Growth of Strain SES-3 with Arsenate and Other Diverse Electron Acceptors

ANNIET M. LAVERMAN,1 JODI SWITZER BLUM,1 JEFFRA K. SCHAFFER,1 ELIZABETH J. P. PHILLIPS,2 DEREK R. LOVLEY,2 AND RONALD S. OREMLAND1*


Received 17 April 1995/Accepted 26 July 1995

The selenate-respiring bacterial strain SES-3 was able to use a variety of inorganic electron acceptors to sustain growth. SES-3 grew with the reduction of arsenate to arsenite, Fe(III) to Fe(II), or thiosulfate to sulfide. It also grew in medium in which elemental sulfur, Mn(IV), nitrite, trimethylamine N-oxide, or fumarate was provided as an electron acceptor. Growth on oxygen was microaerophilic. There was no growth with arsenite or chromate. Washed suspensions of cells grown on selenate or nitrate had a constitutive ability to reduce arsenate but were unable to reduce arsenite. These results suggest that strain SES-3 may occupy a niche as an environmental opportunist by being able to take advantage of a diversity of electron acceptors.

Some microorganisms can use certain metals and metalloids as terminal electron acceptors for respiratory growth (15). Examples of these metals and metalloids include Fe(III), Mn(IV), U(VI), and Se(VI). The reduction of U(VI) to U(IV) and that of Se(VI) to Se0 are important mechanisms for the detoxification of these metals and metalloids in soils (23). Although chemical processes can account for such changes during speciation (6), the ability of microbial assemblages to reduce As(V) to As(III) has also been reported (8). Bacterial strain SES-3 is capable of growth with lactate as the electron donor and As(V) as the electron acceptor (2). Reduction of As(V) to As(III) has been observed across the oxic-anoxic boundaries of stratified lakes and fjords (1, 21, 29, 25, 26). The reduction of As(V) to As(III) has been observed across the oxic-anoxic boundaries of stratified lakes and fjords (1, 21, 29, 25, 26). The reduction of As(V) to As(III) has been observed across the oxic-anoxic boundaries of stratified lakes and fjords (1, 21, 29, 25, 26).

GROWTH EXPERIMENTS. Strain SES-3 was grown in an anaerobic, lactate-based medium described previously (26). Log-phase cultures of SES-3 in tubes containing 10 ml of medium with 20 mM sodium selenate as the electron acceptor were used as inocula (5%, vol/vol) for starter cultures in medium identical in all respects except that it had NaH2AsO4 (5 or 10 mM) in lieu of selenate. Turbidity (A680 = ~0.06) was achieved within 2 to 3 days, at which time the culture was transferred. Five sequential transfers were made to ensure that selenate was diluted out of the medium before growth experiments were conducted. For growth on thiosulfate, a low-sulfate, lactate-based medium of Lovley and Phillips (17) was employed with the inclusion of 4 mM Na2S2O3, omission of NaH2PO4, and the following modifications (grams per liter): K2HPO4 (0.3), KH2PO4 (0.3), MgCl2•6H2O (1.5), cystine-HCl (0.5), yeast extract (1.0), and peptone (2.5). Growth experiments were performed with serum bottles (160 ml) containing 100 ml of medium with an atmosphere of N2-CO2 (4:1) and incubated at 30°C without shaking. Growth in this medium was also tested with sodium nitrate (10 mM), trimethylamine N-oxide (10 mM) and sodium chromate (5 mM) as electron acceptors. In these experiments, cells were grown in crimp-sealed culture tubes (Balch tubes). For growth with Fe(III)-nitrotriacetic acid as the electron acceptor, medium containing the following was employed: NaHCO3 (4.2), NH4Cl (0.3), K2HPO4 (0.225), KH2PO4 (0.225), NaCl (0.46), Na2SO4 (0.24), CaCl2•2H2O (0.15), MgCl2•6H2O (0.4), cystine-HCl (0.25), Na2S•9H2O (0.4), and yeast extract (0.5). The medium also contained 10 ml of vitamin and mineral mixtures (16), the latter modified by inclusion of 0.025 g of Na2WO4. The final pH of the medium was adjusted to 7.3. Fe(III) was added as Fe(III)-nitrotriacetic acid (final concentration, 20 mM) by filter sterilization after autoclaving. Cells were incubated in Balch tubes with 10 ml of medium. Other electron acceptors used in lieu of Fe(III) to test for growth included manganese dioxide (5 mmol of MnO2 per liter of medium) and sodium fumarate (5 mM). Growth on colloidal sulfur (~7 mmol of S0 per liter of medium) was monitored. S0 was generated by combining 50 ml of an acidified 500 mM Na2S solution with 50 ml of an acidified 500 mM Na2SO4 solution under N2. The white S0 precipitate was washed twice with distilled water and then restored to a volume of 50 ml, after which it was pasteurized by being kept at 85°C for 15 min. Five milliliters of the suspension was added to 100 ml of lactate growth medium in 160-ml serum bottles. Growth on Fe(III) was also tested by substituting acetate or acetate plus H2 instead of lactate as the electron donor and carbon source. Growth experiments with O2 as the electron acceptor were done with cultures containing tubes of the lactate-based medium (28) but without reducing agents or other electron acceptors. Tubes were sealed under N2-CO2 (4:1), and oxygen was added aseptically by injection with a syringe to yield final headspace levels of 1, 3, 5, 7.5, 10, 15, and 20%. Tubes were incubated either statically or with constant shaking (200 rpm) at 28°C. The controls run in conjunction with these experiments consisted of medium lacking an electron acceptor or sealed under air instead of the N2 + CO2 + O2 headspace. An anaerobic tube containing 20 mM nitrate as the electron acceptor (but with no reducing agent) was run for comparison with the growth on O2.

MATERIALS AND METHODS

GROWTH EXPERIMENTS. Strain SES-3 was grown in an anaerobic, lactate-based medium described previously (26). Log-phase cultures of SES-3 in tubes containing 10 ml of medium with 20 mM sodium selenate as the electron acceptor were used as inocula (5%, vol/vol) for starter cultures in medium identical in all respects except that it had NaH2AsO4 (5 or 10 mM) in lieu of selenate. Turbidity (A680 = ~0.06) was achieved within 2 to 3 days, at which time the culture was transferred. Five sequential transfers were made to ensure that selenate was diluted out of the medium before growth experiments were conducted. For growth on thiosulfate, a low-sulfate, lactate-based medium of Lovley and Phillips (17) was employed with the inclusion of 4 mM Na2S2O3, omission of NaH2PO4, and the following modifications (grams per liter): K2HPO4 (0.3), KH2PO4 (0.3), MgCl2•6H2O (1.5), cystine-HCl (0.5), yeast extract (1.0), and peptone (2.5). Growth experiments were performed with serum bottles (160 ml) containing 100 ml of medium with an atmosphere of N2-CO2 (4:1) and incubated at 30°C without shaking. Growth in this medium was also tested with sodium nitrate (10 mM), trimethylamine N-oxide (10 mM) and sodium chromate (5 mM) as electron acceptors. In these experiments, cells were grown in crimp-sealed culture tubes (Balch tubes). For growth with Fe(III)-nitrotriacetic acid as the electron acceptor, medium containing the following was employed: NaHCO3 (4.2), NH4Cl (0.3), K2HPO4 (0.225), KH2PO4 (0.225), NaCl (0.46), Na2SO4 (0.24), CaCl2•2H2O (0.15), MgCl2•6H2O (0.4), cystine-HCl (0.25), Na2S•9H2O (0.4), and yeast extract (0.5). The medium also contained 10 ml of vitamin and mineral mixtures (16), the latter modified by inclusion of 0.025 g of Na2WO4. The final pH of the medium was adjusted to 7.3. Fe(III) was added as Fe(III)-nitrotriacetic acid (final concentration, 20 mM) by filter sterilization after autoclaving. Cells were incubated in Balch tubes with 10 ml of medium. Other electron acceptors used in lieu of Fe(III) to test for growth included manganese dioxide (5 mmol of MnO2 per liter of medium) and sodium fumarate (5 mM). Growth on colloidal sulfur (~7 mmol of S0 per liter of medium) was monitored. S0 was generated by combining 50 ml of an acidified 500 mM Na2S solution with 50 ml of an acidified 500 mM Na2SO4 solution under N2. The white S0 precipitate was washed twice with distilled water and then restored to a volume of 50 ml, after which it was pasteurized by being kept at 85°C for 15 min. Five milliliters of the suspension was added to 100 ml of lactate growth medium in 160-ml serum bottles. Growth on Fe(III) was also tested by substituting acetate or acetate plus H2 instead of lactate as the electron donor and carbon source. Growth experiments with O2 as the electron acceptor were done with cultures containing tubes of the lactate-based medium (28) but without reducing agents or other electron acceptors. Tubes were sealed under N2-CO2 (4:1), and oxygen was added aseptically by injection with a syringe to yield final headspace levels of 1, 3, 5, 7.5, 10, 15, and 20%. Tubes were incubated either statically or with constant shaking (200 rpm) at 28°C. The controls run in conjunction with these experiments consisted of medium lacking an electron acceptor or sealed under air instead of the N2 + CO2 + O2 headspace. An anaerobic tube containing 20 mM nitrate as the electron acceptor (but with no reducing agent) was run for comparison with the growth on O2.

Experiments with washed cells. Log-phase batch cultures of SES-3 grown with either selenate or nitrate were harvested and washed as outlined previously (28). Washed cell suspensions (50 ml) were incubated in 160-ml serum bottles with a headspace of O2-free N2. We eliminated the use of a reducing agent in these experiments to prevent chemical reduction of arsenate. Suspensions were tested

* Corresponding author. Mailing address: U.S. Geological Survey, ms 465, 345 Middlefield Road, Menlo Park, CA 94025. Phone: (415) 329-4482. Fax: (415) 329-4463. Electronic mail address: roremalan@mprcamml.wr.usgs.gov.
for the ability to reduce electron acceptors which included (for nitrate-grown
cells) nitrate (5 mM), arsenate (5 mM), and nitrate plus arsenate (2.5 mM each).
Selenate-grown cells were tested for the ability to reduce selenate (5 mM) or
arsenate (5 mM). The ability of nitrate-grown cells to reduce arsinite (5 mM)
was also examined. To evaluate the capacity of SES-3 to reduce U(VI) and
Cr(VI), Fe(III)-grown cells were harvested and suspended in bicarbonate buffer
containing U(VI) or Cr(VI), the loss of which was monitored over time as
previously described (18, 19).

**Analyses.** Selenium and arsenic oxyanions, thiosulfate, lactate, and acetate
were quantified by high-performance liquid chromatography (5) modified by the
addition in series of a second column (Hamilton PRP-X300; 15 by 0.41 cm
[diameter]) to improve resolution of eluted anions. Sulfide was measured by the
methylene blue method of Cline (4), and Fe(II) was measured by the ferrozine
method (17). Cell densities in quantitative growth experiments on Fe(III),
asenate, and thiosulfate, as well as in cell suspension experiments, were deter-
dined by acridine orange direct counts (11). Growth on nitrite, trimethylamine
N-oxide, and chromate was monitored qualitatively by achieving three successive transfers which resulted in either color
changes in the medium during incubation (MnO₂) or turbidity increases (fuma-
rate).

**RESULTS**

**Growth experiments.** Strain SES-3 grew by reducing 5 mM
As(V) while oxidizing lactate (Fig. 1). Approximate balances
were achieved in terms of lactate removed and acetate formed
(Fig. 1A), as well in recovery of As(III) from As(V) (Fig. 1B).
There was no decline in As(III) after prolonged incubation.
The doubling time for growth was 5 h. Controls incubated
without an electron acceptor had about 13% of the growth
observed with As(V) (data not shown). In an uninoculated
control, a small decrease of As(V) (~0.9 mM) occurred over
115 h and was accompanied by an increase in As(III) (data not
shown). Strain SES-3 grew in medium containing 10 mM

![FIG. 1. Growth of strain SES-3 on 5 mM As(V). (A) Symbols: ○, lactate; □, acetate. (B) Symbols: ▲, As(V); △, As(III); +, cells. These results are averages of two cultures, and the bars indicate the spread of values. Absence of a bar indicates that the spread was smaller than the symbol.](http://aem.asm.org/)
As(V) (Fig. 2), but growth was slower (doubling time, 6.7 h) and cell densities were lower. No growth was observed in medium with As(III) as the electron acceptor (data not shown).

Strain SES-3 grew slowly (doubling time, 13 h) on thiosulfate (Fig. 3A). There was a 1:2 stoichiometry between the thiosulfate consumed and the sulfide produced (Fig. 3B). Strain SES-3 also exhibited growth and sulfide production when elemental sulfur was the electron acceptor (Fig. 4).

Figure 5 shows the growth of SES-3 with Fe(III) as the electron acceptor. With H₂ as the electron donor and acetate as the carbon source, cells grew with concomitant formation of Fe(II) (Fig. 5A). No growth or Fe(II) formation was observed when either H₂ or acetate was solely present in the medium (data not shown). Formate could replace H₂ and support growth, provided that acetate was present to serve as a carbon source (data not shown). Growth and Fe(II) formation were also achieved when lactate was the sole electron donor and carbon source (Fig. 5B). A control with no added electron acceptor did not support growth, while another control with no electron donor did not produce Fe(II) (Fig. 5B). SES-3 was also able to grow via Fe(III) reduction when succinate or citrate served as the electron donor (data not shown).

A small amount of growth was observed when O₂ was the electron acceptor in statically incubated cultures (Fig. 6). In comparison, anaerobic growth on nitrate was far more rapid and extensive. Cells incubated under 15 and 20% O₂ eventually achieved higher turbidities after prolonged incubation, reaching maximum A₆₈₀ values after 105 h of 0.14 and 0.12, respectively (data not shown). Growth at lower pO₂ was less extensive, with maximum A₆₈₀ values of 0.065 for 10% O₂ at 72 h and 0.04 for 1% O₂ at 48 h (data not shown). Shaking the cultures to promote the exchange of O₂ did not enhance growth. Indeed, time lags occurred before growth was evident, with the lower-pO₂ samples (1, 3, and 5% O₂) exhibiting 40-h lags, while longer lag periods took place in higher-pO₂ samples (e.g., 95 h for 15% O₂). No such lags occurred in unshaken cultures (Fig. 6). Shaken cultures grown with 15% O₂ achieved the highest turbidity (A₆₈₀ 0.12 at 140 h) with a doubling time of ~20 h. Cells incubated with constant shaking at 20% O₂ grew very poorly (maximum A₆₈₀ 0.03).

Nitrite, trimethylamine N-oxide, fumarate, and Mn(IV) also supported the growth of SES-3 (data not shown). Growth in nitrite medium was characterized by lags of 1 to 3 days before growth was evident, but cultures eventually became turbid (A₆₈₀ 0.22). Cells in trimethylamine N-oxide medium grew poorly (A₆₈₀ 0.06), and weak growth was also noted with fumarate or Mn(IV). Nonetheless, growth on Mn(IV) always resulted in complete clearing of the dark purple medium and accumulation of a white MnCO₃ precipitate. Chromate did not support growth.

Experiments with cell suspensions. Suspensions of nitrate-grown cells completely removed nitrate, as well as the transient intermediate nitrite (Fig. 7A). In contrast, although reduction of As(V) to As(III) occurred immediately, it was slower than nitrate reduction (Fig. 7B). Similarly, Se(VI)-grown cells reduced Se(VI), as well as the transient intermediate Se(IV) (Fig. 8A). Se(VI)-grown cells were also able to reduce As(V) to As(III) (Fig. 8B). There was no clear evidence of As(III) removal by further reduction, although recovery of As(III) was not in balance with the amount of As(V) reduced. When nitrate-grown cells were given nitrate plus As(V), nitrate reduc-

FIG. 3. Growth of strain SES-3 on 4 mM thiosulfate. These results are averages of two cultures, and the bars indicate the spread of values. Absence of a bar indicates that the spread was smaller than the symbol.
tion proceeded as noted before (rate, 1.5 mmol h\(^{-1}\)) and complete removal of both nitrate and nitrite was evident by 1.7 h (data not shown). The poor growth on 5 mM As(V) could not be attributed to the low concentration of As(V) employed because growth on 10 mM As(V) (Fig. 2) yielded even lower cell densities and longer doubling times. The evidence indicates that the accumulated As(III) inhibited further growth. The cell densities on 10 mM As(V) were close to the value reported for strain MIT-13 (2). However, growth of MIT-13 was better at 10 mM than it was at 5 mM As(V). In addition, the cell density of MIT-13 on 5 mM As(V) was fivefold lower than that achieved by SES-3. These observations suggest some physiological differences between the two strains.

The As(V)-to-lactate consumption ratio of strain SES-3 was ~1.63, which was comparable to the value of 1.85 reported for growth on selenate (28). However, assuming a cell dry weight of 1.9 \(\times\) 10\(^{-10}\) mg cell\(^{-1}\) and that 3.3 mmol of lactate per liter is consumed to achieve a density of 9.2 \(\times\) 10\(^7\) cells ml\(^{-1}\) for growth on 5 mM As(V) (Fig. 1), we calculated a molar growth yield of 5.3 g of cells per mol of lactate. This value is below those previously reported (28) for growth on selenate (11.5 g/mol) and nitrate (7.1 g/mol). Furthermore, the energy yield on As(V) can be calculated from published \(\Delta G^0\) values (35, 38) in accordance with the equation:

\[
\text{Lactate}^{-1} + 2\text{HAsO}_4^{2-} + \text{H}^+ \rightarrow \text{Acetate}^{-} + 2\text{H}_2\text{AsO}_3^{-} + \text{HCO}_3^{-}
\]

This gives a \(\Delta G^0\) of ~140.3 kJ/mol of lactate (~23.4 kJ/e\(^{-}\) equivalent), which is considerably less than the values of ~343.1 kJ/mol (~85.8 kJ/e\(^{-}\)) and ~231.3 kJ/mol (~57.8 kJ/e\(^{-}\)) calculated for reduction of Se(VI) to Se(IV) and nitrate to nitrite, respectively (28). Hence, our data confirm the novel observation of the ability of anaerobes to achieve dissimilatory growth on As(V) (2). The poorer growth on As(V) is in accordance with its lower energy yield.

The metabolism of Se(IV) by strain SES-3 differs in some respects from its response to As(III). Neither As(III) nor Se(IV) supports the growth of SES-3. However, although cell suspensions reduced Se(VI) to Se\(^0\) (Fig. 8A), they did not

**DISCUSSION**

The results described here demonstrate that SES-3 is a versatile respiratory organism which has the potential to play several important environmental roles. Although a previous study emphasized its ability to influence the redox chemistry of selenium and nitrogen (28), SES-3 can also reduce a variety of other metals, metalloids, and inorganic electron acceptors. Its ability to use As(V) to support growth is of special interest because biological reduction of As(V) may be important in affecting arsenic geochemistry (8, 13, 22, 23, 30) and there has been only one previous report of dissimilatory As(V) reduction (2).

The highest cell density achieved on As(V) was 9.2 \(\times\) 10\(^7\) ml\(^{-1}\) (Fig. 1). This is about 6.5-fold lower than that achieved by growth on Se(VI) and about 11-fold lower than that achieved by growth on nitrate (28), although the doubling times were roughly comparable for all three electron acceptors (3 to 5 h).

**FIG. 5.** Growth of strain SES-3 on Fe(III). Initial formation of Fe(II) by chemical reduction of Fe(III) with cysteine-sulfide in sterile medium was subtracted, and the data represent only biological Fe(III) reduction. Open symbols represent Fe(II) concentrations, and closed symbols represent cells. (A) With H\(_2\) plus acetate as the electron donor (○, ●); (B) with lactate as the electron donor (▲, △), a control with no electron donor (□), and a control without Fe(III) (▲).
reduce As(III) to As⁰ or As(−III) (Fig. 7B and 8B). Reduction of As(III) to As(−III) is thermodynamically favorable for growth:

\[
3 \text{lactate}^- + 2H_2AsO_4^- \rightarrow 2\text{AsH}_3 + 3 \text{acetate}^- + 3\text{HCO}_3^- + \text{H}^+ \quad (2)
\]

Reaction 2 yields a \( \Delta G_f^\circ \) of \(-138.4 \text{ kJ/mol of lactate} \) (\(-34.6 \text{ kJ/e}^- \) equivalent), and the reduction of As(III) to As⁰ also has a favorable energy yield (\(-166.5 \text{ kJ/mol or } -41.6 \text{ kJ/e}^- \)). However, we did not observe strain SES-3 to carry out these reactions. Strains of \textit{Pseudomonas} and \textit{Alcaligenes} spp. reduced As(V) and As(III) to As(−III) but did not employ them as respiratory electron acceptors for growth (3).

Cell suspensions of both nitrate-grown and selenate-grown cells had a constitutive ability to reduce As(V) to As(III) (Fig. 7 and 8). This is curious because Se(VI)-grown cells cannot reduce nitrate and nitrate-grown cells cannot reduce Se(VI) (28). Hence, either the capacity to reduce As(V) resides in another enzyme system or both nitrate and Se(VI) reductases can reduce As(V).

Strain SES-3 grows on thiosulfate (Fig. 3), as well as on elemental sulfur (Fig. 4), but not on sulfate or sulfite (28). Strain SES-3 has a number of physiological and substrate affinities in common with the sulfur-reducing bacterium \textit{Sulfurospirillum deleyianum}, as well as some \textit{Campylobacter} species and \textit{Wolinella succinogenes}, all of which are members of the epsilon subgroup of cubacteria (31, 36, 37). We previously reported that strain SES-3 is a strict anaerobe (28). Since we have observed growth of SES-3 under aerobic conditions (Fig. 6), we now report that our earlier claim was in error. However, growth of SES-3 on O₂ was sluggish and was maximal at 15% O₂. These are characteristics of a microaerophile as broadly defined (14). Analysis by DNA sequencing of the 16S rRNA has placed strain SES-3 in the epsilon subgroup, with sufficient distance from characterized species to be assigned a new genus name (34).

The reduction of As(V) to As(III) by bacterial respiration may be responsible for one aspect of the biogeochemical cy-
cling of arsenic (1, 21–23, 29, 32). The mobility and speciation of arsenic in sediments, however, is a complex process governed in part by chemical oxidation-reduction reactions and adsorption onto mineral phases (7). Nonetheless, since As(V) is adsorbed to by Fe(III) (9), bacterial reduction of Fe(III) to Fe(II) should result in liberation of bound As(V), which can then be available for reduction (24). The dissipative reduction of As(V) may also have bioremediation implications for the removal of arsenic. If the mobilized As(III) can be sequestered with sulfide to form a precipitate or re-oxidized to As(V) and adsorbed to by Fe(III), then trapping mechanisms are suggested for any As(III) liberated by anaerobic treatment of contaminated soils.

ACKNOWLEDGMENTS

We are grateful to P. T. Vischer, J. Stolz, L. Hochstein, and two unidentified referees for critical reviews of versions of the manuscript. Financial support for A. Laverman was provided by the government of The Netherlands. We thank R. Prins for help in arranging this support. This work was also funded in part by support from the USEPA (IAG DW 14936864-01-1) and by the USDA-NRICG program. Financial support for A. Laverman was provided by the government of The Netherlands. We thank R. Prins for help in arranging this support. This work was also funded in part by support from the USEPA (IAG DW 14936864-01-1) and by the USDA-NRICG program.

REFERENCES