visible precipitation occurred on day #6, when nearly all the As(V) had been reduced; at that point, As(III) and S(-II) began to be removed from solution (Fig. 3B & 3C). The pH measured at the beginning and end of the experiment remained constant at 6.8. The coincident reduction of As(V) and S(VI) by *D. auripigmentum* allows As_2S_3 to precipitate. Neither As(V) nor S(VI) are reduced in the absence of this organism. Precipitation does not occur when As(V) and S(-II) are added to the medium in its absence.

Comparisons with other bacteria. To test whether the metabolism underlying As_2S_3 precipitation was unique, we compared *D. auripigmentum* to MIT-13 (an As(V)-reducer) and to *Desulfobulbus propionicus* (a S(VI)-

reducer). All bacteria were supplied with 1 mM As(V), 14 mM S(VI), and 20 mM lactate. The MIT-13 inoculum came from an As(V)-reducing culture (10 mM As(V), no S(VI)), whereas the *D. propionicus* and *D. auripigmentum* inocula came from S(VI)-reducing cultures (10 mM S(VI), no As(V)). While all three organisms reduced As(V) to As(III) over time, MIT-13 did not reduce S(VI) over the course of the experiment, whereas *D. propionicus* grew vigorously on S(VI) (2.5 mM S(II) appeared in the medium over 5 days; cells increased by an order of magnitude) and *D. auripigmentum* began to reduce S(VI) after As(V) reduction was essentially complete (data not shown). As_2S_3 precipitation occured only in the *D. auripigmentum* cultures when As(III) concentrations had reached approximately 1mM and S(-II) concentrations were still low (0.01 to 0.1 mM) ("a+" symbol Fig. 4). When S(-II) rose to 1mM and above in the *D. auripigmentum* cultures, the precipitate dissolved ("a-" symbol Fig. 4). As_2S_3 formed in neither the MIT-13 cultures ("m-" symbol Fig. 4) nor in the *D. propionicus* cultures ("p-" symbol Fig. 4). *D. propionicus* could not use As(V) as a terminal electron acceptor since it failed to either grow on or reduce As(V) when As(V) was provided as the sole oxidant (data not shown). While *D. auripgimentum* was the only one of the three organisms to precipitate As_2S_3 in these experiments, microbial precipitation of As_2S_3 is not unique to this bacterium *per se*, rather, it is a function of the ability of an organism to reduce both As(V) and S(VI) to appropriate concentrations of As(III) and S(-II). These experiments suggested that S(-II) concentrations between 0.1 mM and 1 mM were necessary to observe As_2S_3 precipitation.

Abiotic As_2S_3 precipitation. To better understand the the chemical constraints on As_2S_3 precipitation implied by our comparative microbial experiments, we constructed a dominance diagram for As_2S_3 in equilibrium with various aqueous As(III) species (Fig. 4). The following reactions were considered:

$1/2\ As_2S_3\ (am) + 3\ H_2O \rightleftharpoons H_3AsO_3 + 3/2\ H_2S$ logK = -11.9 (7)

$3/2\ As_2S_3\ (am) + 3/2\ H_2S \rightleftharpoons H_2As_3S_6^- + H^+$ logK = -5.0 (7)

$H_2As_3S_6^- \rightleftharpoons HAs_3S_6^{2-} + H^+$ logK = -6.56 (28)

$H_3AsO_3 \rightleftharpoons H_2AsO^{3-} + H^+$ logK = -9.29 (24)

$H_2S \rightleftharpoons HS^- + H^+$ logK = -7.02 (21)

The diagram predicts that the solubility of As_2S_3 should increase with pH and with high concentrations of S(-II): the greater the As(III) concentration, the broader the stability region of As_2S_3 with respect to pH and [S(-II)]. The diagram indicates that the stability of As_2S_3 is very sensitive to pH and [S(-II)] over the concentration range relevant to our experiments (pH between 6.5 and 7.0, [S(-II)] between 0.1 and 1.0 mM, [As(III)] = 1mM).

To test whether As_2S_3 could precipitate abiotically at the concentrations predicted by our diagram, we varied [S(-II)] and pH with [As(III)] constant at a concentration of 1 mM in the same medium used in our microbial experiments. We observed the expected pH and [S(-II)] effects: when S(-II) was fixed at 0.1 mM (log [S(-II)] = -4), precipitation occurred at pH $\leq$ 7.0 ('+" symbol Fig. 4) but did not occur at high pH (7.4) ('-" symbol Fig. 4); when pH was fixed at 6.6, precipitation occurred below 1 mM S(-II) (log [S(-II)] = -3), but did not occur at high S(-II) (log [S(-II)] = -2.5) ("-" symbol Fig. 4). We did not observe As_2S_3 precipitation when S(-II) was supplied at 10 µM (log [S(-II)] = -5) even though this concentration is predicted to fall within the stability region of As_2S_3 over a broad pH range when [As(III)] = 1 mM (shaded area Fig. 4), and *D. auripigmentum* cultures precipitated As_2S_3 at these levels ("a+" symbol Fig. 4). The addition of heat-killed cells from clean medium (i.e., lacking precipitates) did not nucleate As_2S_3 formation in these samples. Abiotic precipitation at the As_2S_3 stability boundary (pH = 7.0, [S(-II)] = 0.1 mM , [As(III)] = 1 mM) was kinetically slow: it took several hours

before a precipitate became visible ("∂ " symbol Fig. 4); at low pH, however, the bottles turned yellow within seconds. For the most part, visible As_2S_3 precipitation readily occured abiotically within the expected region of the stability diagram provided As(III) and S(-II) concentrations were high enough to form visible particles (e.g. above 10 μM).

Intracellular and Extracellular Precipitation To investigate whether the precipitation of As_2S_3 was solely an indirect effect of microbial production of As(III) and S(-II) in the external milieu, we prepared thin sections of *D. auripigmentum* a week after the As_2S_3 precipitate first appeared in the medium. Electron micrographs showed small monodisperse particles of As_2S_3 (50 nm) concentrated intracellularly in the vicinity of the cytoplasmic membrane (Fig. 5A, 5B). Extracellular precipitates were observed surrounding the bacteria in great abundance, apparently coating the gram-positive cell wall (Fig. 5B). In one section, a cluster of As_2S_3 particles could be seen attached to the surface of a cell, seemingly surrounded by a membrane (Fig. 5C). Microbial precipitation of As_2S_3 appeared to be something more than a simple extracellular chemical reaction.

Discussion

Precipitation of As_2S_3 is not biomineralization in the strict sense of the word. Unlike the magnetosomes of the magnetotactic bacteria (9), As_2S_3 precipitates are not "boundary organized" but "biologically induced" (as defined by H. A. Lowenstam (18)) and form readily under the appropriate chemical conditions. Abiotic experiments demonstrate that the stability of As_2S_3 is highly sensitive to small changes in pH and S[-II], and microbial experiments confirm this sensitivity by showing that As_2S_3 precipitation by a given organism depends on the rate and extent to which it can reduce As(V) and S(VI). *D. auripigmentum* reduces the majority of As(V) to As(III) before reducing a significant fraction of S(VI) to S(-II) (23). Because of this, there is an interval when [S(-II)] is high enough to promote visible precipitation, yet low enough such that As_2S_3 does not redissolve. In contrast, MIT-13 does not precipitate As_2S_3 because it cannot reduce S(VI). Even *D. propionicus*, which rapidly reduces S(VI) and As(V) (the latter most likely for detoxification purposes, since *D. propionicus* cannot respire As(V)) does not precipitate As_2S_3 because the high concentrations of S(-II) it produces cause the aqueous species $HAs_3S_6^{2-}$ to form rather than As_2S_3. The formation of $HAs_3S_6^{2-}$ also explains why As_2S_3 precipitates dissolve in the *D. auripigmentum* cultures when [S(-II)] approaches 1 mM. In both our microbial and abiotic precipitation experiments, the tendency for the delayed appearance and/or dissolution of the precipitates at the limits of the domain of stability of the solid is likely due to slow kinetics of formation/dissolution at these conditions.

Despite the ability of As_2S_3 to form in the bulk medium as a result of a biologically-induced chemical reaction, significant amounts have also been observed inside the cell. This raises the question of the relationship between

the intracellular and extracellular precipitates. Interestingly, when *D. auripigmentum* is grown on solid medium, its colonies turn bright yellow several days before a yellowish tint appears in the surrounding agar. The sharp yellow definition of the colonies suggests that precipitates that are closely tied to the cell form first, and that additional precipitation in the bulk medium follows as the concentrations of As(III) and S(-II) rise due to diffusion or transport beyond the cell. Confirming this, the electron micrographs show that the particles associated with the membrane and cell wall are uniformly small (50-100 nm in diameter) and that the particles in the bulk medium are significantly larger. This suggests that cell-associated particles may nucleate additional precipitation in the outside milieu. The finding that precipitation occurs in the presence of metabolically active bacteria at concentrations where none is visible in abiotic experiments despite solution supersaturation, reinforces this idea. It is well known that bacteria are capable of creating micro-environments which favor mineralization despite apparently unfavorable conditions in the bulk environment (5). In the case of As_2S_3 precipitation, reduction may take place independently on both sides of the cell membrane, or dead bacteria may release As_2S_3 which then adheres to the outside of living cells. Alternatively, an export mechanism is possible whereby membrane-bound particles could be transported from the membrane through the cell wall to the outside, as suggested by one of the electron micrographs (Fig. 5C). Such a mechanism has been observed in a gram-negative bacterium containing localized peptidogylcan hydrolases (13).

In any case, the intracellular and extracellular As_2S_3 precipitates are more than a mere consquence of an abiotic reaction due to incidental microbial production of As(III) and S(-II). On the contrary, As_2S_3 precipitation is linked to important metabolic processes. Dead As_2S_3-free cells (grown in medium which does not allow the formation of precipitates, and then heat-killed) do not appear to nucleate precipitation. First and

foremost, precipitation is a direct product of respiratory growth on As(V) and S(VI) (23). Localization of the intracellular precipitates to the inside of the cytoplasmic membrane may not be a coincidence, as this is where we would expect As(V) and S(VI) reduction to be coupled to the respiratory chain. Second, precipitation may also serve as a detoxification mechanism for As(III). Precedent exists for bacterial precipitation of toxic metals such as cadmium (3) nickel (8) and selenite (19) both intra- and extracellularly. The high levels of As(III) which accumulate in the medium prior to the formation of As_2S_3 (suggesting cellular export of As(III)) seem to argue against precipitation as a primary mechanism for As(III) detoxification, although in support of this hypothesis, we have observed bacterial growth on 1 and 5 mM As(V) to be better in the presence of S(VI) than without. Perhaps the simplest argument for microbial precipitation of As_2S_3 is based on thermodynamics: the free energy to be gained from As(V) reduction would increase by maintaining a low level of As(III) in solution.

Microbial precipitation of As_2S_3 may have important consequences for arsenic cycling. Over the past two decades, evidence has been mounting that points to a missing sink for As(III) in various aquatic and sedimentary environments (1,20). In areas such as the Upper Mystic Lake in Woburn Massachussetts, from which *D. auripigmentum* originates, interannual variability in the concentration and speciation of arsenic in the hypolimnion has been observed, with the redox state of arsenic often in apparent disequilibrium with those of iron and sulfur (4,15,27). The ability of microorganims to influence such cycles through reduction (2,16) and precipitation of FeAsS (26) has been documented. More recently, Dowdle et. al. (6) suggested that As_2S_3 formation could possibly account for arsenic imbalances in As(V)-reducing sediments. This explanation was thought to be paradoxical, however, given that As(V) reduction inhibited S(VI) reduction in these sediments. The presence of organisms such as *D. auripigmentum* which can reduce both As(V) and S(VI) and precipitate

As_2S_3, would resolve this paradox. In environments with low Fe(II), As_2S_3, rather than FeAsS, may be the dominant sink for As(III) (1). Depending on the ability of As(V) respiring organisms present in a given environment to precipitate As_2S_3, the effect of microbial arsenate reduction should lead in some instances to the dissolution and enhanced mobility of arsenic, and in others, to its removal by precipitation.

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Figure legends

Fig. 1. Cultures before and after the precipitation of As_2S_3. **(A)** The bottle on the left shows the enrichment a few days after inoculation; on the right, the enrichment after two weeks. A synthetic sample of As_2S_3 (Aldrich) lies between the two bottles. Pure cultures of *D. auripigmentum* look the same. **(B)** SEM micrograph of *D. auripigmentum* on day #4. **(C)** SEM micrograph of *D. auripigmentum* with particles of As_2S_3 on day #8.

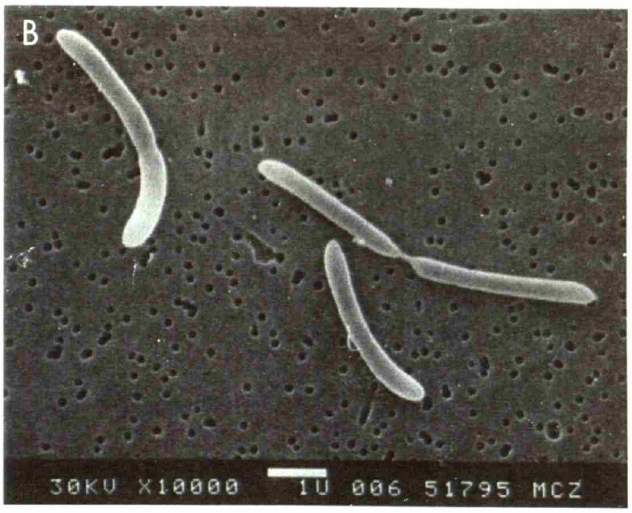
Fig. 2. Mineral analysis. **(A)** A representative EDS X-ray emission spectrum, collected from electron dense particles in a microbial thin-section. Identical spectra were collected from particles of synthetic As_2S_3. The Si, O, and Ni peaks are due to background from the supporting grid. **(B)** The upper left quadrant is a dark field image of a whole-cell mount of *D. auripigmentum*. Moving clockwise, the other quadrants show X-ray maps of As_2S_3, arsenic, and sulfur, respectively. The As_2S_3 map is an overlay of the arsenic and sulfur profiles. The bar represents 1 μm. Maps were acquired on a VG-HB603 STEM.

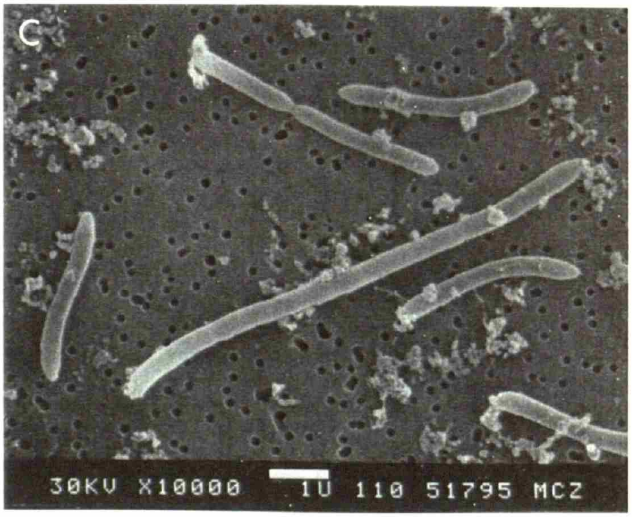
Fig. 3. Redox transformations of As(V) and S(VI). **(A)** As(V) is reduced to As(III) only in the presence of bacteria, and the precipitation of As_2S_3 removes 40-50% of the total arsenic. Error bars represent the range of duplicate cultures. The experiment was performed with an enrichment culture, growing on 10 mM lactate, 1 mM As(V) and 1 mM cysteine. Arrows are drawn where yellow precipitates of As_2S_3 were first observed. **(B)** Reduction of As(V) to As(III) and S(VI) to S(-II) by *D. auripigmentum* growing on 10 mM pyruvate, 1 mM As(V) and 1 mM S(VI).

Fig. 4. Dominance diagram for As_2S_3 precipitation in equilibrium with various As(III) species. Total [S(-II)] assumes the species [H_2S], [HS^-] and [S^{2-}]. "+" precipitation observed abiotically, "-" precipitation not observed abiotically, "∂" precipitation kinetically slow abiotically, and the shaded area indicates precipitation was expected but not observed abiotically. "a+" precipitation observed for *D. auripigmentum*, "a-" precipitation not observed for *D. auripigmentum*, "p-" precipitation not observed for *D. propionicus*, "m-" precipitation not observed for strain MIT-13.

Fig. 5. Transmission electron micrographs of **(A)** intracellular As_2S_3 precipitation **(B)** intracellular and extracellular As_2S_3 precipitation **(C)** As_2S_3 particles associated with a membrane. Bar represents 0.5 μm.

Fig 1





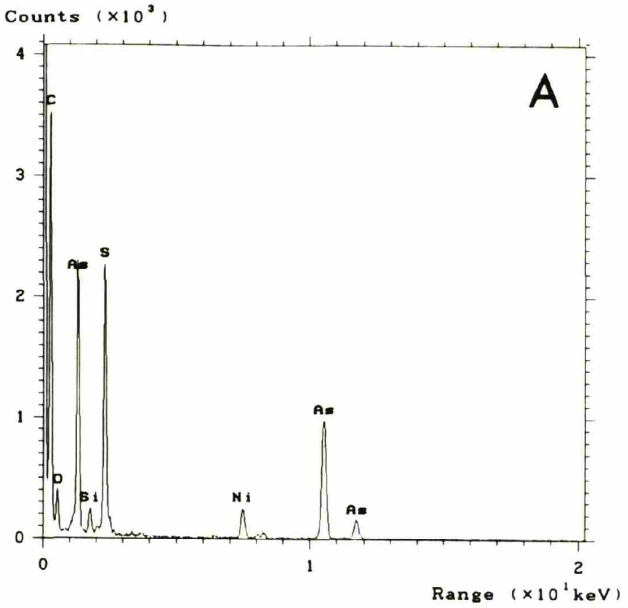


Fig 2

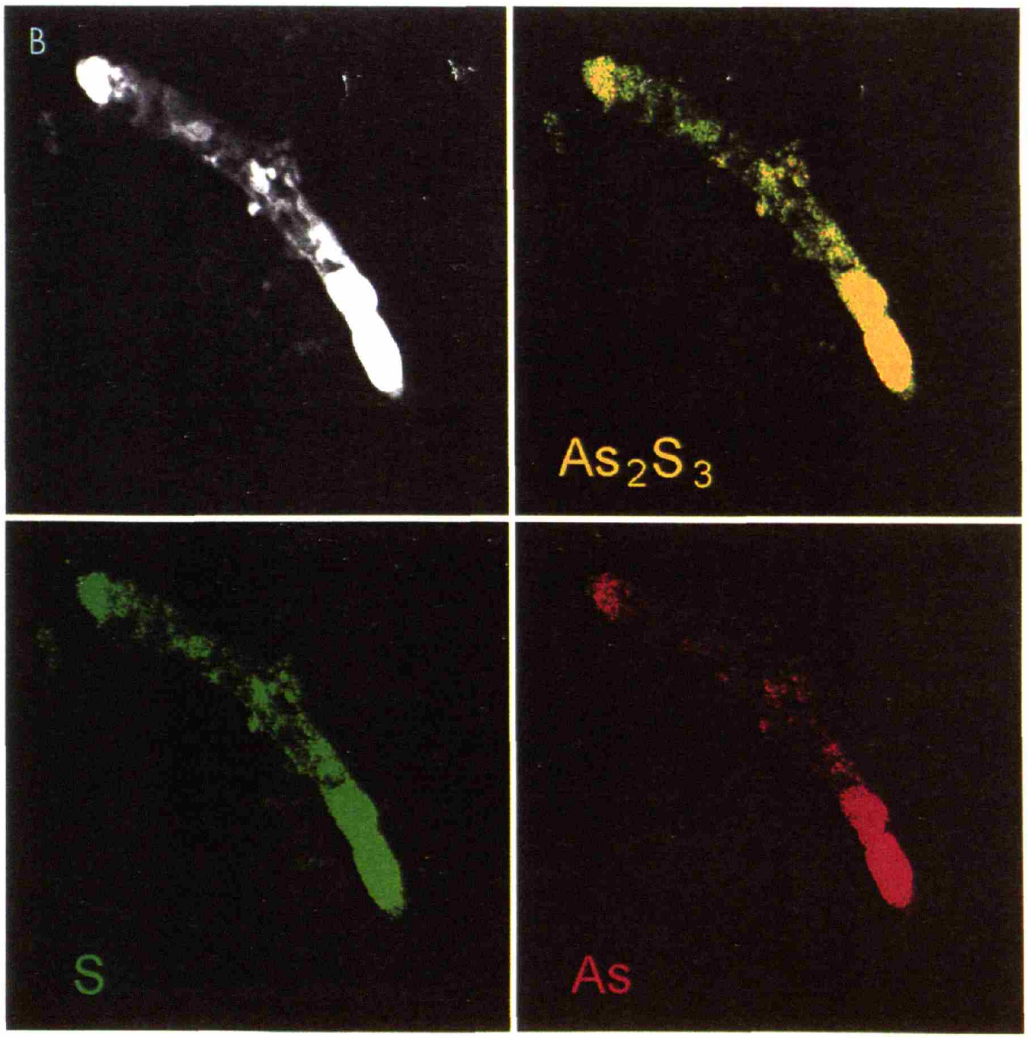


Fig.3

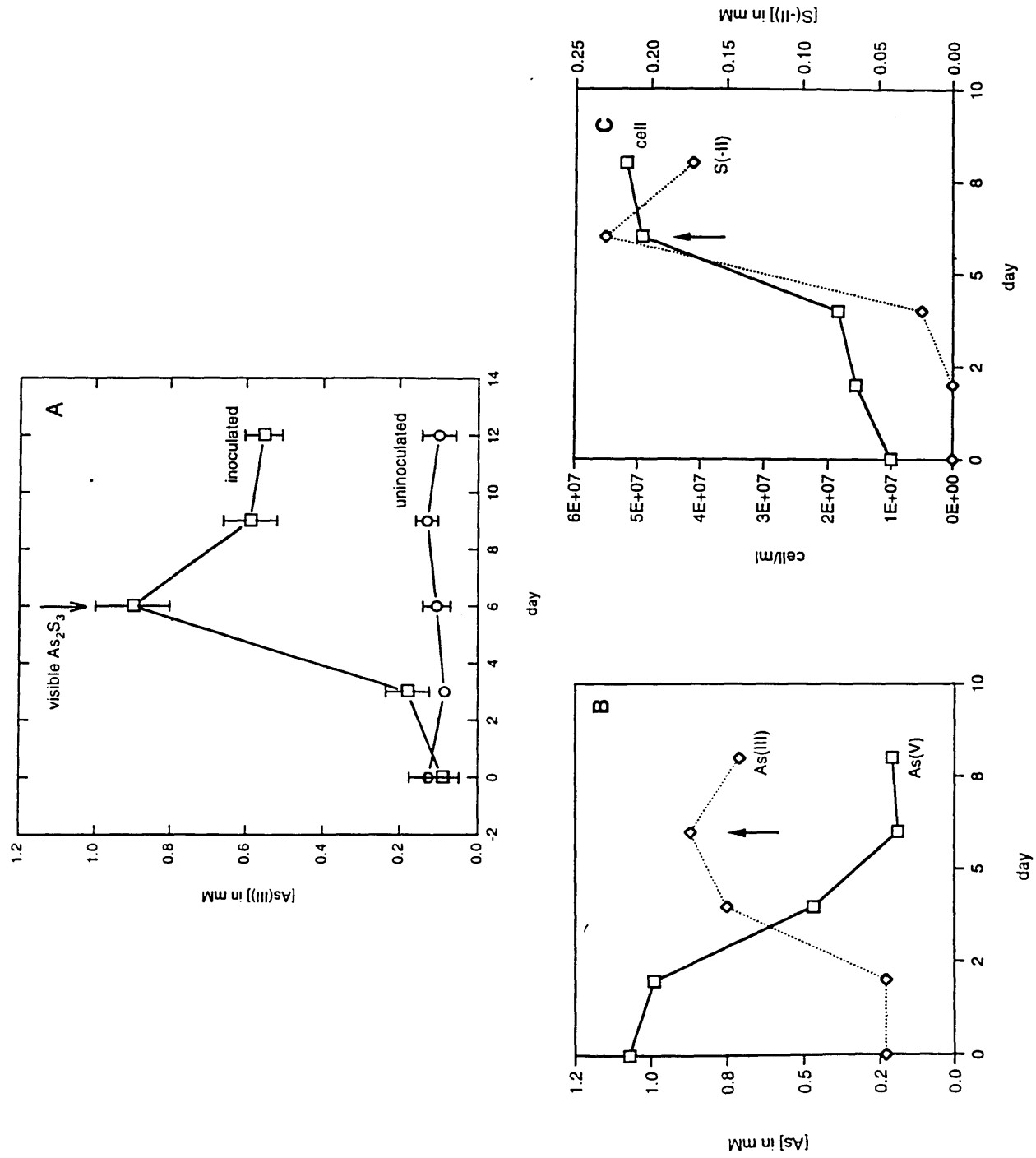

A
visible As_2S_3
inoculated
uninoculated
[As(III)] in mM
day
B
As(III)
As(V)
[As] in mM
day
C
cell
S(-II)
cell/ml
[S(-II)] in mM
day

Figure 4

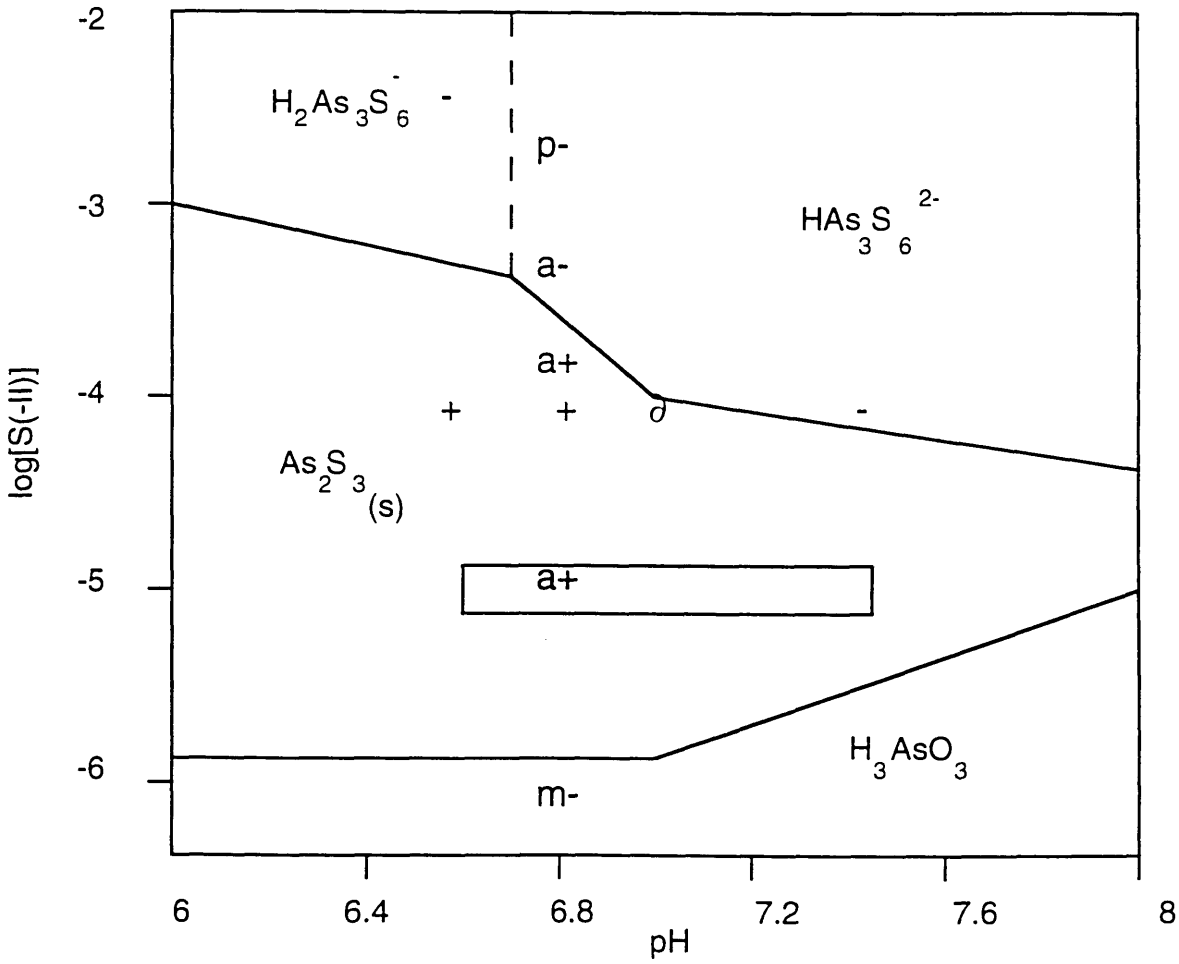
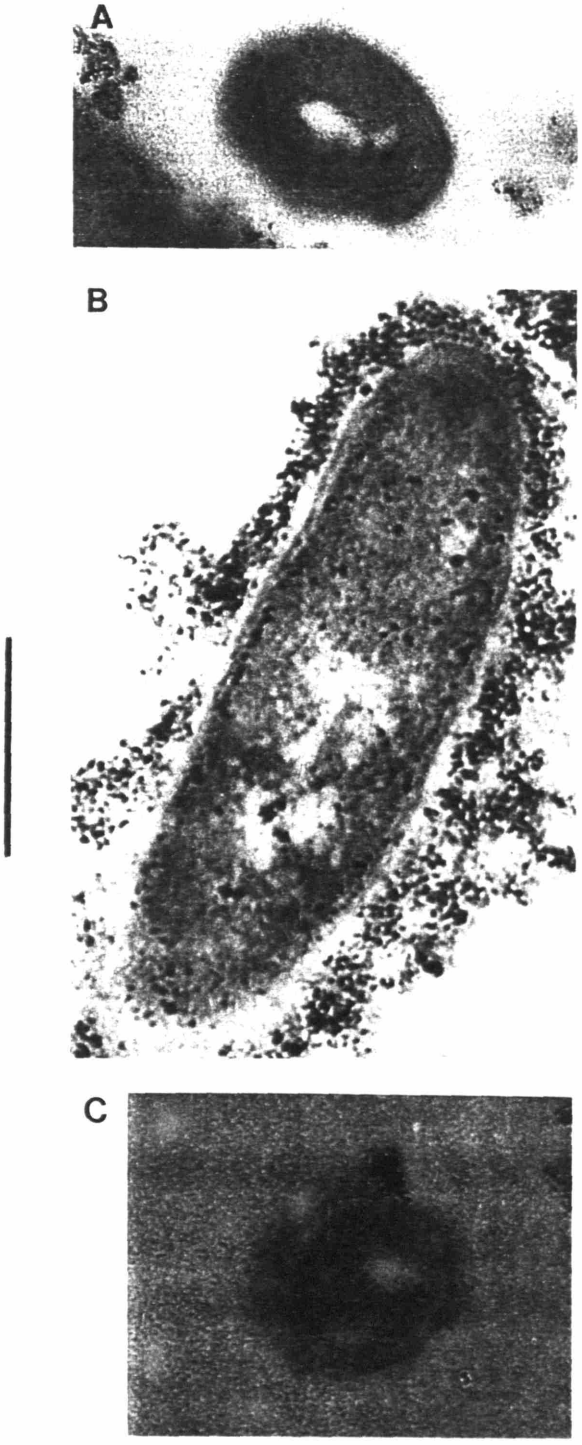

-2
-3
-4
-5
-6
log[S(-II)]
$H_2As_3S_6^-$
-
p-
$HAs_3S_6^{2-}$
a-
a+
+
+
d
-
As_2S_3(s)
a+
H_3AsO_3
m-
6
6.4
6.8
7.2
7.6
8
pH

Fig 5



Chapter 4: Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum aurpigmentum* sp. nov.

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Abstract

A newly discovered arsenate-reducing bacterium, strain OREX-4, differed significantly from strains MIT-13 and SES-3, the previously described arsenate-reducing isolates which grew on nitrate but not on sulfate. In contrast, strain OREX-4 did not respire nitrate but grew on lactate with either arsenate or sulfate serving as the electron acceptor, and even preferred arsenate. Both arsenate and sulfate reduction were inhibited by molybdate. Strain OREX-4, a gram-positive bacterium with a hexagonal S-layer on its cell wall, metabolized compounds commonly used by sulfate reducers. Scorodite [$(FeAsO_4)\cdot 2H_2O$], an arsenate-containing mineral, provided micromolar concentrations of arsenate which supported cell growth. Physiologically and phylogenetically, strain OREX-4 was far removed from strains MIT-13 and SES-3: OREX-4 grew on different electron donors and electron acceptors, and fell within the gram-positive group of the Bacteria whereas MIT-13 and SES-3 fell together in the epsilon subdivision of the Proteobacteria. Together, these results suggest that organisms spread among diverse bacterial phyla can use arsenate as a terminal electron acceptor, and that dissimilatory arsenate reduction might occur in the sulfidogenic zone at arsenate concentrations of environmental interest. 16S rRNA sequence analysis indicated that strain OREX-4 is a new species of the genus *Desulfotomaculum*, and accordingly, the name *Desulfotomaculum auripigmentum* is proposed.

Introduction

Arsenic is relatively abundant in the environment owing to contamination from a variety of anthropogenic sources in addition to its natural occurrence in minerals (Bumbla and Keefer, 1994). Over the past three decades, evidence has been growing that suggests microorganisms play an important role in arsenic's geochemical cycle despite its toxicity. Bacteria and phytoplankton have been described which oxidize (Osborn and Ehrlich, 1976), methylate (Anderson and Bruland, 1991), and reductively detoxify arsenic (Cervantes et al. 1994), and recently, organisms have been found which grow by reducing arsenate (As(V)) to arsenite (As(III)) during anaerobic respiration (Ahmann et al. 1994; Laverman et al. 1995). Indeed, evidence is mounting that dissimilatory arsenate reduction may be an important process in environments where arsenic concentrations are high (Dowdle et al. 1995). For example, arsenic in the contaminated Aberjona Watershed in eastern Massachusetts (from which the isolate described in this study originates) has been measured at concentrations as high as 1 mM (Aurilio 1994).

Previous discussion of arsenate reducing microorganisms has suggested that arsenic may inhibit a variety of ecologically important anaerobic respiratory processes (Dowdle et al. 1996). This conclusion was based upon a study of subsurface aquifer sediments where arsenic was shown to inhibit denitrification (Bradley and Chappelle 1991) and a study of various anoxic sediments which showed that arsenate and/or arsenite dramatically inhibited sulfate reduction and methanogenesis presumably through toxic effects (Dowdle et al. 1996). Furthermore, in the latter study, molybdate was found to have no effect on arsenate reduction, and thus it was argued that sulfate-respiring bacteria were not involved in dissimilatory arsenate reduction in these sediments. This finding fit the physiological characterization of the previously described arsenate-reducing isolates, MIT-13 (Ahmann 1994) and SES-3 (Laverman et al. 1995)--which are unable to reduce sulfate--and allowed the inference to be made that the inhibition of sulfate-reduction by arsenic in these sediments was non-competitive (Dowdle et al. 1996). We now report that a novel arsenate-reducer, strain OREX-4, possesses metabolic abilities that challenge the conclusions drawn regarding the compatibility of dissimilatory arsenate reduction with sulfate reduction.

Because OREX-4 is physiologically and phylogenetically distinct from the two other known arsenate-reducers, it now seems possible that microorganisms which respire arsenate may occupy wider niches than previously believed.

Materials and Methods

Isolation and cultivation.

Surface sediments were taken from the Upper Mystic Lake, located in Woburn, Mass (Aurilio et al. 1994; Spliethoff et al. 1995). Initial enrichments were made in freshwater minimal medium, amended post-autoclaving with sterile 14 mM sodium sulfate (Na_2SO_4), 20 mM sodium lactate and 1 mM cysteine under a N_2:CO_2 atmosphere (80:20 v/v). After several transfers, the enrichment was inoculated into medium where 10mM dibasic sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) was substituted for sulfate. A yellow precipitate formed, which was determined to be As_2S_3 (cysteine was the source of reduced sulfur in this medium) (Newman et al. 1997). The bacterium responsible for this precipitate, strain OREX-4, was isolated by successive colony transfers in agar shake tubes (Pfennig 1981), and maintained on 5-20 mM lactate, 1-10mM arsenate, and 1-14 mM sulfate. In each shake tube series, single yellow colonies were picked for further purification. The medium was buffered at a pH of 6.8 with $NaHCO_3$ (1.9 g/l), and reduced with $PdCl_2$ + H_2 (300 µg/ml) (Aranki 1972). Salts supplied per liter of medium were: 0.14 g KH_2PO_4, 0.25 g NH_4Cl, 0.50 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 1.0 g NaCl, and 0.62 g $MgCl_2 \cdot 6H_2O$. The medium contained per liter: 0.05 mg *p*-aminobenzoic acid, 0.02 mg biotin, 0.05 mg nicotinic acid, 0.05 mg calcium pantothenate, 0.05 mg thiamine HCl, 0.1 mg pyridoxin HCl (B_6), and 0.001 mg cyanocobalamin (B_{12}); trace metals supplied per liter were: 0.001 ml conc. HCl, 0.1 mg $MnCl_2 \cdot 4H_2O$, 0.12 mg $CoCl_2 \cdot 6H_2O$, 0.07 mg $ZnCl_2$, 0.06 mg H_3BO_3, 0.025 mg $NiCl_2 \cdot 6H_2O$, 0.015 mg $CuCl_2 \cdot 2H_2O$, 0.025 mg $Na_2MoO_4 \cdot 2H_2O$, and 1.5 mg $FeCl_2 \cdot 4H_2O$. All preparations and manipulations were performed under strict anoxic conditions using the Hungate technique (Miller 1974). Cultures were incubated in the dark at 25°C unless otherwise noted.

Analyses.

Total arsenic (oxidized sample), arsenate (untreated sample) and background phosphate (reduced sample) were measured directly by the molybdenum blue spectrophotometric assay (Johnson and Pilson 1972); arsenite was indirectly determined by measuring the difference between the oxidized and untreated samples. Sulfate was measured by a slight modification of the barium-sulfate

technique, the $BaCl_2$ gel was prepared by microwaving for convenience (Tabatabai 1974). Sulfide was measured by the methylene blue method (Franson 1981). Samples were taken directly for arsenic and sulfide measurements, but were filtered through a 0.2 μm cellulose filter (Nalgene) before sulfate measurements were taken. Cells were stained with DAPI and counted. Lactate and acetate concentrations were determined by HPLC on a polysulfonate ion exclusion column (Hamilton PRP X 300) with UV detection at 210 nm.

Growth experiments.

The optimal pH and temperature ranges for growth of strain OREX-4 were determined by growing the organism on sodium lactate (10 mM) and sodium sulfate (10mM) and performing direct cell counts. The pH range tested was 6.0-8.0 as set by sterile additions of 0.1 M HCl or 0.1 N NaOH to TES (N-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid) buffered medium (for the pH experiments, the medium was the same as given above, except that TES (4.6 g/l) and $NaHCO_3$ (1 g/l) were used in place of $NaHCO_3$ (1.9 g/l) alone) and measured after autoclaving. The temperature range studied at pH = 6.8 was 17 to 50°C, incrementing by roughly 5°C. Alternative electron donors and acceptors were added to the medium from anoxic sterile stocks to give the concentrations listed in Table 1. Colloidal elemental sulfur (S^o), synthetic MnO_2, and Fe(III) gel were added to the medium as previously described (Blumentals et al. 1990, Lovely and Phillips 1988). Hydrophobic hydrocarbon electron donors (toluene, benzene, and cyclohexane) were prepared in degassed mineral oil at 2% (v/v) (Rabus et al. 1993). In all of the growth experiments, cysteine-HCl (0.025% w/v) was used as the reductant in place of $PdCl_2$.

Growth on an arsenate mineral.

An arsenate-containing mineral, scorodite [$(FeAsO_4)\cdot 2H_2O$], was obtained from the Princeton University mineral collection and ground through a mesh screen to 40-70 μm. A Scintag PAD-V Automated Powder X-ray Diffractometer (XRD) was used to confirm its identity and purity. One gram of the ground sample was added to each culture bottle containing 20 ml

medium , and autoclaved prior to inoculation. XRD analysis confirmed the mineral's identity after autoclaving. Dissolved arsenate and arsenite (samples passed through a 0.2 μm cellulose filter (Nalgene)) and cell growth were measured on day 0 and day 14.

16S rRNA (rDNA) sequencing and phylogenetic analysis.
Nucleic acids were isolated from a cell pellet of strain OREX-4 and the partial 16S rRNA gene was amplified using eubacterial primers 50F and 1492R and sequenced as described previously (Caccavo 1994). Sequencing was performed at the Michigan State University Sequencing Facility. Preliminary identification of strain OREX-4 was obtained using the Blast program (Altschul 1990). Sequences alignments were performed manually or were obtained from the Ribosomal Database Project (Maidak 1996). The phylogenetic tree was inferred using the distance matrix method with the least squares algorithm (De Soete 1983). The method of Jukes and Cantor was used to compute the evolutionary distances (Jukes and Cantor 1969).

Nucleotide sequence accession numbers.
The 16S rRNA sequences of *Campylobacter helveticus* (Cam.helvet), *Campylobacter jejuni* (Cam.jejun5) *Clostridium quercicolum* (C.quercico), *Desulfitobacterium dehalogenans* (Dfi.dehalo), *Desulfobulbus propionicus* (Dbb.propio), *Desulfotomaculum nigrificans* (Dfm.nigrif), *Desulfotomaculum orientis* (Dfm.orient), *Desulfotomaculum ruminis* (Dfm.rumin2), *Desulfovibrio desulfuricans* (Dsv.desulf), *Desulfovibrio vulgaris* (Dsv.vulgar), *Escherichia coli* (E.coli), *Selenomonas lacticifex* (Sln.lactic), and *Staphylococcus aureus* (Stp.aureus) were obtained from the Ribosomal Database Project (Maidak 1996). The 16S rRNA sequences of strain MIT-13 and strain SES-3 and *Desulfitobacterium hafniense* were obtained from Genbank (accession numbers U85964, U41564 and X94975, respectively). The partial 16S rDNA sequence of strain OREX-4 has been submitted to Genbank (accession number U85624).

Transmission electron micrographs. Thin sections of strain OREX-4 grown on lactate and sulfate were cut from pellets embedded in L.R. White resin as

previously described (Newman et al. 1997). Cells were negatively stained with 2% uranyl acetate to visualize the S-layer.

Results

Cell and Colony Morphology.

Strain OREX-4 is a gram-positive, slightly curved, non-motile rod, which does not appear to contain endospores. Cells are approximately 2.5 μm in length and about 0.4 μm in diameter, and electron micrographs show the murein sacculus (peptidoglycan) of strain OREX-4 to be thinner than usual for a gram-positive bacterium (Fig. 1A). A hexagonal surface array (S-layer) is attached to its cell wall (Fig. 1B). On solid medium amended with sulfate but no arsenate, the cells are light gray. On solid medium amended with both arsenate and sulfate, the cells are bright yellow, due to precipitation of As_2S_3 (Newman et al. 1997).

Respiration of arsenate and sulfate.

Strain OREX-4 grew exponentially while oxidizing lactate to acetate when either arsenate or sulfate was provided as the electron acceptor. For cells grown on lactate and arsenate, the average molar ratio (of duplicate cultures) of acetate produced to arsenite produced was 0.47 (0.49-0.46) (Fig. 2); for cells grown on lactate and sulfate, the average molar ratio of acetate produced to sulfide produced was 1.65 (1.73-1.56) (data not shown). When it is considered that some carbon was probably incorporated into cell biomass, these results are in fairly good agreement with the theoretical values of 0.5 and 2 as predicted by the following reactions:

$$CH_3\text{-}CHOH\text{-}COO^- + 2HAsO_4^{2-} + 4H^+ \rightarrow CH_3\text{-}COO^- + 2HAsO_2 + CO_2\,(g) + 3H_2O$$

$$CH_3\text{-}CHOH\text{-}COO^- + 0.5SO_4^{2-} + 0.5H^+ \rightarrow CH_3\text{-}COO^- + 0.5HS^- + CO_2\,(g) + H_2O$$

Final cell numbers of strain OREX-4 were proportional to the arsenate concentration in the presence of 5 mM lactate, further demonstrating its use of arsenate as an electron acceptor (Fig. 3). It appears that higher arsenate concentrations eventually inhibit growth, perhaps because of the build up of high arsenite: growth slowed and the rate of arsenate reduction decreased

after 5 mM arsenate had been reduced in cultures supplied with 10 mM arsenate. Growth on non-fermentable substrates (H_2 + acetate and arsenate; H_2 + acetate and sulfate) further established that arsenate and sulfate are used as electron acceptors (data not shown). Arsenate was never observed to be reduced further than to arsenite.

In all cultures where arsenate and sulfate were added together, we observed arsenate reduction to precede that of sulfate. This was examined by growing OREX-4 in medium amended with 1 mM arsenate, 10 mM sulfate and 20 mM lactate. Arsenate reduction was nearly complete by the time sulfate reduction began (days 2-4) (Fig. 4A). On day 4, As_2S_3 precipitated in the medium, causing small amounts of sulfide to be removed from solution (Fig. 4B) (The arsenite and sulfide present in As_2S_3 are not measured by either the molybdenum blue or methylene blue assay). As time progressed, sulfide levels began to rise again as more and more sulfate was reduced, yet the rate of sulfate reduction was poor, with most of the sulfide going to As_2S_3 (Fig. 4B). Preferential reduction of arsenate to sulfate was also observed when the medium was amended with 5 times the concentration of arsenate (data not shown). A complementary experiment was performed in medium amended with 2.5 mM arsenate, 1 mM sulfate and 10 mM lactate with samples previously grown on arsenate alone, on sulfate alone, and on both arsenate and sulfate. Arsenate reduction rapidly commenced in the bottles that had been inoculated from an arsenate culture, whereas bottles inoculated from sulfate or dual-substrate cultures took slightly longer to reduce the same amount of arsenate (data not shown). More than 1 mM arsenate was reduced before a significant fraction of the sulfate had been depleted, and by the end of the experiment, only 0.5 mM sulfate had been reduced, yet all of the 2.5 mM arsenate had been reduced to arsenite (data not shown).

To verify that arsenate reduction in the presence of sulfate did not result from bacterial sulfide production and then subsequent abiotic reduction of arsenate, we studied the kinetics of arsenate reduction by sulfide over the concentration range of interest. Bottles of 1 and 5 mM arsenate were amended with several concentrations of sulfide (0, 0.3, 1.0 and 3.0 mM), and arsenate reduction was followed over a 2 week period. 1 mM thiosulfate and 1 mM sulfite were also tested for their ability to reduce arsenate. Reduction of arsenate by sulfide was kinetically slow, and thiosulfate and sulfite showed

negligible reduction of arsenate. An apparent second-order rate constant of 20-25 $M^{-1}day^{-1}$ for the reduction of arsenate by sulfide enabled us to fit all experimental data, assuming a 1:1 stoichiometry of arsenate reduced by sulfide. This is shown for the 5 mM arsenate data set in Fig. 5. Assuming k = 25 $M^{-1}day^{-1}$, an initial arsenate concentration of 2.5 mM and an initial sulfide concentration of 0.5 mM, we predict a maximum of 0.2 mM arsenate to be reduced over 10 days. This is well below the concentration of arsenate reduced in our microbial experiments (2.5 mM arsenate reduced in 2 days) given these conditions.

When cultures growing on either 1 mM arsenate and 1 mM sulfate, or 1 mM arsenate alone, were amended with 1 mM sodium molybdate, near total inhibition (95%) of arsenate reduction was achieved (Fig. 6). As expected, complete inhibition of sulfate reduction was also observed for cultures growing on 10 mM sulfate alone (data not shown).

Growth conditions and substrates.

Strain OREX-4 has a temperature optimum between 25-30°C, and a pH optimum between 6.4-7.0. The ability of strain OREX-4 to utilize a wide variety of electron donors and acceptors was tested and compared to those of strains MIT-13 and SES-3 (Table 1). Strain OREX-4 can obtain energy for growth by coupling the reduction of sulfate to the oxidation of pyruvate, ethanol, glycerol, butyrate, formate, and malate, in addition to lactate. Strain OREX-4 can also ferment pyruvate and appears to be capable of autotrophic growth on H_2. For incubation times of approximately one week, strain OREX-4 grows best on pyruvate (10^8 cells/ml) and glycerol (10^8 cells/ml), well on lactate (10^7 cells/ml), but only poorly on butyrate (10^6 cells/ml) when sulfate serves as the electron acceptor, and both the electron acceptor and the electron donor are provided at concentrations of 10mM. In addition to arsenate and sulfate, electron acceptors that support growth include thiosulfate, sulfite, and fumarate.

Growth on Scorodite.

Strain OREX-4 was able to utilize scorodite [$(FeAsO_4)\cdot 2H_2O$] as a source of arsenate for respiratory growth. Scorodite is one of the most abundant arsenate-containing minerals (Azcue and Nriagu 1994), and thus, representative of what bacteria might encounter in the natural environment.

Uninoculated controls show the dissolution of scorodite to be slow: less than 1 mM of arsenate dissolved abiotically over the course of the experiment (14 days). No growth was observed in inoculated controls lacking scorodite (cell numbers remained constant at 2 X 10^6 cells/ml). In cultures amended with 10 mM acetate, 1 atm H_2 and 1 g scorodite, however, cell numbers increased significantly (from 2 X 10^6 cells/ml initially to 2 X 10^7 cells/ml at the end of the incubation) as dissolved arsenate decreased by approximately 300 μM and dissolved arsenite increased by approximately 400 μM. Because growth of OREX-4 by a factor of 10 was greater than expected given these measured concentrations of arsenate and arsenite (compare to Fig. 3, where 1 mM arsenate produces an equivalent increase in cell numbers), it is probable that more arsenate was reduced than could be measured due to adsorption of arsenite onto scorodite.

Phylogenetic analysis.

Phylogenetic analysis of the nearly complete 16S rDNA sequence of strain OREX-4 placed it within the gram positive phylum of the Bacteria (Maidak 1996) (Figure 7). In contrast, 16S rRNA based phylogenetic analysis of the other known arsenate reducers, strain MIT-13 and strain SES-3, has placed these two microorganisms within the epsilon subdivision of the Proteobacteria (unpublished results; Lonergan et al. 1996). Thus, strain OREX-4 represents the first known example of a gram positive microorganism able to obtain energy for growth from the reduction of arsenate.

The closest known relative of strain OREX-4 is *Desulfotomaculum orientis* (96.2% sequence identity, 1020 nucleotides considered). Based on the consistent phylogenetic placement of strain OREX-4 within the *Desulfotomaculum* group of the gram positive phylum regardless of the algorithm used to infer the tree (maximum-likelihood (Felsenstein 1981) or distance-matrix (De Soete 1983), and its phylogenetic relationship and physiological similarity to *D. orientis* as compared to *D. dehalogenans*, we propose that it is a new species within the genus *Desulfotomaculum*: *Desulfotomaculum auripigmentum* (Figure 7).

Discussion

D. auripigmentum is the first example of a sulfate reducing bacterium that can also grow with arsenate as a terminal electron acceptor. No other dissimilatory arsenate reducing bacterium has been found that can also reduce sulfate, although strain SES-3 is capable of reducing other sulfur compounds such as thiosulfate and elemental sulfur (Laverman et al. 1995). *D. auripigmentum* achieves a higher molar growth yield when grown on arsenate (5.6 g cells/mol lactate) than on sulfate (2.3 g cells/mol lactate), assuming a cell dry weight of 2×10^{-10} mg/cell and the measured values of lactate consumed to achieve the observed cell densities for growth on 5 mM arsenate and 10 mM sulfate (Fig. 2). This is not surprising given that arsenate is a better oxidant than sulfate and would correspondingly yield a higher free energy when coupled to the oxidation of lactate to acetate at pH=7 (Peters 1974; Morel and Hering 1993):

$$CH_3\text{-}CHOH\text{-}COO^- + 2HAsO_4^{2-} + 4H^+ \rightarrow CH_3\text{-}COO^- + 2HAsO_2 + CO_2\,(g) + 3H_2O$$

$$\Delta G^{\circ\prime} = -172 \text{ kJ/mol lactate}$$

$$CH_3\text{-}CHOH\text{-}COO^- + 0.5SO_4^{2-} + 0.5H^+ \rightarrow CH_3\text{-}COO^- + 0.5HS^- + CO_2\,(g) + H_2O$$

$$\Delta G^{\circ\prime} = -89 \text{ kJ/mol lactate}$$

The molar growth yield for arsenate is in good agreement with the value found for growth of strain SES-3 on 5 mM arsenate and lactate (5.3 g/mol lactate) (Laverman et al. 1995). When supplied with both electron acceptors, for a time, *D. auripigmentum* reduces arsenate and sulfate concomitantly, yet shows a preference for arsenate. Stoichiometrically, the reduction of arsenate in dual arsenate/sulfate cultures cannot be explained by the abiotic reduction of arsenate by sulfide. For example, in the experiments where 2.5 mM arsenate and 1 mM sulfate were supplied, only 0.5 mM sulfate was consumed over the course of the experiment; even if we assume that all of this was converted to sulfide, stoichiometrically there would still be too little to account for the full reduction of 2.5 mM arsenate (assuming a 2 electron trasfer from sulfide to arsenate). Furthermore, our kinetic data show the

reduction of arsenate by sulfide is too slow to account for our results, regardless of the stoichiometry of the redox reaction

Preference for arsenate raises the question whether sulfate reduction is inhibited by arsenate, an issue of potential importance in contaminated environments. Our data suggest that the pathways for the dissimilatory reduction of arsenate and sulfate are linked in *D. auripigimentum*. Significantly, the rate of arsenate-reduction in cultures amended with both arsenate and sulfate is always faster than that of sulfate reduction, independent of the relative concentrations of arsenate and sulfate or the substrate history of the inoculum. This may reflect the energetic advantage of arsenate over sulfate reduction. Alternatively, the reduction of arsenate to arsenite may be necessary before sulfate is reduced either because arsenate is toxic to the bacterium (and reduction to arsenite, although itself toxic, allows As to be exported) or because it inhibits sulfate reduction biochemically. This last possibility is reinforced by the near total inhibition of arsenate reduction by molybdate. Since molybdate is a known inhibitor of sulfate reduction (Oremland and Capone 1988), the enzyme ATP sulfurylase (which activates the sulfate ion) may also be involved in the reduction of arsenate to arsenite. Of course, it is also possible that by causing ATP sulfurylase to form a futile cycle, molybdate simply forces the cell to run out of energy, and this indirectly affects arsenate metabolism.

In a recent study, the lack of inhibition of arsenate reduction by molybdate was used to argue against the involvement of sulfate-respiring bacteria in dissimilatory arsenate reduction in certain anoxic sediments (Dowdle et al. 1996). The discovery of *D. auripigmentum*, a sulfate reducer which also reduces arsenate, suggests that this might not be true for all environments. In the experiments of Dowdle et al., 8-10 mM arsenic (arsenate or arsenite) completely inhibited sulfate reduction, however, sulfate reduction has been observed in other sedimentary enrichments in the presence of 1.3 mM arsenite (Rittle et al. 1995). In an attempt to reconcile these results, it was suggested that due to its toxicity, the concentration of arsenic determined the degree to which sulfate reduction was inhibited (Dowdle et al. 1996). Our data showing consistent preferential reduction of arsenate to sulfate in *D. auripigmentum*, however, suggest another possible explanation: toxicity aside, arsenate may competitively inhibit sulfate

reduction in some highly contaminated environments because its reduction is more energetically favorable. Indeed, it is possible that dissimilatory arsenate reduction in contaminated sediments differs from community to community, and while some may contain bacteria such as strains MIT-13 and SES-3 (which are unaffected by molybdate and cannot respire sulfate), others may harbor organisms more similar to *D. auripigmentum*. As shown in Table 1, strains MIT-13 and SES-3 fundamentally differ from *D. auripigmentum* in important metabolic capabilities (such as their ability to use nitrate, Fe(III), or sulfate as electron acceptors), and thus, it would not be surprising if the pathway responsible for the dissimilatory reduction of arsenate in *D. auripigmentum* differed from that responsible for this reduction in strains MIT-13 and SES-3. *D. auripigmentum* is a gram-positive bacterium whereas strains SES-3 and MIT-13 are gram negative (Laverman et al. 1995, Ahmann 1996). Precedent for biochemical differences in arsenate reduction between gram-positive and gram-negative bacteria can be found in comparisons between *Staphylococcus aureus* and *Escherichia coli* (Ji et al. 1994, Gladysheva et al. 1994), which couple non-respiratory arsenate reduction to the oxidation of thioredoxin and glutaredoxin, respectively.

The concentration of arsenic in most environments is very low (nM), and thus, we would normally expect only a negligible fraction of the organic carbon pool to be mineralized through dissimilatory arsenate reduction. The capacity to grow by dissimilatory arsenate reduction, however, may provide a selective advantage to microorganisms in some particular environments. In the anoxic hypolimnion of the Upper Mystic Lake (from which *D. auripigmentum* was isolated), significant amounts of both arsenite and arsenate (>200 nM) were found to persist in sulfidic waters (25μM) (Spliethoff et al. 1995). Interestingly, the sampling date when *D. auripigmentum* was retrieved from the surface sediments was the only time during the year when sulfide appeared in the lake, and one of only two times when the hypolimnetic concentrations of arsenite exceeded those of arsenate (Spliethoff et al. 1995). While this chemical profile fits with what we might expect if arsenate-reducing bacteria were active in this environment, clearly more work would be necessary to demonstrate *in situ* microbial arsenate-reduction: since *D. auripigmentum* was isolated from an enrichment culture, it is difficult to draw conclusions about its prevalence and/or arsenate-reducing

activity in the Upper Mystic Lake without further study. Specific molecular probes (both organismal and functional) present one attractive way to build on culture studies such as this one to assess the importance of microbial arsenate reduction in the environment. The ability of *D. auripigmentum* to grow on scorodite (which essentially supplies low concentrations of arsenate), suggests that microbial arsenate-reduction might occur in uncontaminated environments in the presence of natural arsenate-containing minerals. Nonetheless, it is only in highly contaminated environments, such as certain locations in the Abjerjona Watershed where arsenic concentrations have been measured at mM levels (Aurilio 1994), that we would expect dissimilatory arsenate-reduction to significantly contribute to the mineralization of organic matter.

Description of *Desulfotomaculum auripigmentum* sp. nov.

Desulfotomaculum auripigmentum (au.ri.pig.men'tum) sp. nov. N. L. chem. n. *auripigmentum* golden pigment, because it reduces arsenate and sulfate, causing As_2S_3 to precipitate (Newman et al. 1997). *D. auripigmentum* is a freshwater, gram positive, non motile, strictly anaerobic chemoorganotroph with sausage-shaped cells, 2.5µm in length by 0.4µm in diameter. The murein sacculus of *D. auripigmentum* is thinner than usual for gram-positive bacteria, and a hexagonal S-layer is attached to its cell wall. *D. auripigmentum* grows by oxidizing H_2, lactate, pyruvate, butyrate, ethanol, glycerol and malate with the concomitant reduction of sulfate. *D. auripigmentum* is an incomplete oxidizer, producing acetate from its organic substrates. *D. auripigmentum* can also obtain energy for growth by the reduction of thiosulfate, sulfite, and fumarate. *D. auripigmentum* has a temperature optimum of 25-30 °C and a pH optimum of 6.4-7.0 for growth.

The type strain of *Desulfotomaculum auripigmentum*, strain OREX-4, was enriched from freshwater sediment samples taken from the Upper Mystic Lake in Woburn, Mass with lactate as the electron donor and arsenate and sulfate as the electron acceptors. The strain has been deposited in the American Type Culture Collection (ATCC 700205). The 16S rRNA sequence has been deposited in GenBank (accession number U85624).

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Figure legends

FIG. 1. Transmission electron micrographs of strain OREX-4. (A) Thin section profile showing the gram positive cell wall. PG points to the peptidoglycan layer, PM points to the plasma membrane, and SL points to the S-layer. Bar = 200 nm (B) A fragment of the hexagonal surface array (S-layer) of strain OREX-4, negatively stained with 2% uranyl acetate. Bar = 200 nm

FIG. 2. Growth of strain OREX-4 on 10 mM lactate with 5 mM arsenate as the electron acceptor. Growth coincides with the stoichiometric evolution of acetate and the production of arsenite. *Open squares* arsenite production; *open circles* cell density; *filled circles* acetate production. Symbols represent single data points which are representative of duplicate cultures.

FIG. 3. Cell growth in response to increasing concentrations of arsenate. Cultures were grown on 5 mM lactate, and 0, 1, 5, and 10 mM arsenate. *Open symbols* represent values for arsenate concentrations; *closed symbols*, cell numbers. *Squares* = 0 mM arsenate; *diamonds* = 1 mM arsenate; *circles* = 5 mM arsenate; *triangles* = 10 mM arsenate. Symbols represent single data points which are representative of duplicate cultures.

FIG. 4. Preferential reduction of arsenate in comparison to sulfate. Cultures were amended with 20 mM lacate, 1 mM arsenate and 10 mM sulfate. (A) Arsenate reduction to arsenite; *closed triangles* arsenate, *open triangles* arsenite. (B) Sulfate reduction to sulfide; *closed circles* sulfate, *open circles* sulfide. Arrows indicate precipitation of As_2S_3. Symbols represent the means of duplicate cultures, and bars indicate the data range.

FIG. 5. Kinetics of arsenate reduction by various concentrations of sulfide. pH = 6.8, ionic strength = 0.038 M. Data points represent single measurements. *Open squares* 0 mM sulfide, *closed triangles* 0.3 mM sulfide, *closed circles* 1 mM sulfide, *closed triangles* 3 mM sulfide. The lines were fit to the data by modeling the disappearence of arsenate over time using the measured initial concentrations of arsenate and sulfide (1:1 stoichiometry), and $k = 25\ M^{-1}day^{-1}$ as a second-order rate constant.

FIG. 6. Molybdate inhibition of arsenate reduction. Cultures were grown on 10 mM pyruvate and 1 mM arsenate, 1 mM sulfate, and 1 mM molybdate as indicated. *Open squares* arsenate + molybdate, *open circles* arsenate + sulfate + molybdate, *closed squares* arsenate + molybdate. The data are representative of duplicate cultures.

FIG. 7. Phylogenetic tree inferred from partial 16S rDNA sequences showing the placement of *Desulfotomaculum auripigmentum* (strain OREX-4) within the *Desulfotomaculum* group of the gram positive bacteria (Maidak 1996).

The phylogenetic tree was inferred from 913 positions using the least squares algorithm (De Soete 1983) with evolutionary distances computed by the method of Jukes and Cantor (Jukes and Cantor 1969). Bar length represents one evolutionary distance unit.

Fig 1

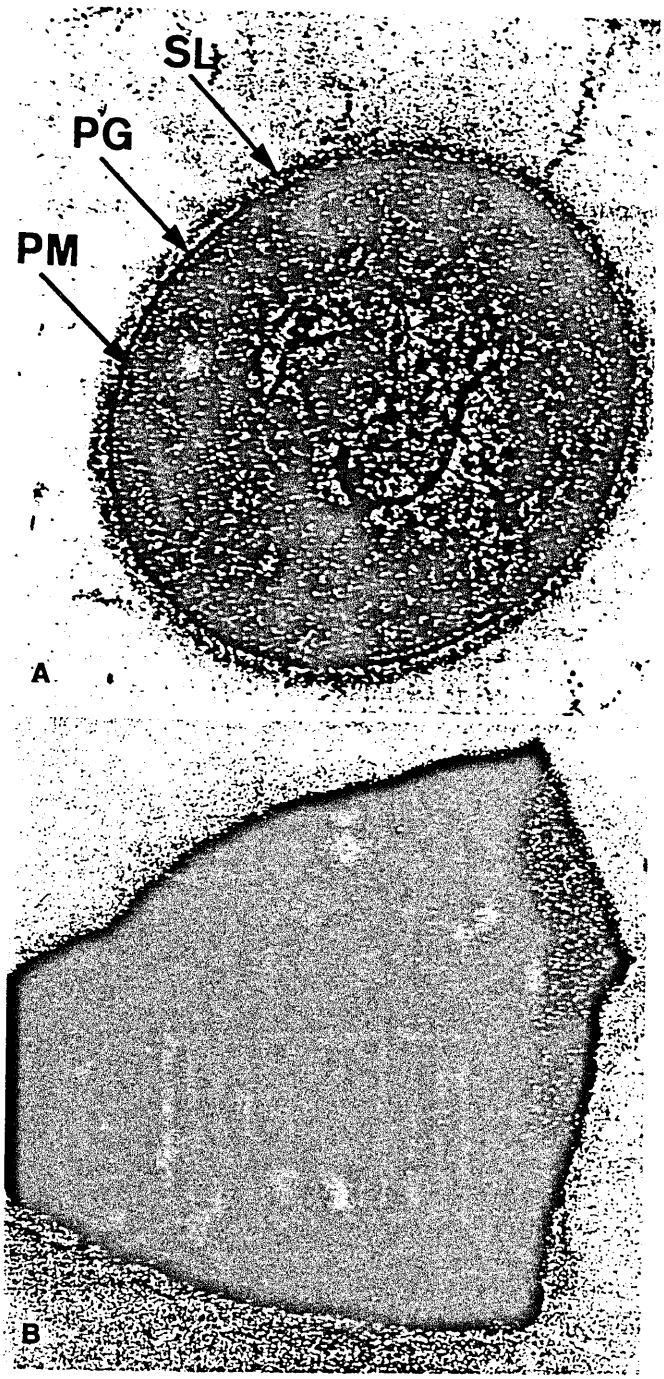


Fig 2.

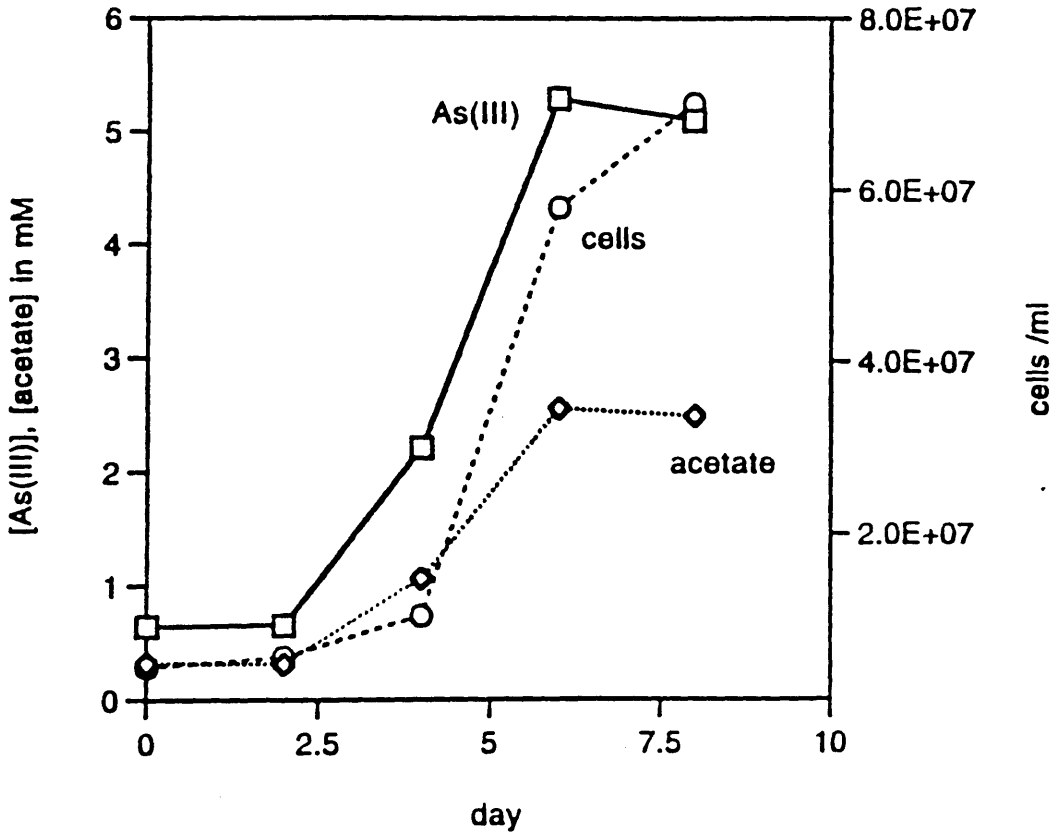

6
5
4
3
2
1
0
8.0E+07
6.0E+07
4.0E+07
2.0E+07
As(III)
cells
acetate
0
2.5
5
7.5
10
day
[As(III)], [acetate] in mM
cells /ml

Fig 3.

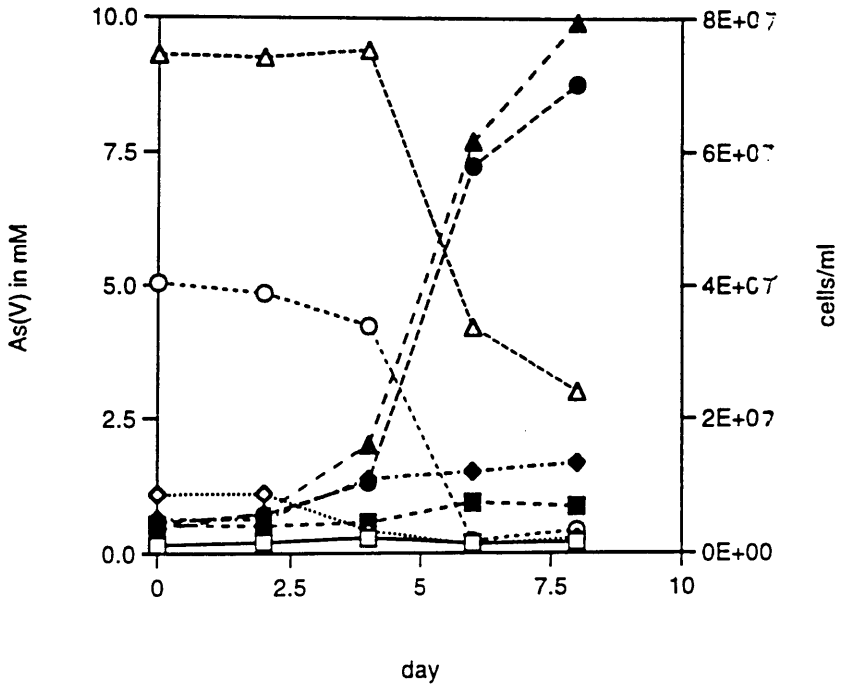
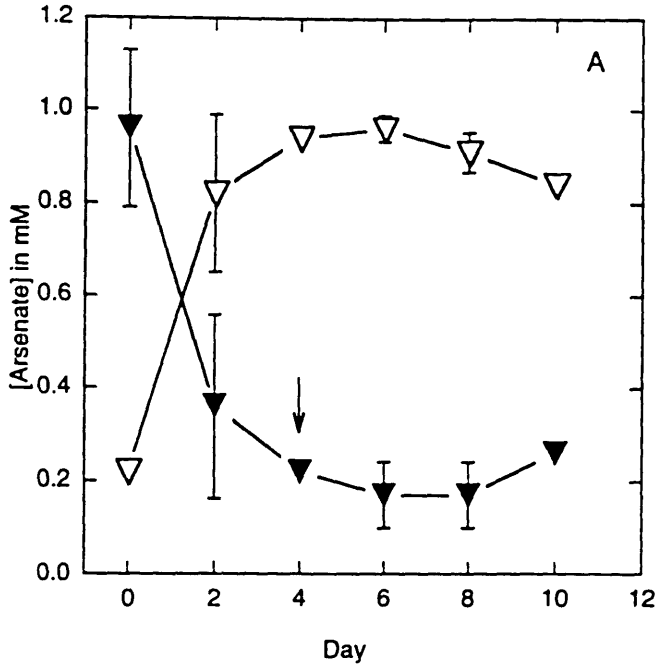

10.0
7.5
5.0
2.5
0.0
As(V) in mM
0
2.5
5
7.5
10
day
8E+07
6E+07
4E+07
2E+07
0E+00
cells/ml

Fig. 4


A
[Arsenate] in mM
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
Day

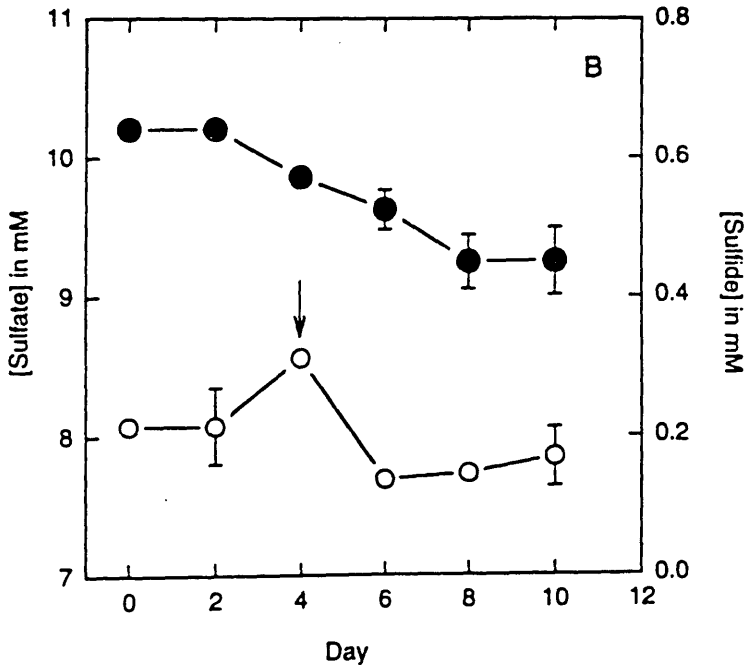

B
[Sulfate] in mM
[Sulfide] in mM
11
10
9
8
7
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
Day

Fig. 5

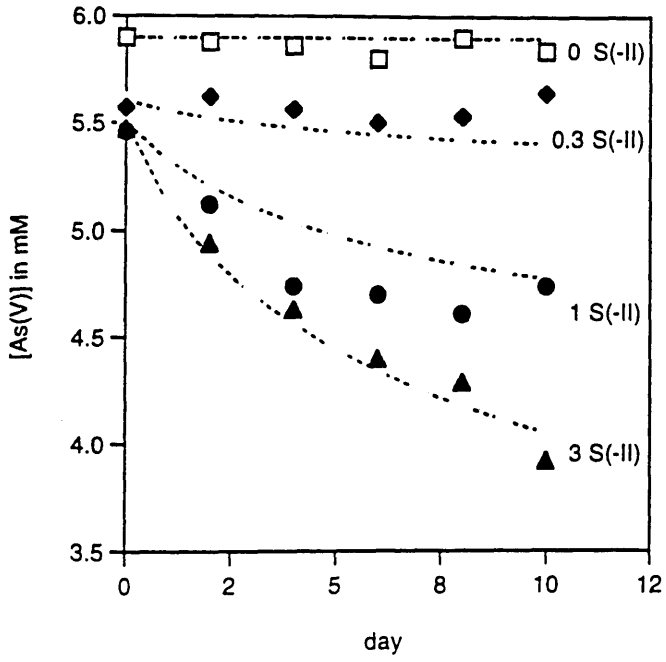


Fig. 6

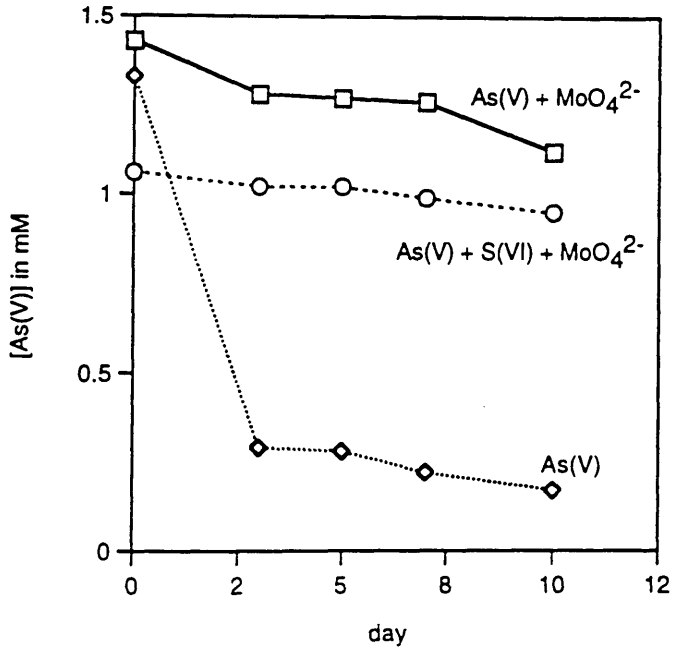


TABLE 1 . Compounds tested as electron donors and electron acceptors for *D. auripigmentum* (strain OREX-4), compared to SES-3 and MIT-13

Electron Donor[a] (mM)	OREX-4	SES-3	MIT-13	Electron Acceptor[b] (mM)	OREX-4	SES-3	MIT-13
Lactate (10)	+	+	+	Nitrate (5)	-	+	+
Acetate (10)	-	-	-	Mn(IV) (20)	-	+	-
H_2 + acetate (1atm +10)	+	+	+	Fe(III) (50)	-	+	0
H_2 (1atm)	+	-	-	Sulfate (10)	+	-	-
Pyruvate (10)	+	0	+	Sulfite (5)	+	0	0
Butyrate (5)	+	0	+*	Thiosulfate (5)	+	+	0
Glucose (10)	-	0	0	S^0 (10)	+	+	-
Formate (10)	-	+*	0	Se(IV) (5)	-	+	-
Succinate (1)	-	+	0	As(V) (5)	+	+	+
Ethanol (10)	+	0	0	Fumarate (20)	+	+	+
Methanol (10)	-	0	0	Malate (10)	-	0	0
Glycerol (5)	+	0	0	DMSO (10)	-	0	0
Yeast extract (1g/liter)	-	0	0				
Casamino acids (1g/liter)	-	0	0				
Methane + acetate (1atm +0.1)	-	0	0				
Benzoate (1)	-	0	-				
Palmatate (1)	-	0	0				
Cyclohexane (1)	-	0	0				
Octane (1)	-	0	0				
Phenol (0.5)	-	0	0				
Toluene (1)	-	0	0				
Fumarate (10)	-	0	+				
Benzene (1)	-	0	0				
Citrate (10)	-	+	0				
Malate (1)	+	0	0				
Propionate (5)	-	0	-				

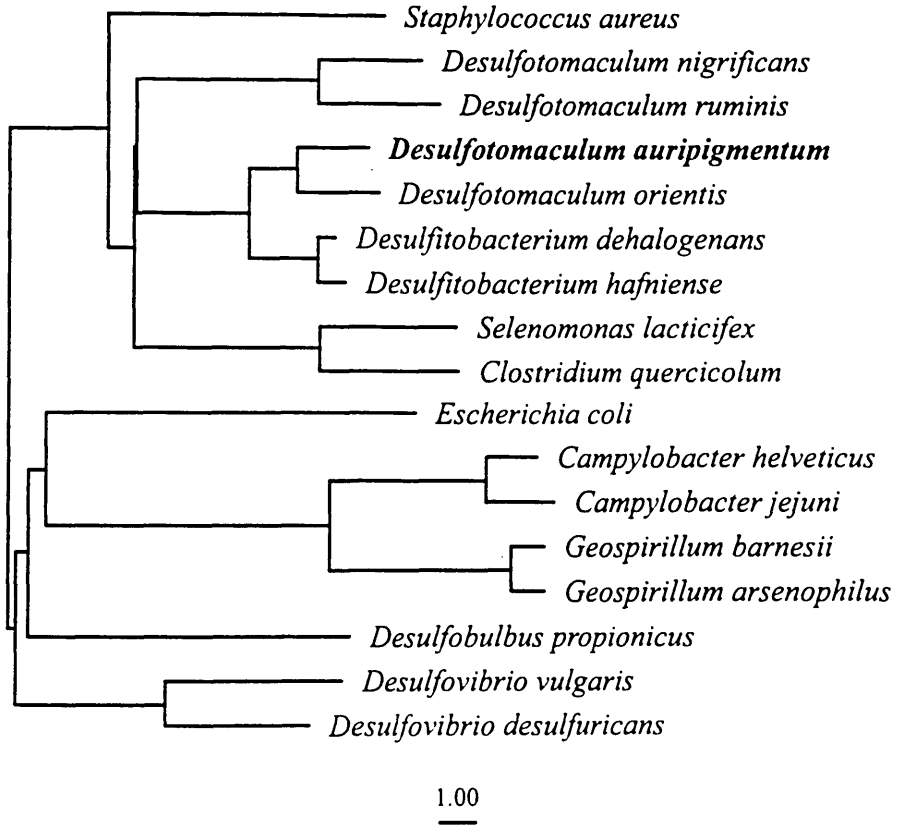
Note: "OREX-4" is given for *D. auripigmentum*, "SES-3" for *G. barnesii*, and "MIT-13" for *G. arsenophilus*

[a] electron donors were tested using sulfate as the electron acceptor (applies only to *D. auripigmentum*)

[b] electron acceptors tested using H_2 + acetate as the electron donor (applies only to *D. auripigmentum*)

* growth occurs only in the presence of acetate 0 = not reported

Fig. 7


Staphylococcus aureus
Desulfotomaculum nigrificans
Desulfotomaculum ruminis
Desulfotomaculum auripigmentum
Desulfotomaculum orientis
Desulfitobacterium dehalogenans
Desulfitobacterium hafniense
Selenomonas lacticifex
Clostridium quercicolum
Escherichia coli
Campylobacter helveticus
Campylobacter jejuni
Geospirillum barnesii
Geospirillum arsenophilus
Desulfobulbus propionicus
Desulfovibrio vulgaris
Desulfovibrio desulfuricans
1.00

Chapter 5: Characterization and purification of dissimilatory arsenate reductase from strain SES-3

to be submitted to the *Proceedings of the National Academy of Sciences*
by

Dianne K. Newman, Ronald S. Oremland, Philip R. Dowdle, François M. M. Morel, and John F. Stolz

Abstract

Several anaerobic bacteria are now known to grow by reducing arsenate while oxidizing an organic substrate. In this study, dissimilatory arsenate reductase, the enzyme catalyzing this reaction, has been characterized and purified from a representative arsenate-reducing organism, strain SES-3. An activity assay was devised based on the coupling of arsenate reduction to the oxidation of methyl-viologen (MV). Dissimilatory arsenate reductase was determined to be membrane-bound, unlike the arsenate reductases of *Escherichia coli* and *Staphylococcus aureus* (ArsC enzymes), which are cytoplasmic and used for reductive detoxification of arsenate but not for growth. Dissimilatory arsenate reductase comprises three highly hydrophobic polypeptides with molecular weights of 65, 31, and 22 kDa, in equimolar proportion. Enzyme activity in washed cell suspensions exhibited a V_{max} for arsenate reduction of approximately 0.1 μmol/min/mg at 25 °C and pH 7.2 and an apparent K_m of 200 μM arsenate. Arsenate-coupled MV oxidation was inhibited by arsenite, phosphate, molybdate and nitrate. Fumarate exhibited strong MV oxidizing activity in arsenate-grown cells, yet other alternative electron acceptors used by strain SES-3 showed only weak activity. Evidence for the presence of a *b*-type cytochrome in cells grown on arsenate was found. No significant cross-reaction with the antibody to *E. coli*'s ArsC was observed. NADH can serve as an electron donor for the arsenate reductase, and Fe-S clusters appear to be prosthetic groups in the enzyme. These studies suggest a novel mechanism of arsenate reduction.

INRODUCTION

Arsenic is a toxic element and ranks high on the EPA's list of hazardous chemicals due to its deleterious effects on cellular metabolism (1). In nature, arsenic exists in primarily two oxidation states: as arsenate (V) and arsenite (III) (2). The mechanisms of its toxicity in these forms is well understood: arsenate uncouples oxidative phosphorylation due to its resemblance to phosphate, and arsenite destabilizes proteins due to its strong affinity for sulfhydryl groups (3). As most organisms are poisoned by arsenic, those which have evolved ways to resist it have a selective advantage in certain environments. Indeed, arsenic resistance plasmids were found to be widespread among antibiotic-era clinical isolates tested in the late 70's: 61% of *Escherichia coli*, 83% of *Klebsiella pneumoniae*, 99% of *Pseudomonas aeruginosa*, and 49% of *Staphylococcus aureus* isolates showed arsenate resistance (4). *E. coli* and *S. aureus* are two prokaryotic organisms in which the molecular mechanisms of arsenic resistance have been well characterized. Both organisms depend on enzymes that reduce arsenate to arsenite before exporting it through either ATP-driven or chemiosmotic transport systems (5). While these reductases (known as ArsC enzymes) exhibit important biochemical differences, both function in the cytoplasm, and neither can be used for growth.

In recent years, various prokaryotes have been discovered which are not only resistant to high levels of arsenate (mM concentrations), but are able to grow on arsenate by using it as a terminal electron acceptor in anaerobic respiration (6-9). While initially this remarkable capability was assumed to be uncommon, it is becoming clear that the ability of microorganims to respire arsenate is far more prevalent than previously believed. Indeed, organisms spread throughout the Bacterial kingdom--from gram positive alkalophiles to gram negative Proteobacteria--are continuing to be identified with this capacity. Arsenate-reduction by microorganisms is now believed to make an important contribution to arsenic's geochemical cycle, and in highly contaminated environments where arsenic concentrations are on the order of thousands of ppm, it seems possible that microbial arsenate-respiration may be a viable pathway for the mineralization of organic carbon (9). Until now, the enzymes responsible for this process have been unknown. Here, we present the first exploration of dissimilatory arsenate reduction on the molecular level for strain SES-3, one of the first arsenate-respiring bacteria to be discovered (10).

MATERIALS AND METHODS

Growth of Bacteria and Preparation of Cell Extracts. Strain SES-3 was grown anaerobically with 20 mM lactate as the carbon source and 5 mM arsenate as the electron acceptor as previously described (9). Cells were harvested after reaching a concentration of 10^8 cells/ml (measured by direct counting) and immediately processed. Three liters of culture were concentrated and resuspended in 10 ml of sodium bicarbonate buffer (2.5g/L, pH 7.2). This concentrated suspension was lysed by sonication (Braun-Sonic L) on ice. After DNase I treatment for 15 min and addition of phenylethyl sulfonyl fluoride (PMSF; 0.2 M in 100% ethanol, 60 µl/10 ml sample), unbroken cells and sediment (e.g. arsenic trisulfide) were separated from the membrane fragments and soluble cytoplasm by centrifugation at 7,000 rcf for 10 minutes at 4 °C. Ultracentrifugation at 50,000 rpm for 90 minutes at 4 °C separated the membrane fragments (pellet) from the soluble cytoplasm (supernatant). Additional PMSF was added, and the fractions were either stored at -20 °C or refrigerated overnight for use the next day. The protein concentration was determined spectrophotometrically with the Bradford assay with bovine serum albumin as the standard.

Purification of the Arsenate Reductase. Aliquots of membrane fractions containing 1 mg/ml of total protein were solubilized with the zwitterionic detergent CHAPS (0.5%). The samples were incubated at room temperature for one hour and then centrifuged at 10,000 rpm for 15 minutes. The solubilized proteins were precipitated from the supernatant with 35% ammonium sulfate and resuspended in 0.1 ml bicarbonate buffer. The arsenate reductase was purified by non-denaturing gel electrophoresis using the CHAPS-solubilized, ammonium sulfate-precipitated fractions. Large format 7.5% acrylamide gels containing 0.5% CHAPS were run in a chilled apparatus (4 °C), and developed with the methyl-viologen assay for As(V)-reductase activity (described below). A single clean band was excised from the gel and electroeluted in Tris-Glycine buffer in a Model 422 Electro-Eluter (Bio-Rad). Visualization of both native and denatured protein bands was achieved either by Coomassie blue or silver staining.

Protein Characterization. The electroeluted purified protein was pooled and concentrated with 10% trichloroacetic acid (TCA). This protein preparation was further denatured by boiling in sodium dodecyl sulfate (SDS) before SDS-PAGE was

performed (12.5% acrylamide separating gel). The resulting subunits were transferred to a PVDF membrane and stained with Coomassie Blue R-250 for automated N-terminal sequencing on an Applied Biosystems model 476A protein sequencer. In addition, the native arsenate reductase was transferred directly to a PVDF membrane for sequencing. Electroelution of native arsenate reductase (without TCA concentration) provided material for analysis on a Hewlett Packard SPQC88 quadrupole electrospray LC/MS system. Western blots were performed on membrane fractions from As(V)-grown cells, using the antibody designed against the ArsC from *E. coli* (gift of B. Rosen, Wayne State School of Medicine).

Analysis of Prosthetic Groups. To search for the presence of prosthetic groups in CHAPS-solubilized membrane fractions of arsenate, fumarate, and nitrate-grown cells, an absorbance spectrum was aquired on a Perkin Elmer Lambda 6 UV/VIS Spectrophotometer. CHAPS-solubilized membrane preparations of arsenate-grown cells were extracted and assayed for FAD and FMN as previously described (11).

Assay of Arsenate Reductase Activity. Enzyme activity was detected by coupling the oxidation of the low potential electron donor, 1,1'-Dimethyl-4,4'-bipyridylium (dichloride)--methyl viologen (MV)--to the reduction of As(V). MV is violet when reduced and clear when oxidized. For detection by non-denaturing PAGE, the gels were submerged in a potassium phosphate buffer (0.1 M, pH-7.2) containing 1 mm MV that had been reduced with dithionite (0.1%) for 5 minutes. The violet-stained gels were then rinsed and exposed to 5 mM As(V) and monitored. Enzymatic arsenate-reductase activity appeared as a clear band of oxidized MV. Spectrophotometric assays were performed in Thurnburg cuvettes, measuring the difference in absorbance at 540 nm between the sample and a control over 10 minutes on a Perkin-Elmer Lambda 2 dual-beam spectrophotometer. The reaction mixture contained 2.2 ml bicarbonate buffer, 50 µl arsenate (20 mM) and 60 µl MV (5 mM), and was sparged with oxygen-free N_2 for 5 minutes and then reduced by the addition of 100 µl dithionite (10 mM). Protein was added to the sample (bicarbonate was added to the control). Enzyme activity was calculated as µmol MV oxidized per min using an extinction coefficient of 4.95 $mM^{-1}cm^{-1}$ as determined by Stolz et. al. (12).

Analysis. Selenium and arsenic oxyanions were quantified by high performance liquid chromatography as previously described (7). Cell densities in cell suspension

experiments were determined by 4',6-diamidino-2-phenylindole (DAPI) counts (13).

Determination of Competitive Inhibition and Substrate Specificity. Arsenite, phosphate, sulfate, molybdate, nitrate, thiosulfate, fumarate, selenate, cyanide, and azide were tested for their ability to competitively inhibit arsenate-coupled MV oxidation and/or serve as alternative substrates in the enzymatically-catalyzed reaction. For the competitive inhibition experiments, 800 μM of the inhibitor was added to a reaction vessel which contained 800 μM arsenate, 200 μM MV, 200 μM sodium hydrosulfite and 300 μg protein in 5 ml bicarbonate buffer. Percent activity was determined by monitoring the change in oxidation of MV over time for each of the samples relative to a control with no inhibitor added. Further inhibition experiments were performed in which the capacity of arsenate, fumarate, and nitrate-grown washed cell suspensions (~ 10^{11} cell/ml) to reduce 5 mM arsenate over a period of 5 hours was measured in the presence of various concentrations of fumarate (0, 5, and 50 mM). In addition to MV, 1mM concentrations of alternative electron donors (NADPH, NADH, $FADH_2$, $FMNH_2$, dithiothreitol (DTT), 2-mercaptoethanol (2-ME)) were tested for their ability to stimulate arsenate reduction in the presence of 200 μg protein from the crude membrane fraction of arsenate-grown cells. In related inhibitor experiments, membrane fractions were incubated with 1 mM *N*-ethylmaleimide (a sulfhydryl inhibitor) for 5 min prior to addition of 1 mM 2-mercaptoethanol (2-ME) followed by addition of 1 mM methyl viologen or dithiothreitol (DTT) and 100 μM arsenate; arsenite production was measured over time relative to controls lacking the inhibitor.

Cytochrome Analysis. The pyridine hemochrome difference spectrum of the cytochrome present in arsenate-grown cells was determined by the method of Smith (14). Pyridine (25%, 500 μl) and NaOH (75 mM, 500 ul) were added to a sample containing 200 μg of total protein in 1 ml of deionized water. Equal volumes of sample were placed in the sample and reference cuvettes, and a background correction was done. The cytochrome in the sample cuvette was fully reduced by the addition of sodium hydrosulfite, and the spectrum was recorded. Horse-heart cytochrome *c* was used as a standard. To determine whether the cytochrome was a *c* -type cytochrome, the presence of covalently bound heme in SDS-PAGE gels loaded with membrane proteins was tested using dimethoxybenzidine by the method of Francis and Becker (15).

Data Analysis. Kinetic constants (K_m and V_{max}) were calculated by fitting initial rate data to the Michaelis-Menten equation as described by Cleland (16) and using the software DeltaGraph.

RESULTS

Bacterial respiration of As(V). Several bacteria can grow by using arsenate as a terminal electron acceptor (oxidant) in anaerobic respiration. Table 1 gives the molar growth yields, Y_M (g cell/mol lactate), for strains SES-3 (7), MIT-13 (6) and OREX-4 (9), which have been calculated from data in the literature that show growth of these organisms on arsenate or other oxidants in the presence of lactate (a common organic substrate used by these bacteria). Generally, ordering the Y_M values for these organisms from greatest to least follows the order of the standard free energy ($\Delta G^{\circ\prime}$) expected from the reaction: nitrate is a more powerful oxidant than arsenate, which is a stronger oxidant than sulfate (Table 1). The biochemical pathway utilized by these organisms to metabolize these oxidants, however, is not always the same. For example, strains SES-3 and OREX-4 are known to incompletely oxidize lactate to acetate, whereas MIT-13 appears to completely mineralize lactate to CO_2. Accordingly, the $\Delta G^{\circ\prime}$ to be gained from As(V) respiration when coupled either to incomplete or complete oxidation of lactate differs by more than a factor of 2. This is consistent with the data which show the Y_M for SES-3 and OREX-4 is almost the same, yet significantly less than that for MIT-13. We chose to study the biochemistry of arsenate reduction in strain SES-3 because of its relative ease to culture and because it can be considered a representative arsenate-reducing organism: phylogenetically, it is closely related to strain MIT-13 (9); physiologically, it carries out a similar respiratory reaction to strain OREX-4 (7,9).

Purification and Characterization of Arsenate Reductase. To study dissimilatory arsenate reductase, an activity assay was devised which coupled arsenate reduction to oxidation of MV (Fig. 1). Both washed cell suspensions (not lysed) and membrane fractions were able to catalyze this reaction, yet neither arsenate reduction nor MV oxidation could occur without the addition of protein.

Using MV oxidation as a proxy for arsenate reduction, a purification scheme was devised to isolate the arsenate reductase. As shown in Table 2, activity was found to be membrane associated, and the specific activity in the membrane fraction was 1.5 times that of whole cells. Given that the ratio of membrane protein to soluble protein in strain SES-3 is roughly 2:1 (unpublished results), this increase in specific activity is in good agreement with our expectations. Because MV has been shown to be incapable of permeating membranes in both oxidation states (17), these results suggest the site where arsenate reductase receives electrons from MV is

periplasmically oriented, as is the case for the nitrate reductase of *E. coli* (18). Solubilization of the membrane protein in CHAPS followed by ammonium sulfate precipitation lead to a further increase in specific activity; separation of this concentrated fraction by non-denaturing PAGE removed most extraneous proteins, thus allowing for isolation and removal of the band exhibiting arsenate-reductase activity (Fig. 2a).

Denaturated arsenate reductase comprises three subunits (Fig. 2b). Both the relative staining intensities of the three polypeptides on SDS-PAGE and the relative strength of the amino acid signal from automated sequencing of the amino-terminus of the native protein, suggest a 1:1:1 ratio of the subunits within the native complex. LC/MS was used to provide a more accurate estimation of polypeptide molecular weights. The subunits were highly hydrophobic (as evidenced by their late elution in reverse-phase HPLC), and preliminary MS analysis revealed their molecular weights to be in good agreement with those determined by SDS-PAGE (Fig. 2c). The native molecular weight of the arsenate reductase complex was determined to be >100 kDa, confirming an $A_1B_1C_1$ subunit configuration. N-terminal sequencing of ~2pmol of the first 15 amino acids of the 31kD subunit revealed the sequence: AGLKNALTG(A)?ATD?. No matches were found for this sequence in a search of standard databases.

The presence of Fe-S groups is suggested by comparing UV/VIS scans of CHAPS-solubilized membrane fractions of arsenate, fumarate, and nitrate grown cells to UV/VIS scans of Fe-S groups in the literature (Fig. 3). Preliminary circular dichroism spectra also confirm their presence (data not shown). While it is possible that the Fe-S clusters are not associated with the reductases--but rather, with other components of the respiratory chain--our evidence suggests they are associated with the reductase, as their presence coincides with the ability of CHAPS-solubilized protein fractions to oxidize methyl-viologen in native gels in the presence of the appropriate substrate. Inactive preparations that do not oxidize methyl-viologen in native gels do not appear to contain these groups. Acid and trypsin-extractable FAD could not be measured in CHAPS-solubilized membranes from arsenate-grown cells. FMN, however, was detected in these samples (approximately 2 nmol/g protein).

Kinetics of Arsenate-Reductase Catalyzed Reactions. Apparent K_m values for arsenate reductase were determined in whole cells of strain SES-3 by the MV activity assay. Initial rates of MV oxidation were determined for a range of arsenate

concentrations in coupled enzyme assays where MV was saturating (Fig. 4a). Fitting these data to the Michaelis-Menten equation provided a V_{max} value of 0.1 μmol/min/mg, and a corresponding K_m value of 200 μM. The specific activity for whole cells listed in the purification table (Table. 2) is 0.15 μmol/min/mg. This value was determined for a different cell preparation at an arsenate concentration well above the K_m, and thus, compares favorably with the V_{max}. The same experiment, done with both whole cells and membrane fractions, suggested a similar apparent K_m when arsenite production rather than MV oxidation was measured (Fig. 4b). Since MV is required at roughly twice the concentration of arsenate to stimulate arsenate reduction (Fig. 1), the V_{max} values measured in terms of MV oxidized (0.1 μmol/min/mg) versus arsenite produced (0.055 μmol/min/mg) are in good agreement, given that different whole-cell preparations were used in the two experiments.

Competitive Inhibition & Substrate Specificity. Various compounds were tested for their ability either to competitively inhibit or serve as alternative substrates for (arsenate-coupled) MV oxidation in whole cells. Of the 10 compounds tested, only arsenite, phosphate, molybdate and nitrate appeared to inhibit this activity (Fig. 5a). Sulfate, thiosulfate, selenate, cyanide and azide did not measurably affect the rate of As-coupled MV oxidation, although fumarate appeared to enhance this rate. When arsenate was left out of the reaction vessel in experiments designed to test the substrate specificity of MV-oxidation, addition of fumarate restored full activity (Fig. 5b). Negligible activity was observed for additions of arsenite, sulfate, phosphate, molybdate, cyanide and azide, yet between 15 and 30% activity was observed upon addition of nitrate, thiosulfate or selenate. Direct reduction of selenate to selenite was not observed in washed cell suspensions which were able to reduce arsenate to arsenite, however (data not shown). Because of the strong activity of fumarate, MV oxidation could not be used as a proxy method to measure competitive inhibition of arsenate reduction by fumarate. Accordingly, we designed kinetic experiments to directly test whether fumarate could inhibit arsenate reduction by measuring the amount of arsenite produced over time by different washed cell suspensions. Fig. 6 shows apparent low-level constitutive ability of both fumarate and nitrate-grown cells to reduce arsenate, with arsenate-grown cells exhibiting 4 times the activity. No significant inhibition of arsenite production over time was observed in the presence of a ten-fold excess of fumarate, in either arsenate or fumarate-grown cells.

Although MV is a convenient redox dye, it is not a normal source of cellular

reducing power. The physiologically relevant reductants, NADH, NADPH, $FADH_2$ and $FMNH_2$ were therefore tested for their ability to stimulate arsenate reduction in cell-free membrane fractions from arsenate-grown cells. In the presence of NADH, 160 μmol arsenate/mg protein was reduced over 15 minutes. NADPH and the reduced thiol compound dithiothreitol (DTT), shown to stimulate the ArsC proteins *in vitro* (*) did not stimulate dissimilatory arsenate reductase. The sulfhydryl inhibitor *N*-Ethylmaleimide (NEM), which strongly inhibits the ArsC proteins (19,20), did not inhibit the dissimilatory arsenate reductase.

Cytochrome Analysis. Spectral analysis revealed the presence of a *b*-type cytochrome in membrane fractions from arsenate-grown cells (Fig. 7). While the absorption maximum at 552 nm was lower than typical for *b*-type cytochromes (554-556), this is likely due to a systematic error in the spectrophotometer (absorption maxima for standard horse heart cytochrome *c* exhibited a similar deviation from the norm). Confirmation that the cytochrome was *b*-type was provided by the lack of covalently-bound heme in SDS-PAGE gels loaded with membrane fractions (data not shown). The absorption spectrum is very similar to that found for fumarate-grown cells (12), which have been shown to be *b*-type in a number of organisms (11,12,18).

DISCUSSION

The dissimilatory arsenate-reductase of strain SES-3 is significantly different from the arsenate reductases (ArsC enzymes) involved in arsenate detoxification in *E. coli* and *S. aureus*. The dissimilatory arsenate reductase is a multimeric membrane protein (> 100 kDa) with a presumptive chromosomal locus, as the ability to respire arsenate is highly stable from one generation to the next and is maintained regardless of whether arsenate is present in the medium. In contrast, the plasmid-encoded detoxifying arsenate-reductases are small cytoplasmic monomers (~ 15 kDa). Dissimilatory arsenate reductase has an apparent Km of 0.2 mM arsenate, while the Km of *E. coli*'s ArsC is 8 mM (20), and the Km of *S. aureus* is 2 mM in a region of comparable enzymatic activity (21). The V_{max} for *E. coli* and *S. aureus* (roughly 1 and 0.5 μmol NADPH oxidized/min/mg, respectively) was determined for purified ArsC enzymes (20,21); the V_{max} for strain SES-3 (approximately 0.1 μmol MV oxidized/min/mg protein) was determined for whole cells. If we assume that the dissimilatory enzyme accounts for only ~2% of the total protein measured in whole cells, this suggests that the V_{max} for the purified dissimilatory enzyme is several times greater than that for the ArsC enzymes. This seems reasonable, given that strain SES-3 not only can survive over a wider range of arsenate concentrations than either *E. coli* or *S. aureus*, but can also use arsenate for growth. The measured whole-cell V_{max} and the specific activity of purified arsenate reductase are most likely lower than the true activity of dissimilatory arsenate reductase *in vivo* due to heat and/or oxidative damage experienced by the enzyme during the experiments.

The mechanism by which the dissimilatory arsenate reductase passes electrons onto arsenate contrasts with how this is accomplished by the ArsC enzymes. The detoxifying arsenate reductases couple NADPH oxidation to arsenate reduction through reduced dithiol cycling: in the case of *E. coli*, glutathione reductase, glutathione, and glutaredoxin participate in the transfer of reducing power from NADPH to arsenate (20); in *S. aureus*, thioredoxin reductase and thioredoxin serve this function (21). In contrast, the dissimilatory arsenate reductase can receive electrons from NADH but not NADPH, and its active site does not appear to involve thiols. Conventional prosthetic groups, such as Fe-S clusters, most likely are responsible for the transfer of electrons to arsenate in strain SES-3. Together, these results suggest a model for enzymatic energy coupling in strain SES-3. The key assumption of this model is that the highly hydrophobic arsenate-

reductase spans the membrane. Our results indicate that MV interacts with the arsenate reductase in the periplasm, as we have observed an approximate 2:1 stoichiometry of MV oxidized per arsenate reduced in whole cell assays. For example, in the case of growth on lactate, a proton-motive force could be generated by the transfer of electrons from a cytoplasmically-oriented lactate dehydrogenase (22) to arsenate reductase (where arsenate is reduced to arsenite on the cytoplasmic side of the membrane) with proton translocation facilitated by menaquinone. The *b*-type cytochrome present in the membrane of arsenate-grown cells might play a role in electron transfer, although *b*-type cytochromes have been found to occur in cells without being directly linked to the respiratory process (18).

Further insight into the novel mechanism of dissimilatory arsenate reduction is provided by the inhibitor studies. Although arsenite is the most potent inhibitor of arsenate-coupled NADPH oxidation by the ArsC enzymes in *E. coli* and *S. aureus*, phosphate inhibits arsenate-coupled MV oxidation nearly as effectively as arsenite in whole cells of strain SES-3, to a lesser degree in *E. coli* (20), but not at all in *S. aureus* (where, on the contrary, phosphate stimulates ArsC activity (21)). Nitrate inhibits arsenate-coupled MV oxidation in strain SES-3 to a lesser extent than either arsenite or phosphate, has no effect on arsenate-coupled NADPH oxidation in *E. coli* (20), yet stimulates NADPH oxidation in S. *aureus* (21). Molybdate also appears to inhibit arsenate-coupled MV oxidation in strain SES-3, which is interesting because it was found to inhibit arsenate reduction in experiments with another dissimilatory arsenate-reducing bacterium, *Desulfotomaculum auripigmentum* (9). Neither cyanide nor azide inhibit arsenate reduction in strain SES-3; this is probably because MV directly interacts with arsenate reductase, thus circumventing a possible inhibitory interaction between cyanide, azide and an associated cytochrome. Direct interaction between a viologen redox dye and a membrane reductase has been observed in the case of fumarate reductase in *Wolinella succinogenes*, a close relative of strain SES-3 (23). Alternatively, it is also possible that the *b*-type cytochrome present in arsenate-grown cells is resistant to cyanide and azide, and thus, if it is involved in the electron transport process, these compounds would have no effect. The failure of cyanide and azide to block Fe(III)-reduction through such a mechanism has been noted in the dissimilatory iron-reducing bacterium *Geobacter metallireducans* (24).

The relationship between fumarate reduction and arsenate reduction in G. barnesii strain SES-3 is of particular interest. While much remains to be done to determine the exact nature of the relationship, our preliminary evidence suggests

the enzymes may be similar, or the regulation of their expression may be linked. The standard potential of the fumarate/succinate couple ($E^{o'}$= 30 mV) is close to that of As(V)/As(III) ($E^{o'}$= 4 mV), and thus, it would not be surprising if similar components of the respiratory chain were present in fumarate and arsenate-grown cells. Cytochrome *b* is one component of the fumarate reductase in *W. succinogenes* (FrdC, 25 kD) (25). Because the difference spectrum of the *b*-type cytochromes present in membrane fractions of fumarate and arsenate-grown cells of strain SES-3 is strickingly similar (12), it seems possible that the 25 kD component of the dissimilatory arsenate reductase is a *b*-type cytochrome. Furthermore, our experiments show that arsenate-grown whole cells can oxidize MV at the same rate when arsenate or fumarate is provided as the substrate. Such activity is not observed with fumarate as the substrate for membrane fractions of strain SES-3 grown on nitrate, thiosulfate, or selenate (12). This suggests that either fumarate reductase is expressed to high levels in arsenate-grown cells, or, the arsenate reductase has a high affinity for fumarate. Our kinetic experiments suggest the former is more likely to be true, as high levels of fumarate did not inhibit the rate of arsenite production significantly. The different migration patterns of fumarate and arsenate reductases on native gels support this interpretation, suggesting they are different enzymes.

From an environmental perspective, perhaps the most important aspect of this work is that it allows us to begin to interpret field data on the molecular level. While similarities and/or differences between biochemistry studies such as this one and experiments in natural systems must be approached with caution, it is interesting to note that among the alternative electron acceptors used by the arsenate-reducing isolates, fumarate is the only one (apart from arsenate) they all have in common (9). Furthermore, the partial inhibition by nitrate of dissimilatory arsenate reductase activity in strain SES-3 matches the finding that nitrate retarded dissimilatory arsenate reduction in anaerobic salt marsh sediments (26). However, in these same sediments, phosphate did not inhibit arsenate reduction (26), and in different sediments from an arsenic-polluted site, nitrate did not inhibit arsenate reduction (27). Variation in the microbial community from site to site almost certainly explains these differences, and provides an incentive for more biochemical and molecular genetic work to be done with a variety of environmental isolates. Indeed, it is only through comparative biochemical and molecular genetic work that generalizations can be made about dissimilatory arsenate reduction both with repect to its biochemistry and its evolutionary relationship to the detoxifying reductases.

This study of the dissimilatory arsenate reductase of strain SES-3 provides a first step in this direction, however, and it is our hope that overexpression and purification of this enzyme will allow us to begin to approach these issues in the near future.

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Figure legends

Table 1. Molar growth yields on arsenate relative to other oxidants

Figure 1. Methyl Viologen (MV) oxidation is coupled to arsenate reduction by a 2:1 stoichiometry. A representative data set is shown.

Table 2. Purification of dissimilatory arsenate reductase.

Fig. 2 (a) Native gel, showing band of clearing corresponding to the oxidation of methyl-viologen when arsenate is added as the substrate **(b)** the subunit profile of purified dissimilatory arsenate reductase **(c)** confirmation of molecular weight by mass spectrometry.

Fig. 3 (a) UV/VIS spectrum of CHAPS-solubilized membranes from arsenate-grown cells at pH 7 **(b)** UV/VIS spectrum of [2Fe-2S] ferredoxin in the oxidized (__) and reduced (----) states at pH 8. The peaks between 400-500 nm are characteristic of Fe-S clusters. Data for (b) are taken from Metal Ions in Biological Systems vol. 27, Helmut and Astrid Sigel (eds)., Marcel Dekker: New York, p. 299

Fig. 4 (a) Rate of arsenate-coupled MV oxidation in whole cell suspensions over a range of arsenate concentrations **(b)** Rate of arsenite production in whole cell suspensions over a range of arsenate concentrations.

Fig. 5 (a) Inhibition of arsenate-coupled MV oxidation by arsenate-grown whole cell suspensions in the presence of various oxyanions **(b)** Ability of arsenate-grown whole cell suspensions to oxidize MV in the presence of alternative electron acceptors. Errors bars reflect the standard deviation (n=3).

Fig. 6. Rate of arsenite production over time for whole cell suspensions. Solid lines indicate arsenate grown cells, dotted lines indicate fumarate-grown cells, dashed lines indicate nitrate-grown cells. Solid circles indicate the experiment was performed in the presence of 5 mM arsenate. Open circles indicate 5 mM arsenate + 50 mM fumarate. Diamonds indicate 5 mM arsenate + 50 mM nitrate. Error bars reflect the data range of duplicate samples.

Fig. 7. Cytochrome *b* spectrum for membranes from arsenate-grown SES-3 cells.

Table 1. Molar growth yields on arsenate relative to other oxidants

Organism	Oxidant	Y_M	$\Delta G^{\circ\prime}$	Reaction
SES-3	selenate	11.5	-347	$2SeO_4^{2-} + 2H^+ + CH_3CHOHCOO^- = 2HSeO_3^- + CH_3COO^- + CO_{2(g)} + H_2O$
SES-3	nitrate	7.1	-311	$0.5NO_3^- + H^+ + CH_3CHOHCOO^- = 0.5NH_4^+ + CH_3COO^- + CO_{2(g)} + 0.5H_2O$
SES-3	arsenate	5.3	-172	$2HAsO_4^{2-} + 4H^+ + CH_3CHOHCOO^- = 2HAsO_2 + CH_3COO^- + CO_{2(g)} + 3H_2O$
SES-3	thiosulfate	2.1	-48	$0.5S_2O_3^{2-} + CH_3CHOHCOO^- = HS^- + CH_3COO^- + CO_{2(g)} + 0.5H_2O$
OREX-4	arsenate	5.5	-172	$2HAsO_4^{2-} + 4H^+ + CH_3CHOHCOO^- = 2HAsO_2 + CH_3COO^- + CO_{2(g)} + 3H_2O$
OREX-4	sulfate	2.3	-89	$0.5SO_4^{2-} + 0.5H^+ + CH_3CHOHCOO^- = 0.5HS^- + CH_3COO^- + CO_{2(g)} + 0.5H_2O$
MIT-13	arsenate	7.0	-394	$6HAsO_4^{2-} + 13H^+ + CH_3CHOHCOO^- = 6HAsO_2 + 3CO_{2(g)} + 9H_2O$

Calulations for Y_M (g cell/mol lactate) assume a cell dry weight of 1.9×10^{-10} mg/cell
Calculations for $\Delta G^{\circ\prime}$ (kJ/mol lactate) assume pH = 7; PCO_2 = 1 atm

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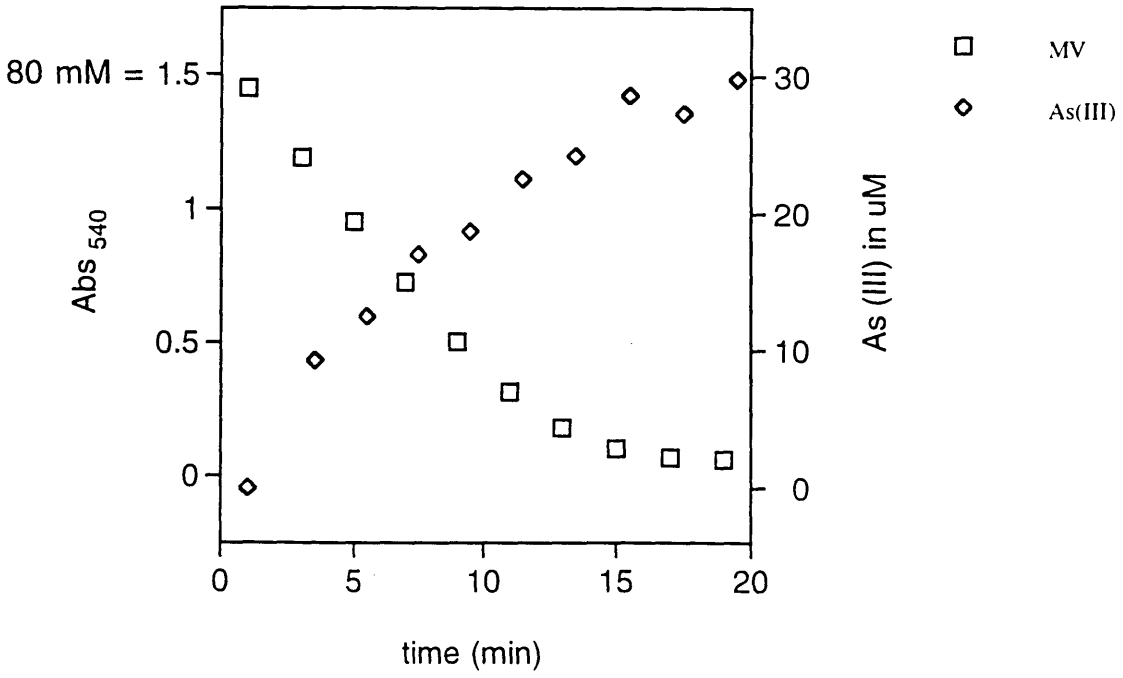
Calculations for $\Delta G^{\circ\prime}$ (kJ/mol lactate) assume pH = 7; PCO_2 = 1 atm

Fig. 1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20
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20
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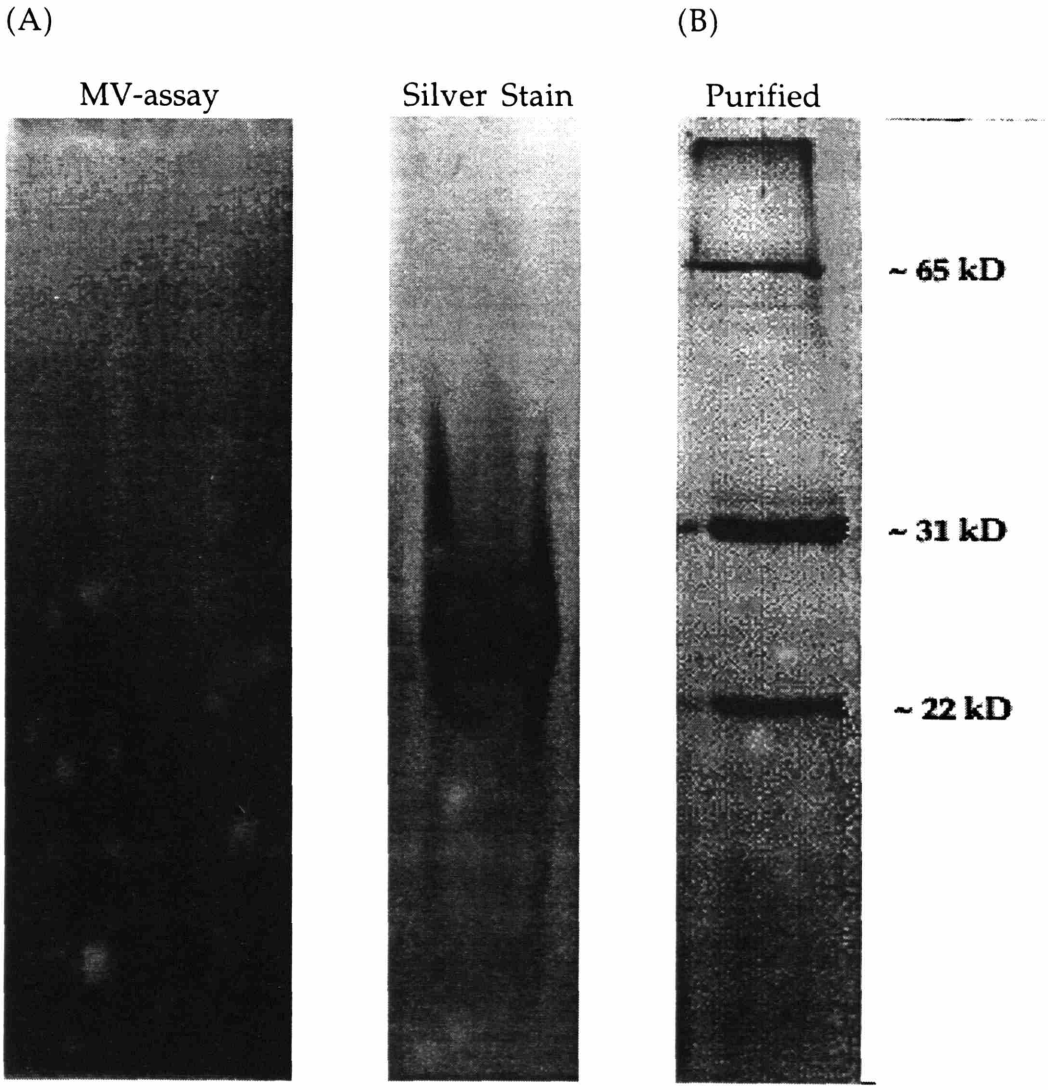
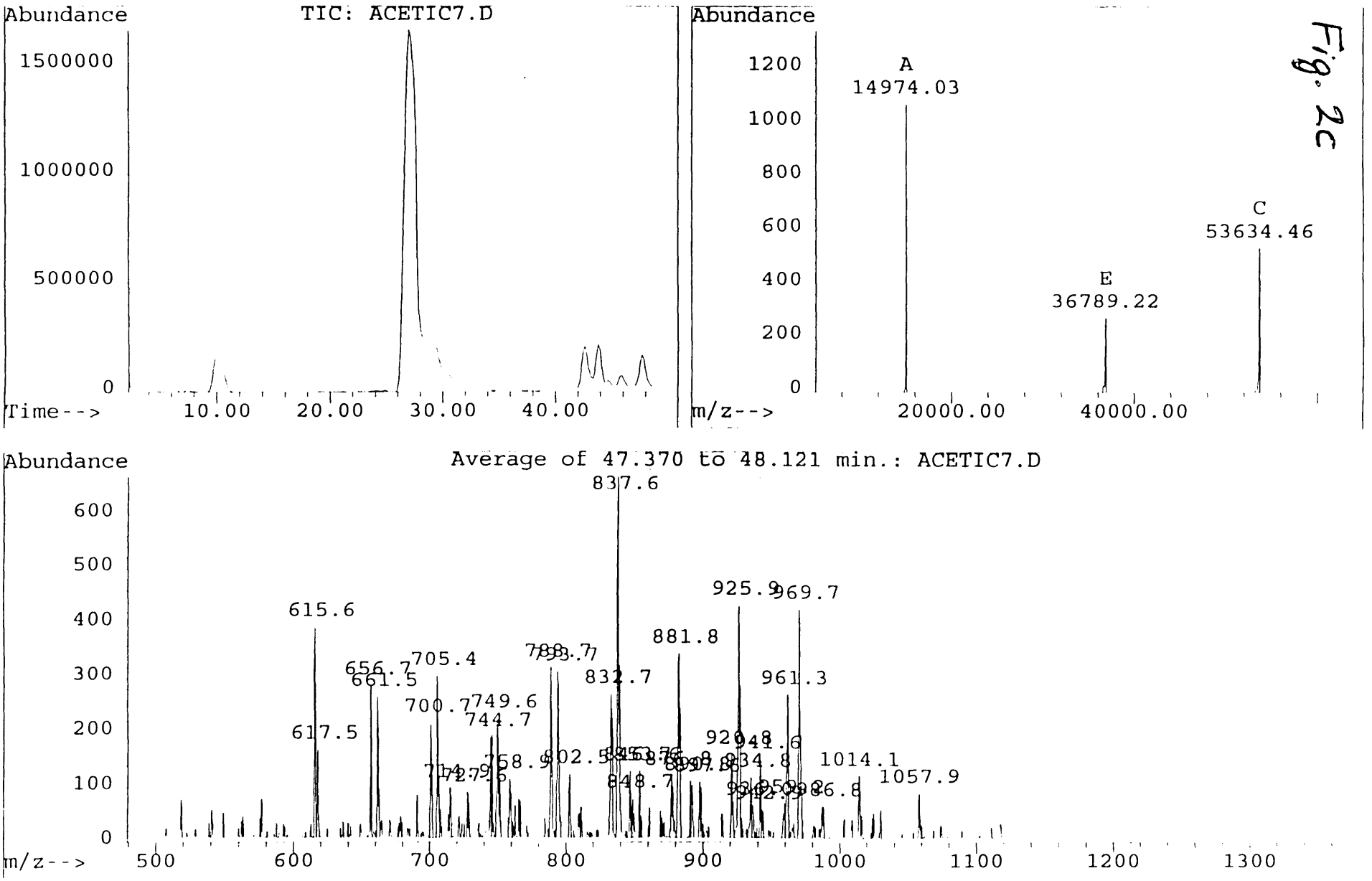
As (III) in uM
MV
As(III)

Table 2. Purification of Arsenate Reductase

Fraction	Total protein (mg)	Total activity (μmol/min)	Specific Activity (μmol/min/mg)	% Recovery (protein/total initial protein)
Whole Cells	0.25	0.038 ± 0.006	0.15 ± 0.03	100
Membrane	0.18	0.041 ± 0.004	0.23 ± 0.02	67
Cytoplasm	0.32	0.003 ± 0.0007	0.01 ± 0.002	33
cs 35% Mem	0.02	0.012 ± 0.001	0.60 ± 0.03	50

Fig. 2

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53634.46
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Abundance
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100
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615.6
925.9
969.7
881.8
705.4
656.7
661.5
832.7
961.3
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744.7
617.5
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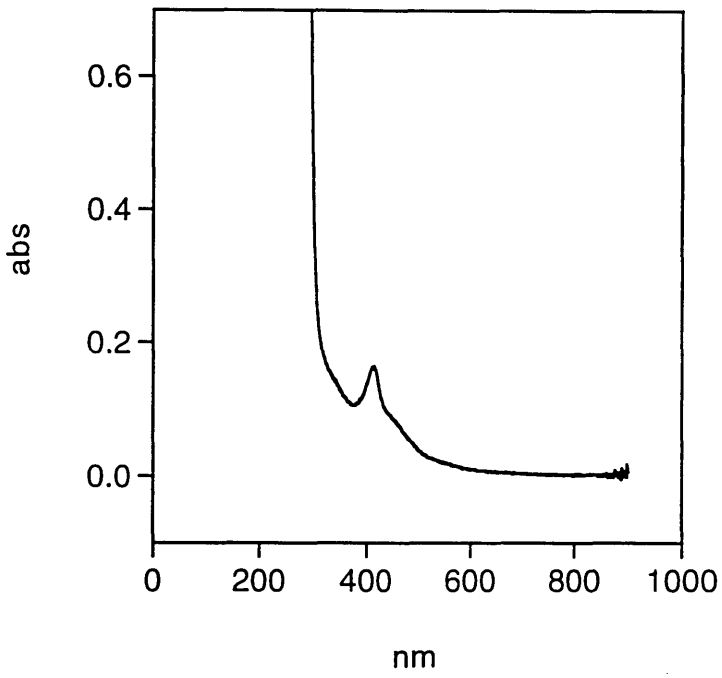
Fig. 2c

901

Fig. 3a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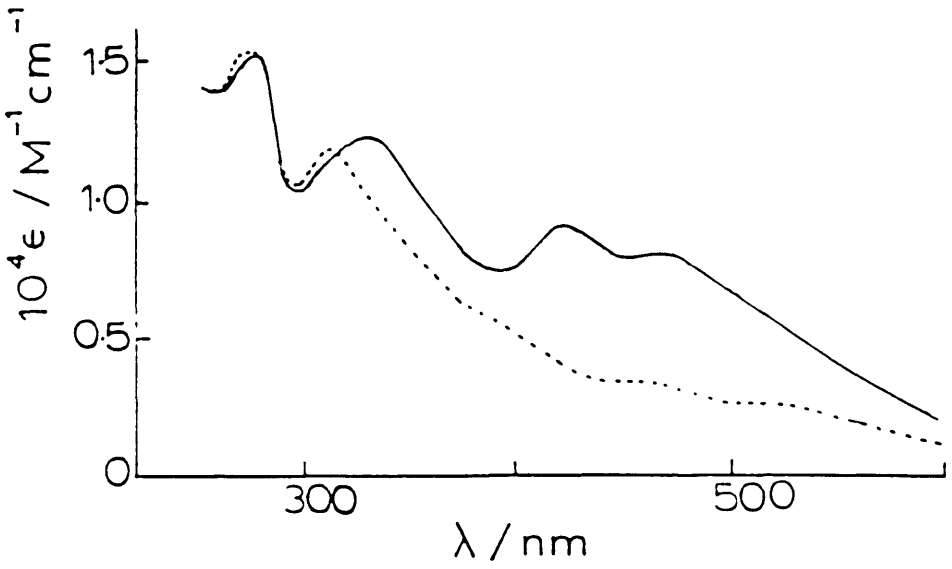
3b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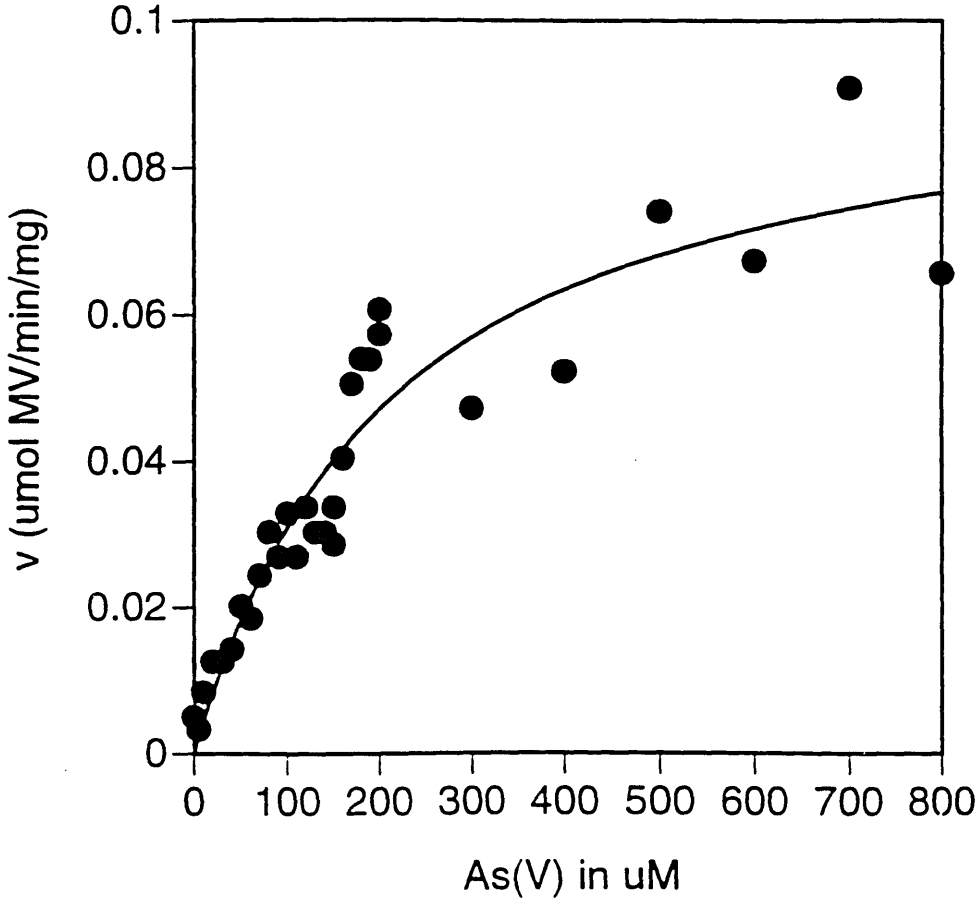
λ / nm

Fig 4a


0.1
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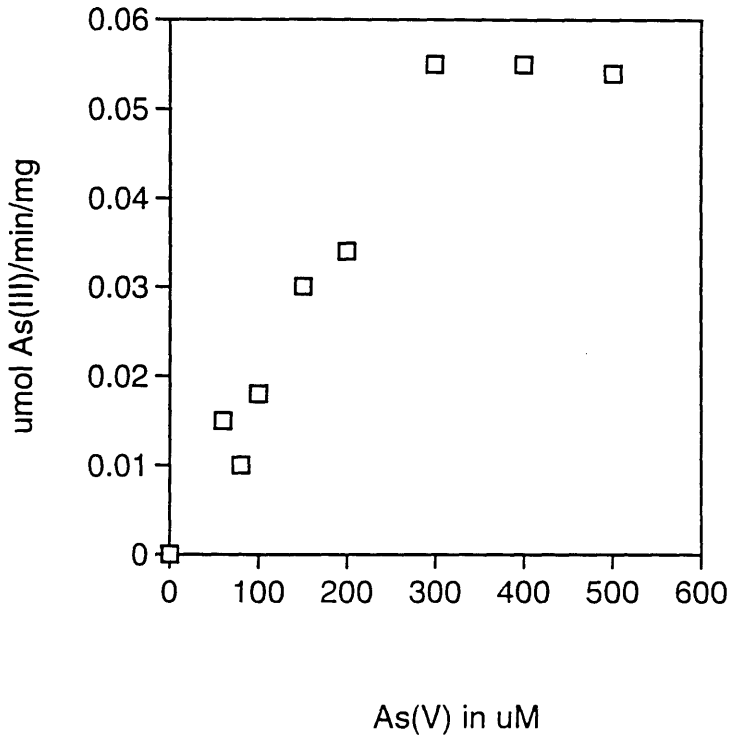
600
700
800
As(V) in uM

Fig. 4b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0.05
0.04
0.03
0.02
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umol As(III)/min/mg
0
100
200
300
400
500
600
As(V) in uM

Figure 5a

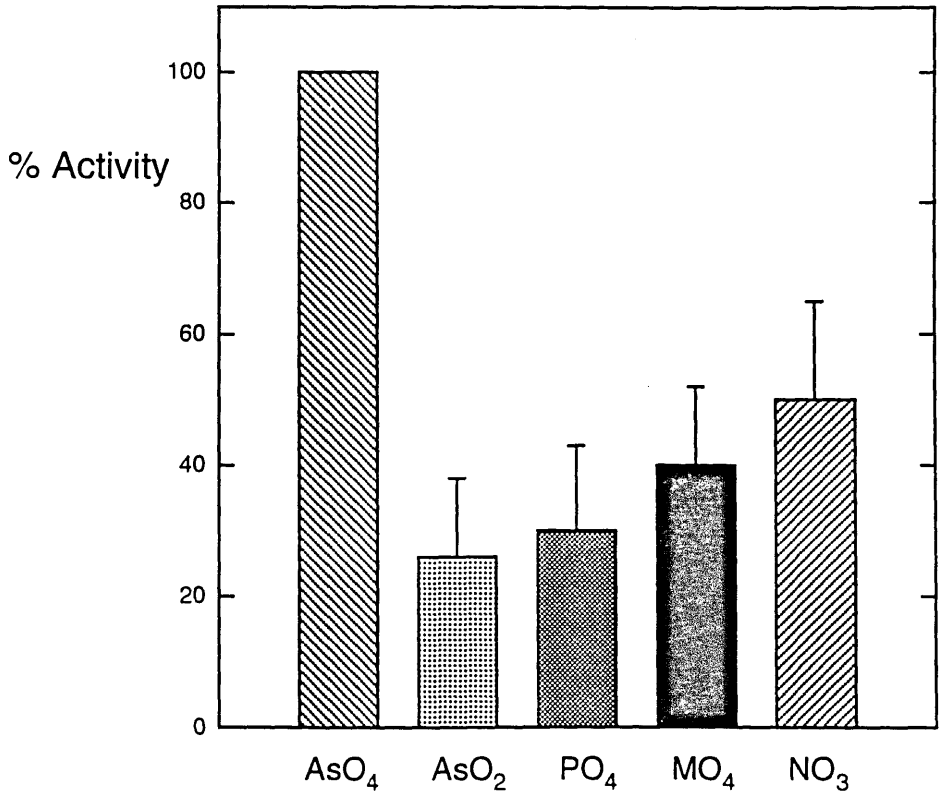


Figure 5b

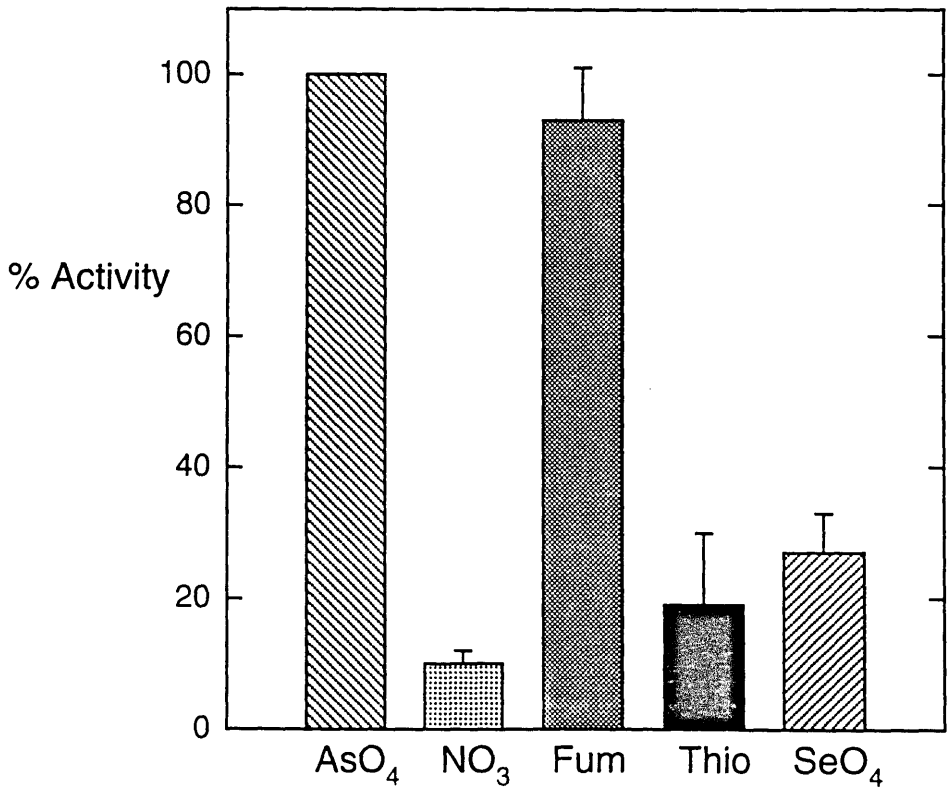


Fig. 6

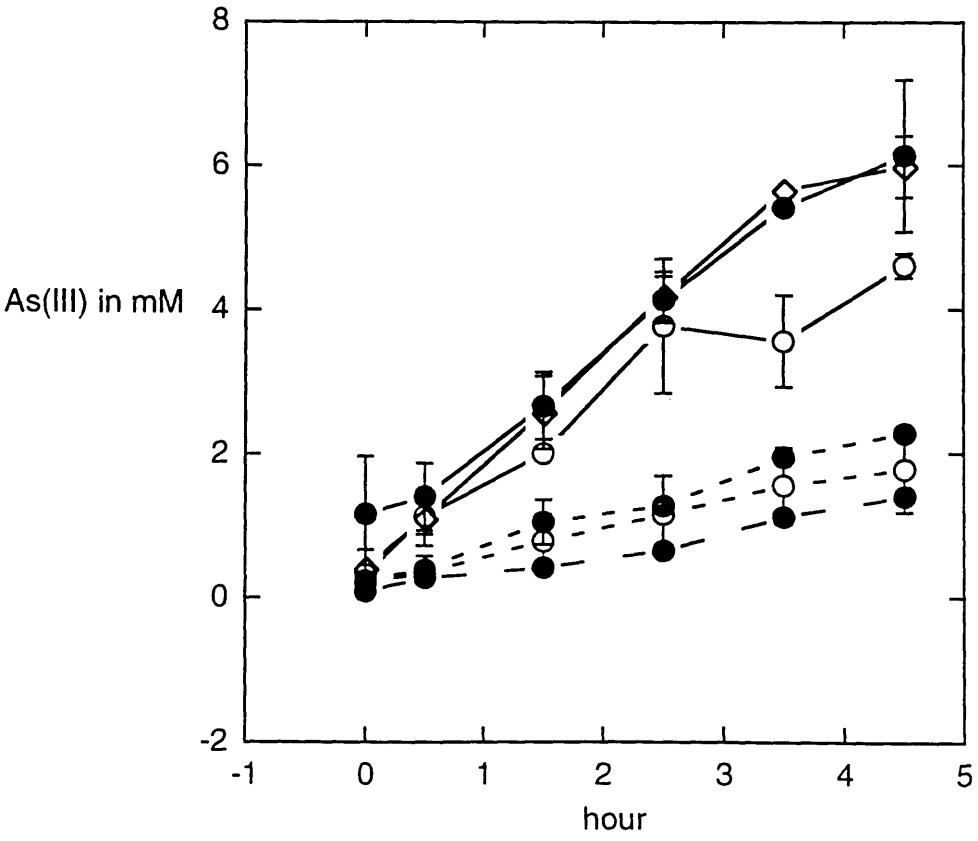
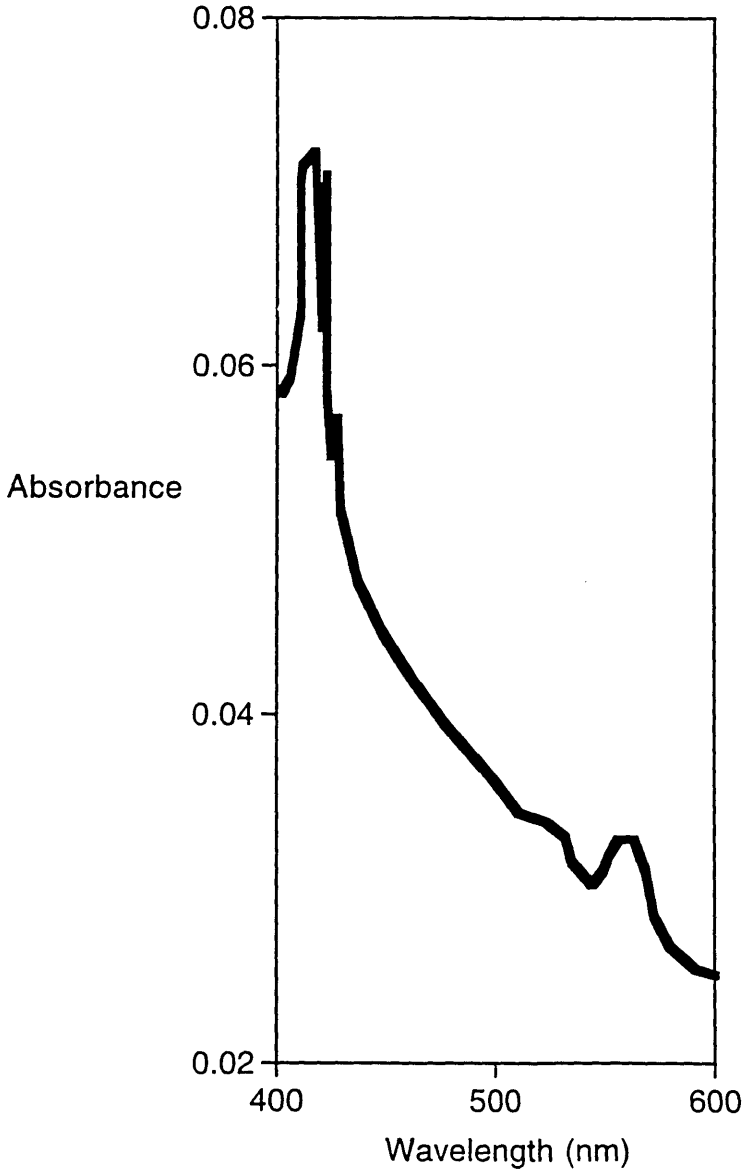


Fig. 7



Chapter 6: The ability of bacterial consortia from arsenic-contaminated soils to mobilize arsenate

to be submitted to *Environmental Science and Technology*
by

Dianne K. Newman, Jonathan O. Sharp, and François M. M. Morel

Abstract

Respiration of arsenate is now known to be performed by diverse bacterial isolates as well as microbial consortia from uncontaminated sediments. Recently, the ability of arsenate-reducing microbes to catalyze the dissolution of arsenic from iron arsenate was demonstrated. In this study, we examined whether bacteria indigenous to arsenic-polluted soil could reduce and mobilize arsenate. In a first series of experiments, soil bacteria were grown in defined medium that was spiked with a high concentration of sodium arsenate. Arsenate-reduction in the resulting enrichment culture was measured with varying carbon sources and O_2 concentrations. Glucose and microaerophilic conditions appeared to stimulate arsenate-reduction. Potential alternative electron acceptors were examined for their ability to inhibit arsenate reduction. Nitrate did not affect arsenate reduction, sulfate and MnO_2 slightly retarded it, whereas ferric iron significantly delayed arsenate reduction. In a second series of experiments, arsenate reduction in polluted soils was examined directly. In soil-slurries which contained nutrient media, more arsenate was liberated from the soil than in controls lacking nutrients or in controls that had been poisoned with formaldehyde, and microbial activity rapidly reduced the dissolved arsenate to arsenite. These results suggest that indigenous soil bacteria readily mobilize arsenate under a variety of conditions. This may be particularly important in sites that are both contaminated with arsenic and rich in organic matter.

INTRODUCTION

High concentrations of arsenic (hundreds-thousands ppm) have been measured in soils contaminated as a result of human activities. Typically, arsenic has accumulated to dangerous levels as a result of disposal of industrial wastes, mining processes, arsenical pesticide usage, and application of fertilizers (1). Due to the known toxicity and wide distribution of arsenic, it now ranks second on the ATSDR/EPA priority list of the top 20 hazardous substances (2). While anthropogenic inputs of arsenic into the environment are projected to decrease in the future, past production and release of arsenic-containing substances has left a legacy of contaminated sites in need of remediation (1). Currently, precipitation of arsenic by the addition of ferric iron with subsequent fixation by mixing with lime or Portland Cement is the primary method used to treat these sites (3).

In the past few years, the ability of physiologically and phylogenetically disparate microorganisms to grow by reducing arsenate to arsenite under anaerobic conditions has been described. While arsenate reduction is also performed by bacteria for detoxification purposes, arsenate-respiring bacteria thrive in the presence of high arsenic concentrations whereas organisms which only perform reductive detoxification do not. Dissimilatory arsenate reducers have been isolated from a variety of environments, ranging from the Aberjona Watershed in eastern Massachusetts (4,5), the Massie Slough marsh in western Nevada (6), and the Ballarat Goldfields in Australia (7), to Mono Lake in eastern California (8). In addition to these pure cultures, anaerobic consortia from both freshwater and saline environments have been found to engage in dissimilatory arsenate reduction (9). Accordingly, the ability to respire arsenate--once thought to be an unusual attribute--is coming to be seen as a widely distributed function among organisms in the environment.

This study was carried out with soil bacteria from a Superfund site in eastern Pennsylvania, which was placed on the National Priority List in the mid 1980's and is currently being remediated in accordance with the Resource Conservation and Recovery Act (RCRA) (3). Arsenic concentrations in the wastes at this site have been measured as high as 157,000 ppm, well in excess of the arsenic action level for treatment as defined by the EPA (>1,000 ppm); leaching into the groundwater has resulted in concentrations which exceed

the Maximum Contaminant Level (MCL) by about a factor of 3,000 (3). Organic contamination accompanies arsenic pollution at this site, with principal contaminants including: tetrachloroethene (PCE), trichloroethene (TCE), benzene, pyrene, and aniline (3).

Our goal was to determine whether bacteria native to this site could reduce arsenate to arsenite. Specifically, we were interested in comparing the conditions under which arsenate-reduction was performed by these bacteria to the conditions described for consortia from unpolluted sites (9). As a consortium native to a Superfund site in eastern Massachusetts has recently been shown to mobilize arsenic from iron arsenates in batch culture (10), we were also motivated to test whether an indigenous bacterial consortium operating under different growth conditions could mobilize arsenate directly from polluted soil.

MATERIALS AND METHODS

Soil Samples. Soil used in this study derived from an arsenic-polluted Superfund site in eastern Pennsylvania. A U.S. Standard Sieve #14 was used to separate out particles larger than 1.5 mm. Soil passing through the sieve was used in the experiments. Inorganic arsenate was the primary form of arsenic in the soil (3).

Cultures and Medium. In all of the experiments except the slurries described below, freshwater minimal medium was used for cultivation as previously described (5). Sodium arsenate was added to the medium (5 mM final concentration) to enrich for arsenate-reducing bacteria in the presence of 0.2 g soil. Sulfide was chosen as the reductant for the medium (0.025% w/v) to permit a rapid visual indication of arsenate reduction by the formation of As_2S_3 (11). Depending on the experiment, glucose, lactate, pyruvate, and acetate were added to the medium to give final concentrations of 10 mM. For inhibition experiments, nitrate, Mn(IV), Fe(III), and sulfate were added to the medium in the form of $NaNO_3$, MnO_2, Fe(III)-citrate, and Na_2SO_4 to final concentrations of 5 mM. For experiments with O_2 , either 5 ml or 10 ml air was injected into the 15 ml N_2-CO_2 headspace (80:20), or the headspace was flushed fully with air after which the serum bottles were capped with impermeable butyl rubber stoppers. Some samples were capped with porous

lids, permitting constant contact with the air. All samples were incubated in the dark at room temperature (25 °C).

Agar plates (2% w/v) were poured under an atmosphere of N_2:CO_2:H_2 (85:5:10) in an anaerobic chamber. The medium was supplemented with 10 mM lactate, 5 mM arsenate, and 0.025% sulfide. Attempts to isolate a facultative arsenate reducing bacterium were made by picking colonies which appeared positive for arsenate reduction (signaled by formation of a yellow colony) to be subsequently streaked for aerobic and anaerobic incubation. Several transfers back and forth between aerobic and anaerobic plates were performed before final testing for arsenate reduction in liquid medium.

Slurry Preparation. Arsenate reduction in 2.5% slurries (w/v) was monitored in batch cultures under 5 different conditions: 1.) water liquid phase, anaerobic, 2.) nutrient liquid phase (10 mM glucose, 0.5 g/L yeast exract, 2.4 g/L sodium bicarbonate), anaerobic, 3.) nutrient liquid phase, in contact with the air through a syringe inserted in the butyl rubber stopper, 4.) nutrient liquid phase, 7% enrichment-culture inoculum, anaerobic, 5.) nutrient liquid phase + 4% formaldehyde (v/v), 7% inoculum, anaerobic. Bottles were incubated at room temperature with constant orbital shaking. As(V), As(III), Fe(II), and S(-II) measurements were taken for the liquid phase. Cell densities were monitored by direct 4',6-diamidino-2-phenylindole (DAPI) counts.

In separate slurry experiments, 12% (w/v) slurries were prepared by combining 11g dry soil with 90 ml liquid in 100 ml anaerobic serum bottles. Either ultra pure water, nutrient medium B (20 mM glucose, 0.5 g/L yeast extract, 2.4 g/L sodium bicarbonate), nutrient medium C (20 mM glucose, 20 mM fumarate, 0.5 g/L yeast extrac, 2.4 g/L sodium bicarbonate), or nutrient medium B plus 4% (v/v) formaldehyde, comprised the liquid portion. Bacteria from the enrichment culture provided a 5% inoculum. Bottles were initially sealed with 1 atm air and were incubated at room temperature with constant orbital shaking. Periodically, the soil was allowed to settle, arsenic measurements were made for the liquid phase, and 70 mls of liquid were removed and replaced with fresh liquid. These exchanges were made on a benchtop open to the air.

Analytical Procedures. For dissolved arsenic concentrations, total arsenic (oxidized sample), arsenate (untreated sample), and background phosphate (reduced sample) were measured directly by the molybdenum blue spectrophotometric assay (12); arsenite was indirectly determined by measuring the difference between the oxidized and untreated samples. Sulfide was measured by the methylene blue method (13). Ferrous iron was measured by the ferrozine assay (14,15). All samples were filtered through a 0.2 um cellulose filter (Nalgene) before measurements were taken.

RESULTS AND DISCUSSION

Biological Arsenate Reduction. Soil bacteria rapidly reduced As(V) when grown anaerobically in the presence of 10 mM lactate. Bacteria which were autoclaved or poisoned with 4% formaldehyde at the onset of the experiment did not reduce arsenate (Fig. 1). These results indicate that arsenate reduction was biological. The rates of reduction compare favorably to those found for dissimilatory arsenate-reducers in pure and mixed cultures (4,5,6,10).

O_2 Stimulation. Because the rate of abiotic arsenite oxidation is slow (16), we would expect to be able to measure arsenate-reduction in oxic samples if enzymatic arsenate-reduction was occurring. Samples grown in tubes allowing constant contact with the air experienced little growth when cultured in minimal medium containing arsenate and lactate, and arsenate reduction was not observed in these samples (data not shown). Microaerophilic conditions, however, appeared to stimulate growth and reduction in minimal arsenate/lactate medium (Fig. 2). Initial concentrations of oxygen ranging from 0.26 - 0.09 mM yielded faster arsenate reduction rates than the anaerobic control. These results suggest that strict anaerobic conditions--used in previous experiments with arsenate reducers (4-7,9,10)--are not necessary to achieve rapid microbial arsenate reduction. However, as arsenate reduction did not occur in fully aerated samples and our attempts to isolate a facultative arsenate-reducer were unsuccessful, it seems likely that the bulk of arsenate reduction was carried out by anaerobic bacteria. This is consistent with what is known about the physiology of previously described arsenate-reducing isolates (4-7), although the possibility that a facultative arsenate-reducing bacterium was present in the consortium cannot be ruled out. Most likely, microaerophilic stimulation of arsenate reduction was due to complex interactions among various species present in the consortium: for example, organisms which could use oxygen for catabolism may have produced metabolites that stimulated the arsenate reducers.

Organic Carbon Stimulation of Arsenate Reduction. Glucose was found to stimulate arsenate reduction in the enrichment culture more effectively than lactate, pyruvate, or acetate (Fig. 3). Arsenate reduction in the presence of acetate--a non fermentable substrate--provides additional evidence that

dissimilatory arsenate reduction was occuring. Arsenate reduction was nearly complete by day 4 for glucose-grown cultures, by day 9 for lactate and pyruvate-grown cultures, and by day 13 for acetate-grown cultures. This difference is not surprising, given that the energy to be gained from the oxidation of these substrates is greatest for glucose and least for acetate (17). These results contrast slightly with those described for salt marsh sediments, in which glucose and lactate were found to stimulate arsenate reduction to the same extent (9). This difference suggests that the microbial populations which reduce arsenate differ from site to site, but the general finding that organic carbon stimulates arsenate reduction appears to apply regardless of the environment. Such stimulation might be particularly relevant in sites which are both polluted with arsenic and rich in organic matter, whether from anthropogenic sources or natural materials.

Inhibition Studies. Previous experiments with consortia from uncontaminated sites considered whether microbial arsenate-reduction could be inhibited by common soil constituents (9). Such inhibition studies have both ecological and applied relevance. Accordingly, we performed similar inhibition experiments using glucose as the carbon source to reduce the necessary incubation times. To simplify the interpretation of our results, we performed these experiments under anaerobic conditions. Contrary to the finding that nitrate strongly inhibited microbial arsenate reduction in uncontaminated salt marsh sediments, nitrate did not appear to inhibit arsenate reduction by the contaminated-soil bacteria used in this study (Fig. 4). As the mechanism for nitrate inhibition is believed to be due to its use as a preferred electron acceptor (9), these results seem to confirm the finding that not all arsenate-reducing bacteria have the capacity to reduce nitrate (5), and provide further evidence that arsenate reduction is performed by different organisms from site to site.

Slight inhibition of arsenate reduction occured in the presence of Mn(IV) and SO_4. No reduction of Mn(IV) was apparent in our cultures (as evidenced by the lack of appearance of $MnCO_3$, a black solid which forms readily when Mn(IV) is reduced to Mn(II) given the conditions of our medium). Because MnO_2 is known to adsorb and oxidize arsenite (*), chemical oxidation provides a reasonable explanation for the slight inhibition of arsenate-reduction observed in cultures containing MnO_2. The apparent

inhibition of arsenate reduction by sulfate is surprising, however, given that no measurable sulfide was produced in our cultures. This implies that sulfate-reduction was not a competitive process in the consortium. The mechanism for sulfate inhibition of arsenate reduction in this instance is unclear.

Fe(III) was by far the most potent inhibitor of arsenate reduction . Upon the addition of Fe(III)-citrate, a rusty orange color appeared in the medium, indicating that $Fe(OH)_3$ had precipitated. This color remained throughout the first week of the experiment, but disappeared shortly before the appearance of As_2S_3, implying that Fe(III) reduction occured prior to arsenate reduction (Fig. 4). To test the hypothesis that the consortium harbored iron reducers, we grew the bacteria in the presence of Fe(III) as the sole electron acceptor. Fe(II) appeared in the medium over time, with the eventual formation of a black precipitate (iron-sulfide) (data not shown). As As(V) is known to have a high affinity for iron hydroxides (19), it seems likely that some of the arsenate may have adsorbed onto the $Fe(OH)_3$ particles, making it unavailable for reduction until Fe(III) reduction occurred. Notably, however, the formation of $Fe(OH)_3$ did not appear to decrease the amount of arsenate in solution, as has been observed by others (9). While this is somewhat puzzling, one possible explanation is that even though our method for measuring dissolved arsenate could not discriminate between $Fe(OH)_3$ -associated and truly dissolved arsenate (samples were not filtered before measuring in this experiment), the microbial population could only access the dissolved arsenate. In contrast to recent studies that showed iron reduction was not necessary for the dissolution and reduction of ferric arsenate (10), this study seems to indicate that iron reduction was required to make arsenate available for reduction, again, pointing to differences in the arsenate-reducing community from one site to another.

Mobilization of Arsenic from Soil Slurries. While the previous experiments were perfomed in chemically defined medium for clarity of interpretation, it was necessary to determine whether arsenate-reduction could also occur in an undefined but natural system. Accordingly, we designed two different slurry experiments where contaminated soil was used as the sole source of arsenic. The first experiment was performed with a 2.5% slurry in batch culture over a period of 50 days. Rapid arsenate reduction was observed in a culture which

was in partial contact with the air (Fig. 5), however similar reduction was not observed in a duplicate sample (data not shown). This is probably due to the heterogeneous nature of the microbial population in the soil: the soil was not well mixed before distribution to the various experimental bottles. Nevertheless, in anaerobic samples that were inoculated with bacteria from the enrichment culture, arsenate reduction proceeded at similar rates in all samples (Fig. 5). Uninoculated anaerobic samples exhibited slower rates of arsenate reduction, but after 50 days, significant reduction had occurred relative to the controls. These results compare favorably with those which showed that strain MIT-13 could moblize arsenic from contaminated sediments in batch culture (10). On average, however, the bacterial consortia from our soils produced an order of magnitude more As(III) over time than strain MIT-13 alone (10). This may be due to the fact that more As(V) was available to the organisms from these soils than from those used in experiments with MIT-13, or due to differences in the medium (here, glucose was provided as a carbon source; in the MIT-13 experiments, acetate and H_2 served as electron donors).

Because our experiments with the enrichment culture indicated microaerophilic conditions could stimulate arsenate reduction (Fig. 2), the rapid arsenate reduction observed in the batch slurry partially open to the air was not unexpected. To pursue this, we performed a second slurry experiment, this time in semi-continous culture with a greater percentage of soil. The soil was thoroughly mixed before being dispensed into the experimental bottles to minimize artifacts resulting from heterogeneous bacterial populations. By periodically replacing the liquid in the bottles on a benchtop open to the air, microaerophilic conditions were maintained in the system. Similar nutrients were supplied to foster the growth of the indigenous population, although in one experimental set (set C), fumarate was also added as recent evidence points to a connection between arsenate and fumarate reduction (20). Averaging the total As(V) and As(III) concentrations measured in the liquid phase during the washes revealed that significantly greater amounts of As(III) were produced in sets containing nutrient media than in those containing only water or those poisoned with formaldehyde (Fig. 6a). Furthermore, fumarate appeared to stimulate release of As(V) from the soil (Fig. 6b). These results suggest that arsenate reduction by indigenous soil bacteria is relatively insensitive to low concentrations of

oxygen, and that mobilization of arsenate from soil may be driven by the type of organic carbon available.

With arsenate-reducing bacteria having now been enriched and isolated from both contaminated and pristine sites and from freshwater and saline environments, it is clear that microbial arsenate reduction is widespread and readily performed by diverse populations. Organic carbon appears to stimulate microbial arsenate reduction in every case, and because organic pollution often co-occurs in sites that are contaminated with arsenic, it seems possible that microbes native to these sites may be able to use these compounds (or their breakdown products) to drive arsenate reduction, as is known to be true for Fe(III)-reducers (21). This study, in contrast with previous studies of arsenate-reducing organisms (4-7,9,10), indicates that microbial arsenate-reduction is relatively insensitive to low concentrations of oxygen. This is important to recognize, as low concentrations of oxygen may be present in contaminated soils or sediments. Furthermore, this study suggests that the types of organisms involved in arsenate reduction differ from site to site: common soil constituents such as nitrate and sulfate appear to inhibit arsenate reduction to different degrees depending on the consortium, and iron-reducing bacteria appear to drive arsenate reduction to greater or lesser extents depending on the ability of the arsenate-reducing population to access arsenate when it is bound to iron solids. The ability of microbes to drive dissolution of arsenate through reduction of both iron and arsenate has the potential to mobilize arsenic. Given that the addition of ferric iron is the primary method currently used to remediate arsenic-contaminated sites, such microbial action may be problematic.

ACKNOWLEDGMENTS

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Figure legends

Fig 1. Biological As(V)-Reduction. Error bars represent the range of duplicate cultures.

Fig. 2. O_2 Stimulation of As(V)-Reduction. Error bars represent the range of duplicate cultures.

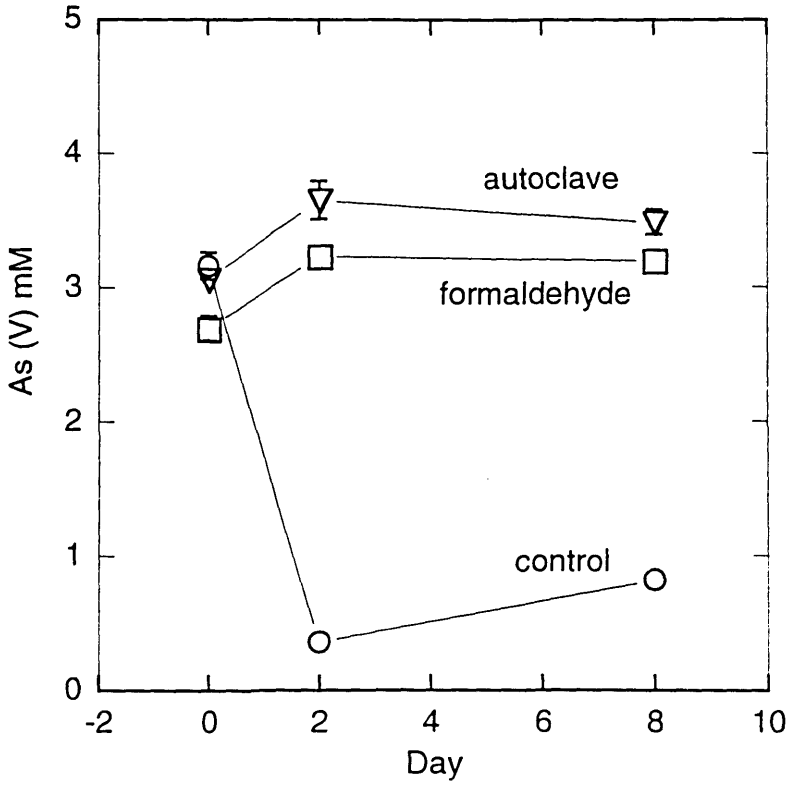
Fig. 3. Organic Carbon Stimulation of As(V)-Reduction. Error bars represent the range of duplicate cultures.

Fig. 4. Inhibition of As(V)-Reduction. Error bars represent the range of duplicate cultures.

Fig. 5. Batch Culture 2.5% Slurry Experiment. Representative data sets are shown for quadruplicate samples, in all cases but for those samples in partial contact with the atmosphere as decribed in the text. W=water; S=soil; N=nutrients; syr=syringe; B=inoculum; for=formaldehyde

Fig. 6. Semi-Continuous Culture 12.2% Slurry Experiment. **a.)** Dissolved As(III) measured for each wash **b.)** Dissolved As(V) measured for each wash. a1 = water; b1 = nutrient medium B; c1 = nutrient medium C; d1 = nutrient medium B + 4% formaldehyde. Data are representative of duplicate cultures.

Fig. 1


5
4
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0
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4
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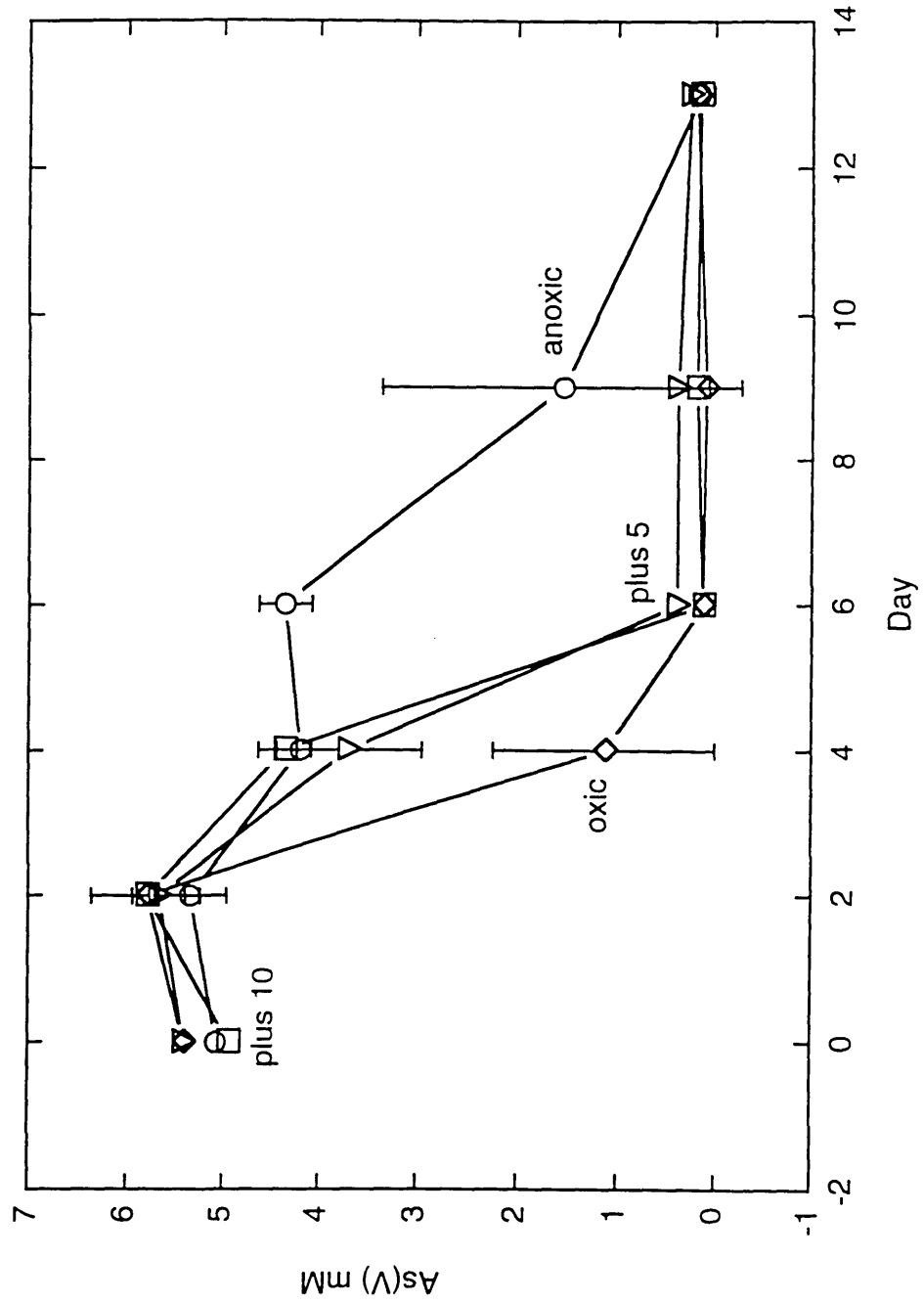
8
10
Day
autoclave
formaldehyde
control

Fig. 2


As(V) mM
Day
anoxic
plus 5
oxic
plus 10
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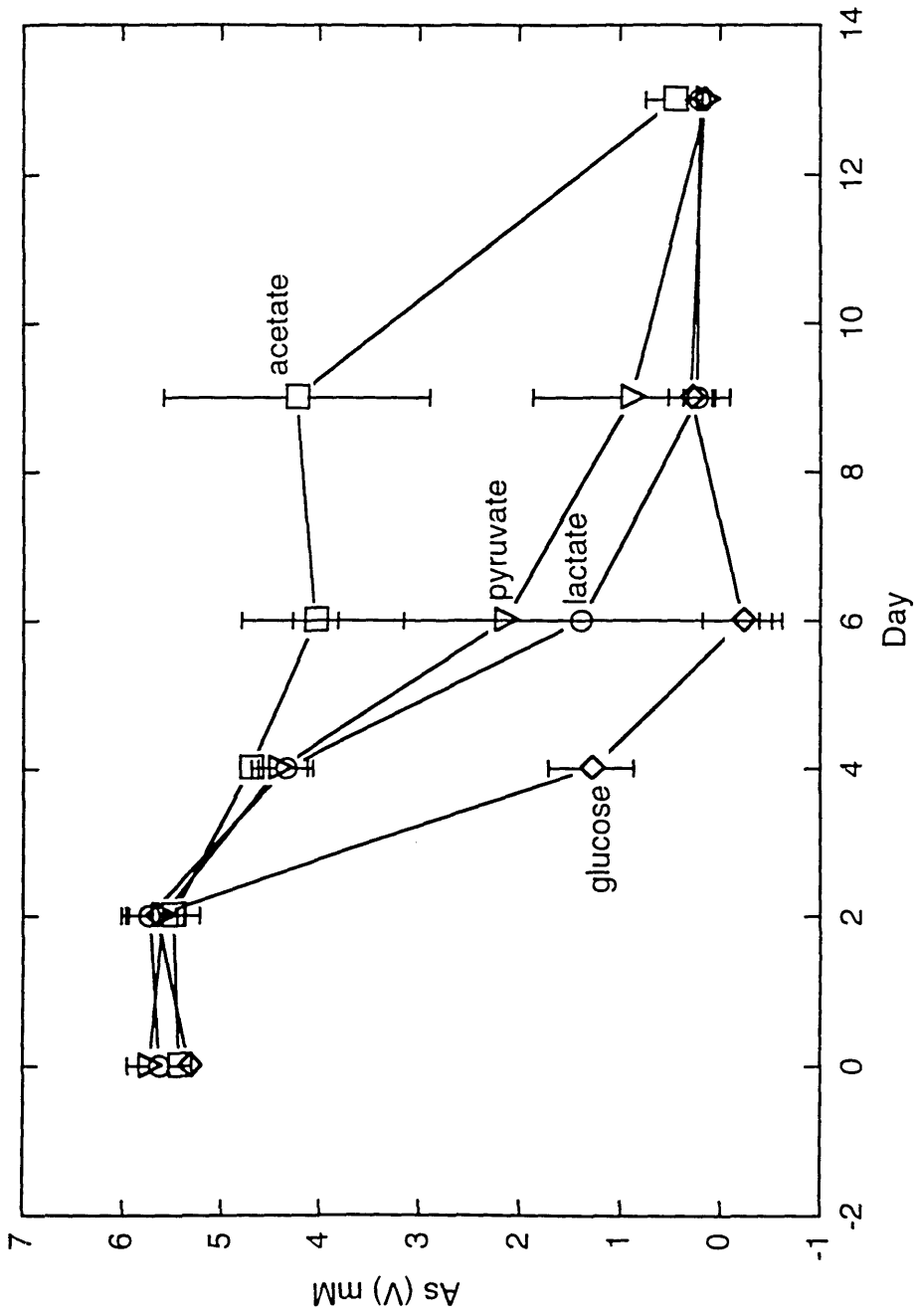
2
4
6
8
10
12
14

Fig. 3


As (V) mM
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6
5
4
3
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0
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2
4
6
8
10
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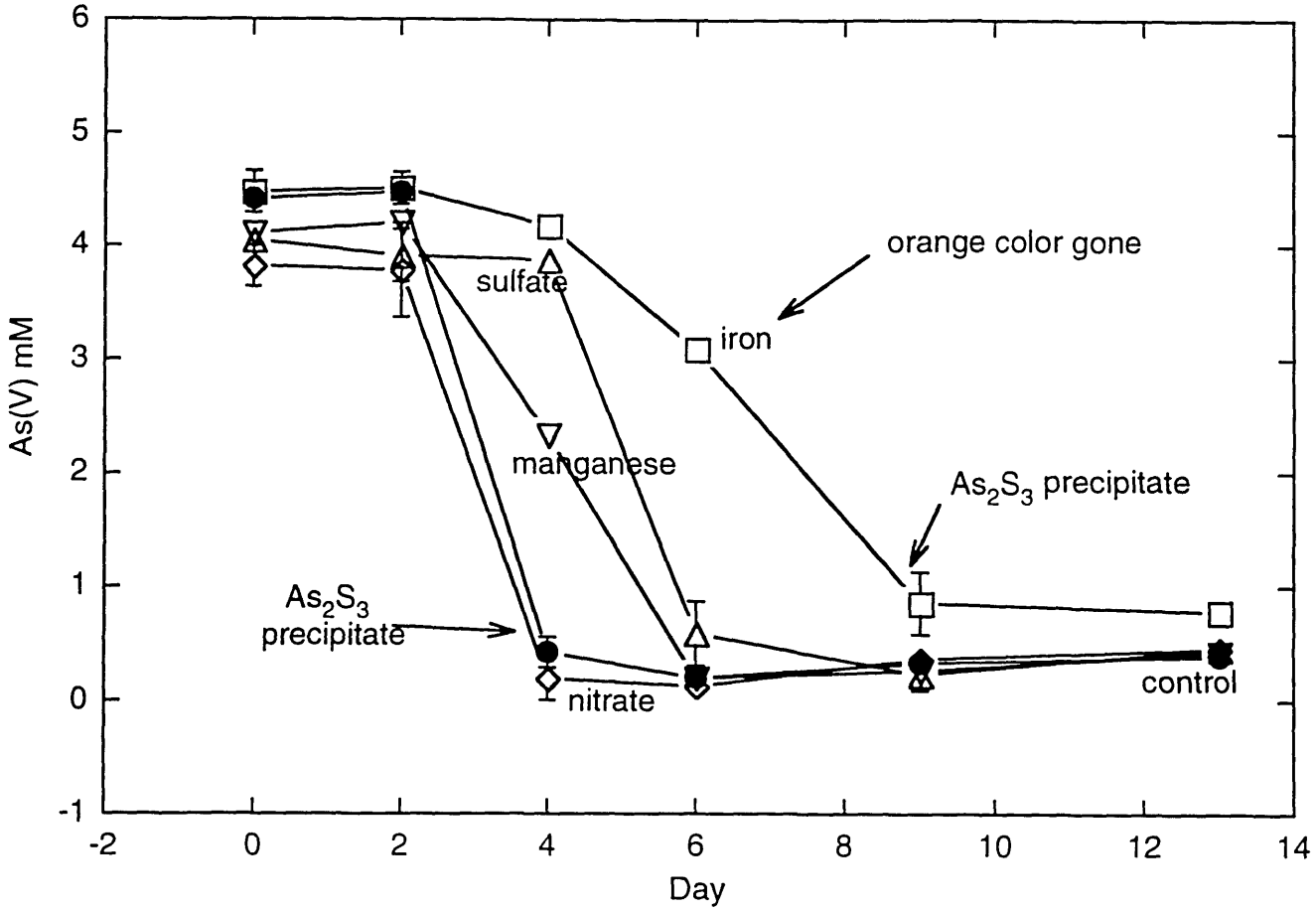
Day
acetate
pyruvate
lactate
glucose

Fig. 4


As(V) mM
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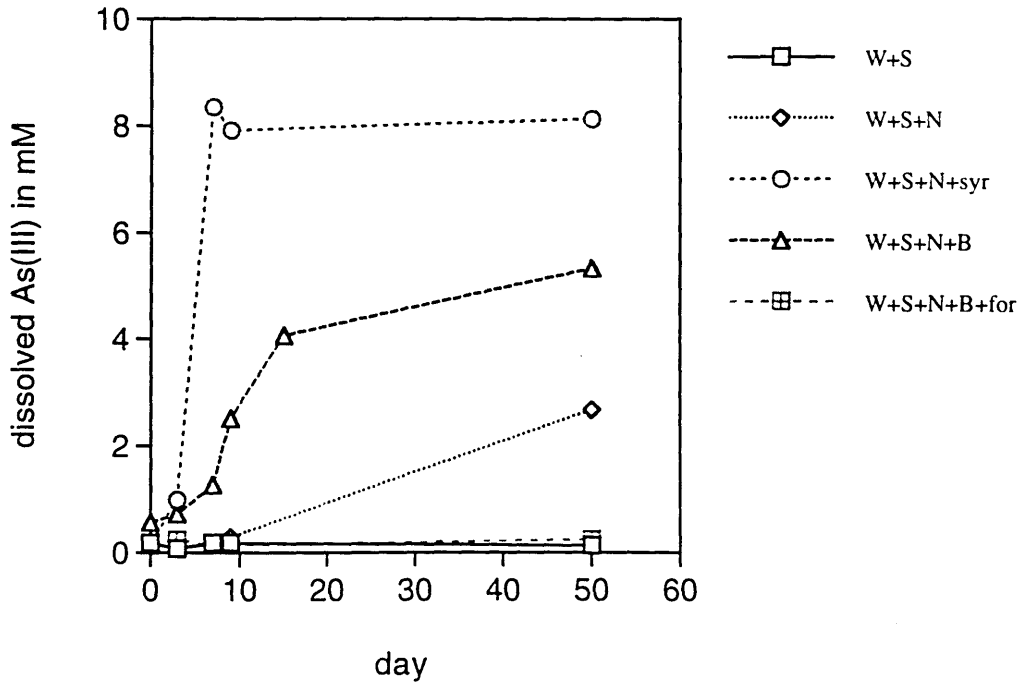
iron
sulfate
manganese
As_2S_3 precipitate
As_2S_3 precipitate
nitrate
control

Fig. 5


10
8
6
4
2
0
0
10
20
30
40
50
60
dissolved As(III) in mM
day
W+S
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W+S+N+syr
W+S+N+B
W+S+N+B+for

Fig. 6a

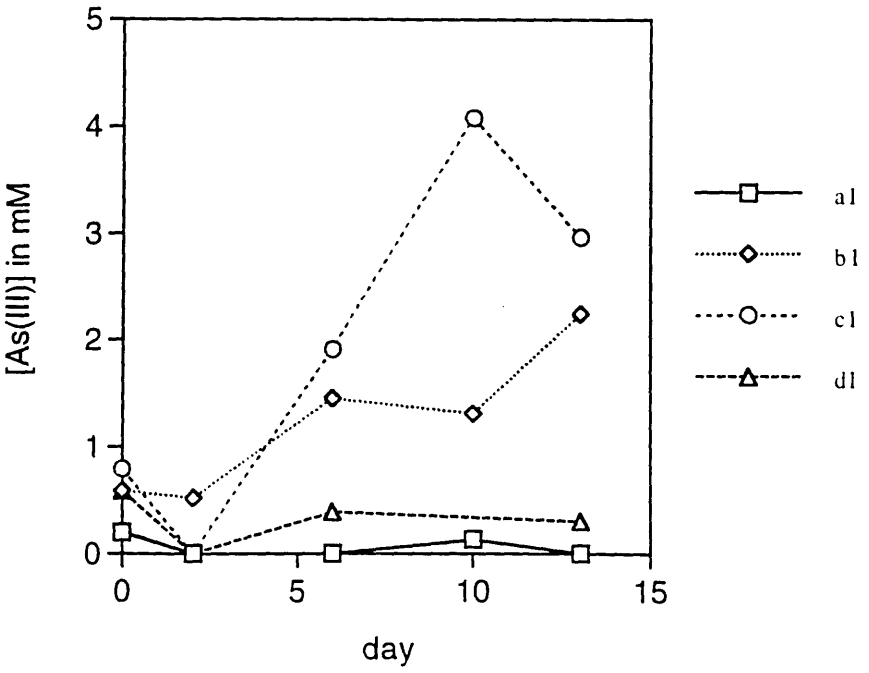
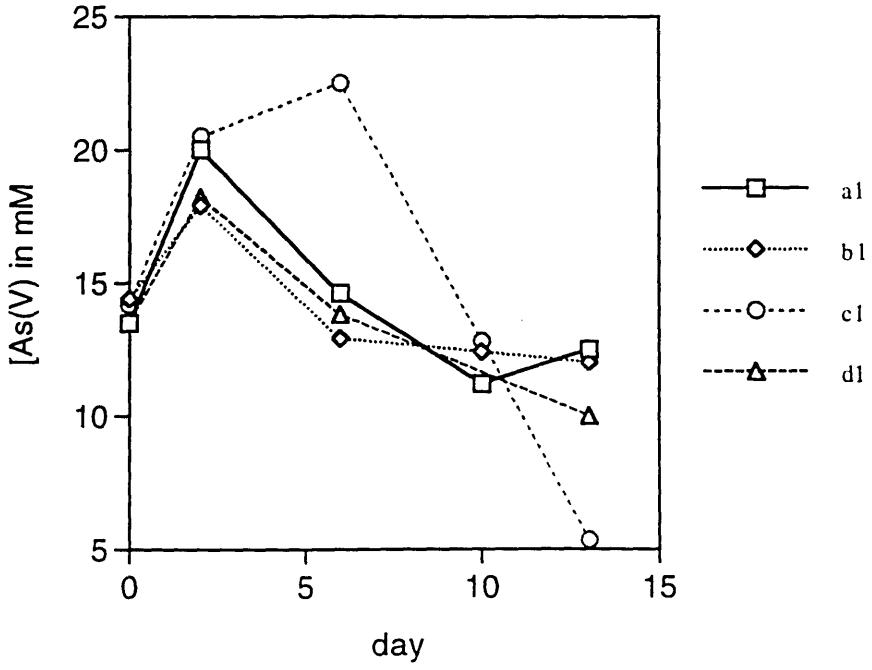


Fig. 6b



Appendix: The Kelvin Effect

Figure 4 in Chapter 3 contains a stability diagram for the precipitation of As_2S_3. As the particles precipitated by the bacteria are very small, there was some question as to whether the boundaries on this diagram would change significantly if the enhanced solubility of colloids was considered in the calculation. Accordingly, the following rough calculation was done to take into account the Kelvin effect (Stumm and Morgan, 1984). While the solubility increases slightly, it does not increase significantly enough to alter any of the interpretations discussed in the text. Lines have been marked on the lower portion of the diagram to show where the boundary would fall considering the Kelvin effect. The same assumptions about species and concentrations have been made as described in the text.

The solubility of the colloids relative to larger particles is given by:

$$\log K_{sp}\,(r = 10\text{ nm}) = \log K_{sp}\,(r = \infty) + \frac{2(mw)(\gamma)}{2.303(RT\varrho r)}$$

where K_{sp} (r = 10 nm) is the solubility product of the 10-nm colloids
K_{sp} ($r = \infty$) is the solubility product of large particles
mw is the molecular weight
γ is the mineral/water interfacial suface energy
R is the gas constant
T is the absolute temperature
ϱ is the mineral density
r is the colloidal radius

The following values have been used:

$\log K_{sp}$ ($r = \infty$) = -11.9 (given for amorphous As_2S_3 by Eary, L.W. 1992. *Geochem. Cosmochim. Acta.* 56:2267-2278)
mw = 246.02 g/mol
γ = assumed to be 30×10^{-7} J cm^{-2}
R = *8.31* $Jmol^{-1}\,deg^{-1}$
T = 298.15 deg
ϱ = 3.5 g cm^{-3} (average of measured and calculated values for As_2S_3 from the *Encyclopedia of Minerals*, ed. Roberts, W.L, Rapp Jr., G.R., and Weber, J.)
$r = 1 \times 10^{-6}$ cm (taken as the value from STEM images of the precipitates)

Solving the equation, we find $\log K_{sp}$ (r = 10 nm) = -11.826

REFERENCES

Gschwend, P.M. and Reynolds, M.D., 1987. *J. Contaminant Hydrology* 1:309-327.
Stumm, W., and Morgan, J.J., 1984. Aquatic Chemistry. Wiley-Interscience, New York, NY.

STEM image of colloidal particles of As_2S_3.

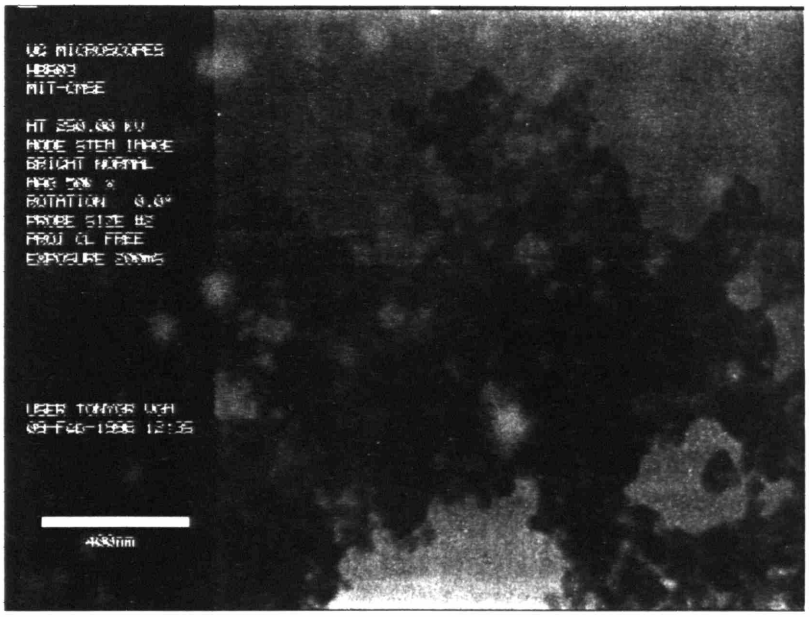


Figure 4 (Chapter 3) stability diagram showing movement of bottom boundary considering the Kelvin effect.