

# **Oxidation of Arsenite by *Agrobacterium albertimagni*, AOL15, sp. nov., Isolated from Hot Creek, California**

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An arsenite-oxidizing bacterium, *Agrobacterium albertimagni* strain AOL15 (ATCC BAA-24), was isolated from the surface of aquatic macrophytes collected in Hot Creek, California. Under laboratory conditions, whole cell suspensions of AOL15 oxidized arsenite with a  $K_s$  of  $3.4 \pm 2.2 \mu\text{M}$  and a  $V_{\text{max}}$  of  $1.81 \pm 0.58 \times 10^{-12} \mu\text{mole} \cdot \text{cell}^{-1} \cdot \text{min}^{-1}$  (or  $0.043 \pm 0.017 \mu\text{mole} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ ). The  $K_s$  value for AOL15 is the lowest value to date reported for whole cell suspensions and is comparable to ambient concentrations of arsenic of  $2.7 \mu\text{M}$  reported for Hot Creek, indicating that AOL15 can oxidize arsenite under ambient conditions. Previous studies at this site revealed a rapid *in situ* oxidation of geothermally-derived arsenite while field incubation studies demonstrated that this oxidation was bacterially mediated. The isolation of the arsenite oxidizer AOL15 from this environment supports these previous observations. Arsenite does not support chemolithoautotrophic growth of AOL15 and toxicity studies with AOL15 showed that arsenite (at 5 mM) is toxic to AOL15, yet arsenate concentrations as high as 50 mM do not show any toxic effects. These results suggest that the oxidation of arsenite by AOL15 is a detoxification mechanism.

**Keywords** arsenite-oxidation, *Agrobacterium albertimagni* strain AOL15, arsenite, arsenic, redox, microbial oxidation

Geothermal waters often contain elevated concentrations of arsenic and can be an important source of arsenic to both surface and groundwater (Welch et al. 1988). In the eastern Sierra Nevada, California, the concentrations of arsenic in Hot Creek, a tributary of the Owens River, average  $2.7 \mu\text{M}$  as a result of inputs of geothermal water containing arsenic in concentrations in excess of  $13 \mu\text{M}$  (DWR 1967; Eccles 1976). Arsenic in the geothermal water at Hot Creek occurs primarily in the +III oxidation state (arsenite) but, after mixing with meteoric surface water, undergoes rapid *in situ* oxidation to As(V) or arsenate (Wilkie and Hering 1998), which is thermodynamically favored in oxic surface waters (Ferguson and Gavis 1972; Cullen and Reimer 1989). Field incubation studies demonstrated that the oxidation was associated with an aquatic macrophyte, *Potamogeton pectinatus*. Rapid arsenite oxidation was observed in the presence of the plant or of particulate material detached from the plant surfaces by shaking. Inhibition by antibiotics and filtration suggested that arsenite oxidation was mediated by bacteria colonizing the surface of the aquatic macrophytes (Wilkie and Hering 1998).

To date, several microorganisms are known to catalyze transformations of arsenic including oxidation, reduction, and methylation (Cullen and Reimer 1989; Dowdle et al. 1996; Newman et al. 1998; Stolz and Oremland 1999). Arsenite-oxidizing bacteria have been isolated from many different environments (i.e., cattle-dipping fluids, soil, sewage, mines and mine waters, and aquatic environments) and have been studied under laboratory conditions. Table 1 summarizes the different studies on arsenite-oxidizing isolates or consortia. Although the ambient concentrations of arsenic were not reported for many of these studies, the concentrations used in the laboratory studies were highly elevated in some cases (e.g., 100 mM for isolates from cattle-dipping fluids) (Green 1918; Turner 1949; Legge 1954; Legge and Turner 1954; Turner 1954; Turner and Legge 1954). In the studies where whole-cell or enzyme kinetics were examined, Michaelis-Menten  $K_s$  or  $K_m$  values range from 2 to  $1540 \mu\text{M}$  (Osborne and Ehrlich 1976; Phillips and Taylor 1976). In the current study, the ambient concentrations of arsenic are near  $2.7 \mu\text{M}$ , and the experimental concentrations of arsenic are low ( $2\text{--}450 \mu\text{M}$  for kinetic studies) relative to other reports of arsenite oxidation.

Although numerous studies have reported on arsenite oxidation, its physiological role is not always well understood or described. In two studies, arsenite-oxidizing strains isolated from mine waters, *Pseudomonas arsenitoxidans* (Ilyaletdinov and Abdrashitova 1981) and strain NT-26 (Santini et al. 2000), were shown to be capable of chemolithoautotrophic

**TABLE 1** Summary of studies of arsenite oxidizers

Microorganism	Isolation environment	Ambient [As]	Experimental [As] (mM)	K <sub>m</sub> (μM)	V <sub>max</sub>	Reference
<i>Bacillus arsenoxydans</i>	Cattle-dipping fluids	NR	20-100	NR	NR	1
<i>Pseudomonas arsenoxydans</i>	Cattle-dipping fluids	NR	20-100	450 <sup>†</sup>	100 μM/mg N/h	2-5
<i>Xanthomonas arsenoxydans</i>					31 μM/mg dry wt./h	
<i>Achromobacter arsenoxydans</i>						
Soil consortium	Soils	NR	2.5-40	NR	NR	6
<i>Alcaligenes faecalis</i>	Raw sewage	NR	10-20	450 <sup>†</sup>	0.05 μmol/mg protein/min	7
				2 <sup>†</sup>		
<i>Alcaligenes</i> strain	Soil	NR	0.24-2.4	1540 <sup>†</sup>	6.7 μl O <sub>2</sub> /min (ca. 10 <sup>10</sup> cells)	8
<i>Pseudomonas arsenitoxidans</i>	Mine water	NR	13.3-26.7	NR	NR	9
Seawater consortium	Coastal seawater	<70 nM	0.0013	70-144 <sup>†</sup>	3.6-9.2 μM/hr	10
AsI-As6	Mine water	27-173 μM	0.67-6.7	NR	NR	11
<i>Sulfolobus acidocaldarius</i>	—*	NA	1	NR	NR	12
<i>Alcaligenes faecalis</i>	—*	NA	0.20	NR	0.023 μmole/mg protein/min	13
purified enzyme						
ULPAsI	Contaminated water	0.47 mmol/kg dry wt.	1.33	NR	NR	14
NT-25, NT-26	Gold mine arsenopyrite rock	NR	5-10	NR	NR	15
<i>Agrobacterium albertimagni</i> strain AOL15	Macrophyte surfaces	2.7 μM	0.002-5	3.4 ± 2.2 <sup>†</sup>	(1.81 ± 0.58) × 10 <sup>-12</sup> μmol/cell/min	**
					0.043 ± 0.017 μmol/mg protein/min	

\*Previously isolated bacterium, <sup>†</sup>whole cells, <sup>‡</sup>cell extracts, \*\*this study, NR = not reported, NA = not applicable.

(1) Green 1918; (2) Turner 1949; (3) Turner 1954; (4) Turner and Legge 1954; (5) Legge 1954; (6) Quastel and Scholenfield 1953; (7) Phillips and Taylor 1976; (8) Osborne and Erlich 1976; (9) Ilyaletdinov and Abdrashitova 1982; (10) Scudlark and Johnson 1982; (11) Wakao et al. 1988; (12) Sehlin and Lindstrom 1992; (13) Anderson 1992; (14) Weeger et al. 1999; (15) Santini 2000.

growth, deriving energy from the oxidation of arsenite. In other cases, arsenite oxidation is generally presumed to be a detoxification mechanism without experimental observations of any toxic effects. This explanation is commonly offered because differential levels of toxicity are associated with arsenite and arsenate. The toxicity of arsenite results from its affinity for sulfhydryl groups; various enzymes can be inactivated when arsenite binds to the cysteine residues of these proteins (Ferguson and Gavis 1972; Summers and Silver 1978). Arsenate, an analogue for inorganic phosphate, exerts a lower level of toxicity by substituting for phosphate in membrane transport systems and in ATP (Summers and Silver 1978).

Here, we examine the kinetics and physiological role of arsenite oxidation by the strain *Agrobacterium albertimagni*, strain AOL15. The kinetic studies demonstrate that, at the ambient concentrations of arsenic at Hot Creek, AOL15 oxidizes arsenite at about half of its maximum rate. In trying to assess the physiological role of AOL15 and possible explanations for its arsenite oxidizing capability, observations from laboratory and field incubation studies are compared but with the caveat that this organism is not a dominant member of the microbial community attached to the Hot Creek plant surfaces (based on molecular level analyses of the biofilm).

## Materials and Methods

### Isolation Procedure

In August 1997, fresh samples of *Potamogeton pectinatus* were collected in sterile polypropylene bottles, transported to the laboratory on ice, and stored at 4°C for 1 day. Clippings of the plant samples were then placed into a sterile, 15-ml polypropylene centrifuge tube with 5 ml of deionized water. The centrifuge tube was vigorously mixed with a vortex mixer and 1 ml of the resulting suspension was inoculated into a liquid citrate medium (which was selected to provide a chemically well-defined, simple growth medium). The medium was amended with 30  $\mu\text{M}$  arsenite and within 1 week, the resulting turbid medium showed a complete oxidation of the arsenite to arsenate. At this point, the culture was transferred to fresh arsenite-amended citrate medium and, after several transfers, an enrichment culture was established. To increase selectivity for arsenite-resistant microorganisms, arsenic concentrations in the enrichment cultures were gradually increased from 30  $\mu\text{M}$  to 500  $\mu\text{M}$  during the isolation process. A serial dilution of the liquid culture was plated and all resulting colonies were transferred to liquid media to screen for arsenite oxidation using the  $\text{KMnO}_4$  method (described next).

### Media

The medium used for the establishment of the enrichment culture and eventual isolation of arsenite-oxidizing microorganisms was a minimal medium with citrate as the carbon source, herein referred to as citrate medium (composition [g/L]:  $\text{KH}_2\text{PO}_4$  (0.75),  $\text{K}_2\text{HPO}_4$  (0.61),  $\text{NH}_4\text{Cl}$  (0.67),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2),  $\text{CaCl}_2$  (0.023),  $\text{FeCl}_3$  (0.0024),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.003),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.001), sodium citrate (3.64) with 30  $\mu\text{M}$  arsenite for isolation, and a final concentration of 500  $\mu\text{M}$  arsenite for the pure culture). After isolation, the citrate medium was modified to improve growth by the addition of 1 g/L tryptone to the defined medium. A mannitol-based medium [modified from Kerr (1992)] was also used for growth studies, herein referred to as mannitol medium (composition [g/L]:  $\text{K}_2\text{HPO}_4$  (0.5),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.4),  $\text{NaCl}$  (0.2), yeast extract (0.3), L-glutamate (2.0), and mannitol (10.0) with variable final concentrations of arsenite ranging from 0 to 5 mM). For variable pH growth studies, the phosphate-buffered medium consisted of 10%

Luria-Bertani (Difco) with pH ranging from 4 to 13 (desired pH obtained by addition of citric acid, hydrochloric acid, tris, glycine, or sodium hydroxide).

### ***DNA Extraction, 16S rDNA Sequencing and DNA-DNA Hybridizations***

For the extraction of genomic DNA, a 100-ml cell suspension was centrifuged at 10,000 × g for 15 min and resuspended in 4 ml dilute Tris-EDTA buffer (0.01 M Tris, 0.005 M EDTA). The sample was treated sequentially with (a) lysozyme (1 mg/ml for 30 min at 37°C) followed by addition of 1 ml of concentrated Tris-EDTA buffer (0.21 M Tris, 0.08 M EDTA), (b) SDS, sodium dodecyl sulfate (1% solution for 60 min at 60°C), and (c) proteinase K and RNAase (0.5 mg/ml and 0.01 mg/ml, respectively, for 45 min at 37°C). The DNA was separated from the bulk solution using a series of phenol and chloroform extractions. The DNA was then precipitated using a combined ethanol and 0.3 M sodium acetate solution, vacuum dried, redissolved in Tris-EDTA buffer (20 mM Tris and 5 mM EDTA), and stored at -20°C [method modified from Ausubel et al. (1992) and Gerhardt et al. (1994)]. Purified genomic DNA from liquid-grown cultures were quantified and ~10 ng of DNA was used as the template for PCR amplification. Universal primers [S-D-Bact-0011-a-S-17 (5' to 3': GTTTGATCCTGGCTCAG) and S-D-Bact-1492-b-A-16 (5' to 3': TACCTTGT-TACGACTT)] (Kane et al. 1993; Wheeler et al. 1996) were used to amplify the 1.4-kb PCR fragment per protocols established by Ruimy et al. (1994). Directly following purification on Qiagen columns (Qiagen, Valencia, California), amplicons thus generated were sequenced. The identity of a given PCR product was verified by sequencing at the DNA Sequencing Core Facility at the Beckman Institute of the California Institute of Technology using the dideoxy chain termination method with Sequenase DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and with an ABI 373A automatic sequencer as recommended by the manufacturer (Perkin-Elmer Corp., Foster City, California). The phylogenetic relationships of organisms examined in this study were determined by comparison of individual 16S rDNA sequences to existing sequences in the public database (GenBank). The resulting 1,412 base-pair sequence was analyzed using MacVector™, GENETYX-MAC 8.0, AssemblyLIGN™, Genetic Data Environment (GDE) version 2.0, Phylip version 3.6 (J. Felsenstein and the University of Washington, Seattle [public domain]). DNA-DNA hybridizations were carried out with biotinylated DNA by fluorometric hybridizations in microdilution wells as described previously (Ezaki et al. 1989; Satomi et al. 1997, 1998).

### ***Nucleotide Accession Numbers***

The nucleotide sequence for strain AOL15 reported here has been deposited with GenBank nucleotide sequence databases under accession number AF316615.

### ***Transmission Electron Microscopy***

Standard methods for transmission electron microscopy (TEM) were used for AOL15 samples. Cells grown in mannitol medium were pelleted and fixed in a 2.5% glutaraldehyde solution and stained with saturated uranyl acetate for TEM based on the procedures outlined by Robinson et al. (1987). Samples were observed on a Philips EM 201 transmission electron microscope at 80 kV.

### ***Growth and Toxicity Studies***

In determining growth rates and doubling times, mannitol medium was used exclusively. Cell numbers were determined microscopically using the fluorochrome DAPI to stain cells.

Stained cells were retained on 0.2- $\mu\text{m}$  pore filters before viewing and counting (Kepner and Pratt 1994). In determining the ability of AOL15 to grow in variable pH or NaCl media, positive results were reported for solutions with minimum optical density of 0.25 at 600 nm. Plate counts were used to determine cell numbers in experiments monitoring growth and oxidation concurrently. For the variable salt content growth studies (%NaCl), the medium consisted of neutral pH (pH 7) solution of 1% tryptone with salt content (%NaCl) ranging from 1 to 10%. To determine if AOL15 was capable of chemolithoautotrophic growth by arsenite oxidation, a medium was prepared with bicarbonate as the carbon source and arsenite at 1 or 5 mM as the energy source (Santini et al. 2000), herein called bicarbonate medium.

The ability of AOL15 to grow anaerobically in mannitol medium with nitrate (5 mM), ferric iron (20 mM), sulfate (5 mM), sulfite (5 mM), thiosulfate (5 mM), arsenate (5 mM), fumarate (20 mM), malate (10 mM), and DMSO (10 mM) as terminal electron acceptors was tested at 30°C.

For the carbon-source utilization characterization of AOL15, API 20 NE strip tests (BioMerieux S. A., Marcy l'Etoile, France) and Biolog GN microplates (Biolog Inc., Hayward, California) were used. Results for AOL15 were compared with those for *Agrobacterium tumefaciens* (ATCC #23308) and *Blastobacter aggregatus* (ATCC #43293), two species that are phylogenetically related to AOL15.

To determine the toxic effects of arsenite and arsenate, LB plates with variable concentrations of either arsenite or arsenate ranging from 10  $\mu\text{M}$  to 50 mM were prepared. Cultures of AOL15, *A. tumefaciens*, and *B. aggregatus* were plated and incubated at 30°C and colony formation was monitored.

## Analyses

In kinetics experiments, arsenite oxidation was monitored by measuring As(III) concentrations over time. Arsenic (III) and As(V) in filtered samples were separated using anion exchange columns filled with AG 1-X8 resin, dry mesh size 100–200 in the acetate form (Bio-Rad, Hercules, CA) as previously described and validated (Wilkie 1997; Wilkie and Hering 1998). Total arsenic concentrations were measured in the initial filtered samples and arsenite concentrations were measured in samples processed through the anion exchange columns. Arsenic concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) on a Perkin-Elmer ELAN 4000.

A qualitative  $\text{KMnO}_4$  screening technique was used to monitor the consumption of arsenite and production of arsenate in culture growth media. Thirty milliliters of 0.01 M  $\text{KMnO}_4$  were added to 1 ml of the culture medium containing 500  $\mu\text{M}$  total arsenic. A pink color for the resulting mixture indicated that arsenate (which does not react with permanganate) was present and thus was a positive result for arsenite oxidation. A clear or orange colored solution indicated that arsenite (which reacts with permanganate) was present and thus was a negative result for oxidation.

Additionally, AOL15, *A. tumefaciens*, and *B. aggregatus* were sent for fatty acid methyl ester (FAME) analysis (Microbial ID, Inc., Newark, Delaware). Fatty acids were separated by high-resolution gas chromatography and the resulting fatty acid profiles were analyzed as previously described (Ringelberg et al. 1994).

## Kinetics of Arsenite Oxidation

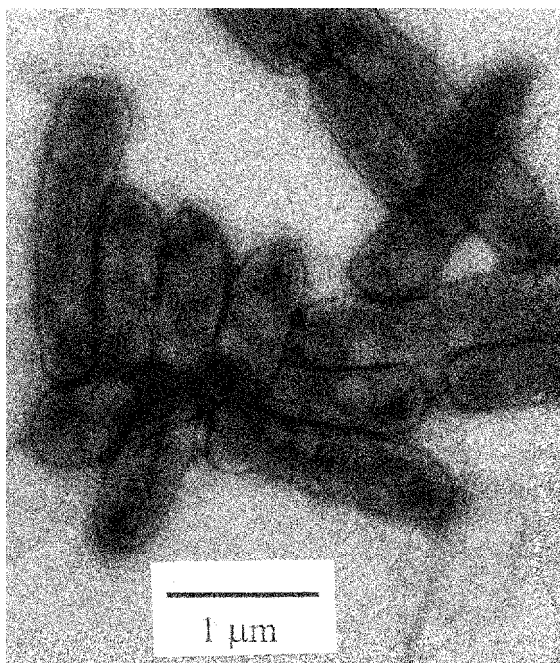
To determine the kinetic constants for whole cell suspensions ( $K_s$  and  $V_{\text{max}}$ ), cultures of AOL15 were used. In a standardized procedure, frozen stocks of AOL15 were used to inoculate into mannitol medium without arsenite at a dilution of 1:50 and allowed to grow for

24 h. After 24 h, the fully-grown culture was transferred into mannitol medium with 500  $\mu\text{M}$  arsenite at a dilution of 1:1,000. After 24 h of growth in the presence of arsenite, the cultures reached late exponential/early stationary phase and were harvested by centrifugation at  $12,000 \times g$  for 20 min at  $4^\circ\text{C}$  with two wash steps using 10 mM HEPES buffer at pH 7. The washed cells were resuspended in HEPES buffer and allowed to equilibrate to  $30^\circ\text{C}$ ; kinetic experiments were initiated by the addition of arsenite. Samples were collected, sterilized by filtration ( $0.2 \mu\text{m}$  filter), and analyzed for arsenite and total arsenic by the anion exchange method and ICP-MS described before. Kinetic parameters were determined from a plot of initial rate vs. initial substrate concentrations using Scientist (Micromath Scientific Software). The variability associated with cell counts resulted in a 32% error in the  $V_{\text{max}}$  value and a calculated error of 65% in  $K_s$ . For data sets with low initial substrate concentrations, initial rates were calculated from  $K_s$  and  $V_{\text{max}}$  values fit to the individual data sets. To compare  $V_{\text{max}}$  values with literature values, protein concentration determinations were made using the Bio-Rad DC protein assay (Hercules, California).

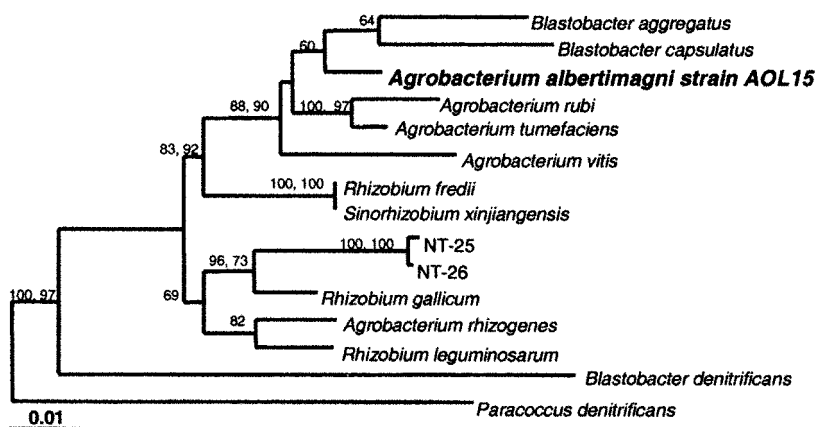
## Results and Discussion

### *Isolation and Identification of AOL15*

The isolation procedure described here yielded two strains capable of arsenite oxidation. *Agrobacterium albertimagni* strain AOL15 (Figure 1) was selected for further study after 16S rDNA analysis revealed that these strains were 100% identical. A polar flagellum was observed by microscopy and AOL15 was found to be motile in mannitol medium but immotile in citrate medium (a similar observation was also made for Green's arsenite oxidizer) (Green 1918).



**FIGURE 1** Negatively stained transmission electron micrograph of AOL15 grown in mannitol medium without arsenite (stained with uranyl acetate).



**FIGURE 2** Phylogenetic tree of AOL15. Numbers at branch points are bootstrap values for a distance- (first value) and parsimony- (second value) based analysis. Single bootstrap values are for distance-based analysis only. Accession numbers: *Blastobacter aggregatus* (X73041), *Blastobacter capsulatus* (X73042), *Agrobacterium albertimagni* strain AOL15 (AF316615), *Agrobacterium rubi* (D12787), *Agrobacterium tumefaciens* (D12784), *Agrobacterium vitis* (D01258 and D14502), *Rhizobium fredii* (D12792), *Sinorhizobium xinjiangensis* (D12796), NT-25 (AF159452), NT-26 (AF159453), *Rhizobium gallicum* (U86343), *Agrobacterium rhizogenes* (X67224), *Rhizobium leguminosarum* (U29386), *Blastobacter denitrificans* (S46917), *Paracoccus denitrificans* (X69159).

Phylogenetic analysis by 16S rDNA (mask: comparing 1,329 of the 1,412 aligned base pairs) showed that AOL15 was most closely related to *B. aggregatus* and *A. tumefaciens* (Figure 2) with 97.0 % and 97.7 % sequence identity, respectively. Of the previously described arsenite oxidizers, the chemolithoautotrophic strains NT-25 and NT-26 are the closest to AOL15 (94.2% similarity) and are included in the phylogenetic tree. DNA-DNA hybridizations confirmed that AOL15 and *B. aggregatus* were more closely related (30% similarity) than AOL15 and *A. tumefaciens* (10–15% similarity) but that AOL15 was a new species (less than 70% identical) (Wayne et al. 1987). However, because the genus *Blastobacter* may be reclassified in the near future (as an *Agrobacterium*) (Hugenholtz et al. 1994), we suggest that strain AOL15 be grouped with the genus *Agrobacterium*.

The phenotypic profiles showed that AOL15 was metabolically similar to both *Agrobacterium tumefaciens* and *Blastobacter aggregatus*. All three strains (AOL15, *A. tumefaciens*, and *B. aggregatus*) were positive for esculin hydrolysis,  $\beta$ -galactosidase activity, and oxidase activity and were negative for indole formation, glucose fermentation, arginine dihydrolase activity, and gelatinase activity. All three strains utilized the same 41 carbon sources of the 95 tested. In the FAME analysis, the relatedness of the three species (*A. tumefaciens*, *B. aggregatus*, AOL15) was determined by applying a Euclidean distance method to analyze the fatty acid profiles (Microbial ID, Inc., Newark, Delaware). This analysis revealed that, of these three strains, AOL15 and *B. aggregatus* were more closely related than AOL15 and *A. tumefaciens* or *B. aggregatus* and *A. tumefaciens*.

### Growth and Toxicity Studies

AOL15 was grown at three different temperatures (25, 30, and 37°C) with varied concentrations of arsenite. An attempt was also made to grow the culture without arsenic at 4

**TABLE 2** Doubling time for AOL15

Temp. (°C)	[As(III)] ( $\mu\text{M}$ )	Doubling time (h)
25	0	2.0
25	5	1.9
25	500	2.0
30	0	1.3
30	50	1.3
30	500	1.6
37	0	1.5

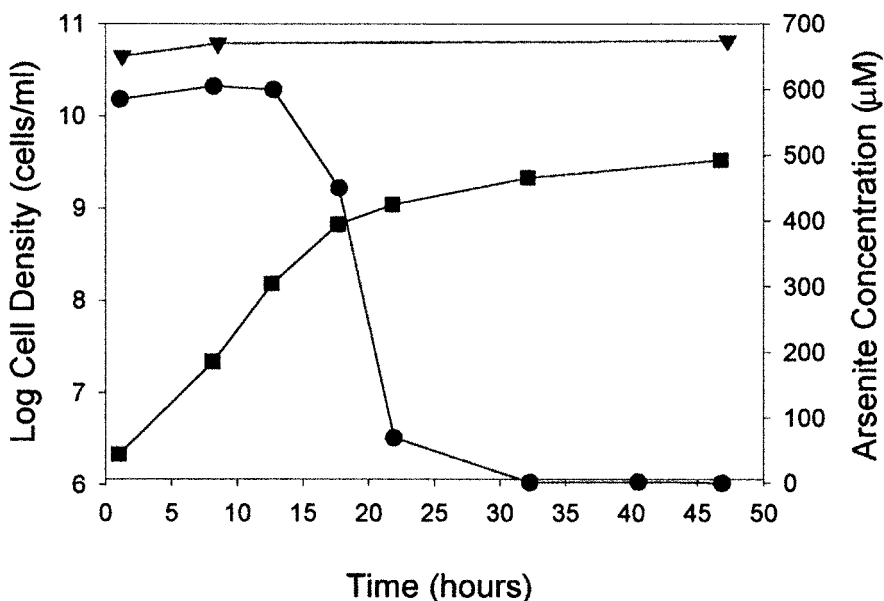
and 42°C. The optimal growth temperature was 30°C (which is comparable to field temperatures ranging from 23 to 28°C) with a doubling time of 1.3 h in mannitol medium when grown without arsenic (Table 2). Only slight differences in the growth rates were observed with variations in arsenite concentration up to 500  $\mu\text{M}$ . However, cultures grown at 1 mM and 2.5 mM arsenite mannitol medium exhibited significant lag periods before entering exponential growth phase (data not shown). This lag suggests that higher concentrations of arsenite are toxic to AOL15. Consistent with this hypothesis, it was observed that AOL15 could grow on solid media in the presence of 50 mM arsenate (the highest level tested) but growth was not observed in the presence of 5 mM arsenite. Although the strains *A. tumefaciens* and *B. aggregatus* (which are closely related to AOL15) were tolerant to elevated concentrations of arsenite, neither strain was capable of oxidizing arsenite (as determined by the permanganate assay). *A. tumefaciens* could grow in the presence of 500  $\mu\text{M}$  arsenite but not at 5 mM. *B. aggregatus* could grow at 400  $\mu\text{M}$  arsenite but not at 500  $\mu\text{M}$ . In the literature, the highest levels of tolerance reported by Green (100 mM arsenite) and Turner (60 mM arsenite) are significantly higher than the levels observed here (Green 1918; Turner 1949).

Arsenite did not support chemolithoautotrophic growth of AOL15 in bicarbonate medium. Growth was observed, however, when the bicarbonate medium was amended with 500  $\mu\text{M}$  mannitol. In addition, growth experiments with gradual increases of arsenite did not show increases in cell numbers compared with parallel batches grown without arsenite. Thus, arsenite oxidation does not appear to be linked to chemolithoautotrophic growth, rather, given the toxic affects of arsenite, it appears to be a detoxification mechanism for AOL15.

AOL15 was not able to grow using any of the tested terminal electron acceptors other than molecular oxygen nor did it exhibit fermentative growth. This is consistent with the reports on the *Agrobacterium* genus, although some strains have been reported that are capable of anaerobic respiration in the presence of nitrate (Kersters and De Ley 1984). Optimal growth of AOL15 was observed at neutral pH (pH 7 and 8), comparable to field conditions (pH 8.3), and at low salt conditions (less than 2% NaCl although no concentrations below 1% were tested).

### **Arsenite Oxidation**

The growth of AOL15 and the redox speciation of arsenic were monitored simultaneously. As shown in Figure 3, AOL15 grew exponentially for 15 h and then reached stationary phase. The decrease in the measured arsenite concentration shows that oxidation occurred during the late exponential phase, once the culture had reached a high cell density. Oxidation of 585  $\mu\text{M}$  arsenite (i.e., 290  $\mu\text{mole}$ ) was complete within 24 h. Arsenite oxidation in short term assays (<5 h) was not observed in suspensions of washed cells from cultures grown

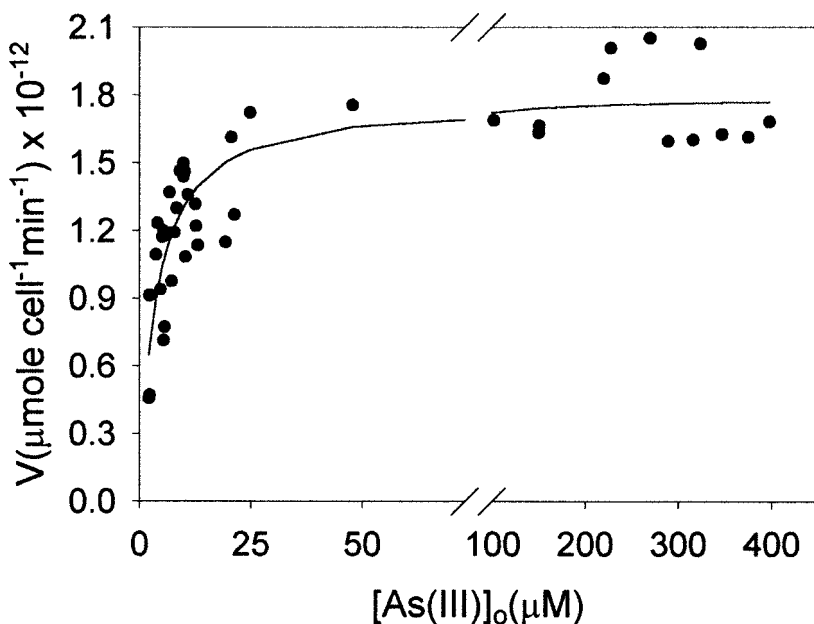


**FIGURE 3** Growth of AOL15 in the presence of approximately 500  $\mu\text{M}$  (585  $\mu\text{M}$ ) arsenite in mannitol medium at 30°C (representative data set). Symbols: (■) cell growth measured by plate counts, (▼) arsenite in control experiments of mannitol solution without bacterial cells, (●) arsenite in mannitol solution with growing cells. Lines are included only as a guide.

in the absence of arsenite (data not shown) but cells that had previously been grown in the presence of arsenite were able to oxidize arsenite immediately. This capacity to oxidize arsenite was not lost even after 10 consecutive transfer and growth cycles in arsenite-free medium. This implies that arsenite oxidation is an inducible activity that is likely to be encoded on the chromosome.

The kinetic parameters for arsenite oxidation by AOL15 were determined using washed and resuspended whole cells harvested at late exponential/early stationary phase. The values of the kinetic parameters for arsenite oxidation are  $K_s = 3.4 \pm 2.2 \mu\text{M}$  and  $V_{\max} = 1.81 \pm 0.58 \times 10^{-12} \mu\text{mole} \cdot \text{cell}^{-1} \cdot \text{min}^{-1}$  ( $0.043 \pm 0.017 \mu\text{mole} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$ ) (Figure 4). Further kinetic studies with cells harvested in the exponential growth phase and in stationary phase showed comparable rates of arsenite oxidation (data not shown). Thus, the apparent localization of arsenite oxidation to the late exponential/early stationary phase (Figure 3) is attributable to the fact that the number of cells present in the early part of the growth experiment was not sufficient to oxidize arsenite at a detectable rate.

The  $K_s$  value for AOL15 is substantially lower than previously reported values (ranging from 70  $\mu\text{M}$  to 1540  $\mu\text{M}$ ) for arsenite oxidation by whole cell suspensions (Table 1). The arsenic concentration in Hot Creek averages 2.7  $\mu\text{M}$ , thus the  $K_s$  value observed here is close to the conditions experienced by AOL15 in its natural setting. The protein-normalized  $V_{\max}$  reported here ( $0.043 \pm 0.017 \mu\text{mole} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ ) compares very well with the  $V_{\max}$  reported for whole cell studies of *Alcaligenes faecalis* ( $0.05 \mu\text{mole} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ ) (Phillips and Taylor 1976) and for the cell-free extracts of *A. faecalis* ( $0.023 \mu\text{mole} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ ) (Anderson et al. 1992). The arsenite oxidase from *Alcaligenes faecalis* is the only enzyme for arsenite oxidation that has been purified and characterized. It has been described as an oxomolybdenum enzyme and its activity has been localized to the periplasmic space of the bacterial cell (Anderson et al. 1992; McNellis and Anderson 1998). It has also been



**FIGURE 4** Oxidation rate as a function of initial arsenite concentration  $[As(III)]_o$ . Data points (●) fit with theoretical line with  $K_s = 3.4 \pm 2.2 \mu\text{M}$  and  $V_{\max} = 1.81 \pm 0.58 \times 10^{-12} \mu\text{mole} \cdot \text{cell}^{-1} \cdot \text{min}^{-1}$ .

suggested that arsenite oxidation is a resistance mechanism that occurs by a periplasmic electron transfer chain with arsenite oxidase as the first protein in the transfer sequence (Anderson et al. 1992; McNellis and Anderson 1998).

#### *Implications of Arsenite Oxidation at Hot Creek*

For AOL15, arsenite is clearly more toxic than arsenate and the enzymatic transformation of arsenite to the less toxic form may be a mechanism for resistance and detoxification. This is in contrast with the arsenic resistance (in Gram-negative and Gram-positive bacteria) associated with the *reduction* of arsenate to arsenite. In these cases, arsenite is exported from the cell into a low-arsenite external medium along a favorable chemical gradient (Rosen 1996; Silver 1996). In Hot Creek, however, the elevated concentrations of arsenite in the external cell environment could result in passive diffusion of arsenite (as the uncharged species  $H_3AsO_3$ ) into the cell. In this case, arsenite oxidation could minimize arsenic uptake into the cell.

The potential contribution of a microorganism like AOL15 to the rapid in situ oxidation observed at Hot Creek can be illustrated by comparing the kinetics of oxidation in the field and in laboratory studies with AOL15. In field incubation studies, pseudo first-order oxidation of arsenite was observed both at the ambient arsenite concentration and in samples spiked with  $2 \mu\text{M}$  arsenite with an average pseudo first-order rate constant of  $0.050 \text{ min}^{-1}$ . For Michaelis-Menten kinetics, pseudo first-order kinetics are expected for the initial substrate concentration  $[S]_o < K_s$ , then

$$V = \frac{V_{\max}[S]}{K_s} \quad (1)$$

and the pseudo first-order rate constant corresponds to  $V_{\max}/K_s$ . In the field study, this assumption is not entirely valid since  $[\text{As(III)}]_0$  is less than the  $K_s$  for AOL15 at the ambient concentration of ca.  $0.5 \mu\text{M}$  (Wilkie 1997) but is comparable to  $K_s$  in the spiked sample. For illustrative purposes, however, Equation 1 can be used to estimate the cell density required to achieve the pseudo first-order rate constant observed in the field incubations. With the values of  $V_{\max}$  and  $K_s$  for AOL15, that estimated cell density is  $10^8$  cells/mL. This value is not a direct representation of ambient conditions because it is an estimate of the number of cells shaken off the plant surface rather than of the number of planktonic cells present in the creek or in association with the biofilm on the surface of the plants. However, it is a reasonable estimate of the number of cells required to account for the observed oxidation and can be even more informative if the density of the biofilm is determined in the future. In addition, prior experiments by Wilkie and Hering (1998) demonstrated comparable oxidation rates with the intact biofilm on the plant surface and with the surface material shaken off the plants and the plant itself removed.

Microorganisms other than AOL15 may contribute wholly or in part to the arsenite oxidation observed in the field. Subsequent field experiments suggest that the  $K_s$  determined for AOL15 is representative of the biofilm community but that the  $V_{\max}$  for AOL15 is lower than that for the dominant arsenite oxidizers in the community (Salmassi 2001). It is likely that other oxidizers present on the surfaces of the macrophytes were not selected in these isolations because the arsenic concentrations in the enrichment media (30 and  $500 \mu\text{M}$ ) were 10 to 170 times the ambient concentrations. A community analysis using molecular techniques (genomic DNA extraction, amplification using universal bacterial primers, generation of clone library, and sequencing) did not show AOL15 to be a dominant member of the attached microbial community; other arsenite oxidizers can be isolated from this community under conditions closer to the natural environment (Salmassi 2001).

### **Description of *Agrobacterium Albertimagni* Strain AOL15 sp. nov**

*Agrobacterium albertimagni* is named after the Dominican scholar Albertus Magnus, who was the first person to describe arsenic.

AOL15 (Figure 1) is a Gram-negative rod,  $1.5 \mu\text{m}$  long by  $0.5 \mu\text{m}$  wide. When the microorganism is grown in mannitol medium, one polar flagellum is observed by transmission electron microscopy and the species is motile although it is immotile in citrate medium.

Strict aerobic; positive for esculin hydrolysis,  $\beta$ -galactosidase activity, and oxidase activity but negative for indole formation, glucose fermentation, arginine dihydrolase activity, and gelatinase activity. From the assimilation tests, able to utilize D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, maltose, L-malic acid, dextrin, D-arabitol, D-fructose, L-fucose, D-galactose, gentiobiose,  $\alpha$ -D-glucose, m-inositol,  $\alpha$ -D-lactose, lactulose, D-melibiose,  $\beta$ -methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, methyl pyruvate, mono-methyl succinate, D,L-lactic acid, L-aspartic acid, L-glutamic acid, L-ornithine, L-proline, L-pyroglutamic acid, urocanic acid, inosine, uridine, glycerol, glucose-1-phosphate, and glucose-6-phosphate.

Optimal growth occurs at  $30^\circ\text{C}$  on mannitol, at neutral pH (7 or 8) and at low % NaCl (1–4). The strain can oxidize arsenite but does not appear to grow chemolithoautotrophically.

Phylogenetically the species is 97.0% identical to *Blastobacter aggregatus* and 97.7% identical to *Agrobacterium tumefaciens*. Based on DNA-DNA hybridizations, AOL15 is 30 to 31% identical to *Blastobacter aggregatus* and 10 to 15% identical to *Agrobacterium tumefaciens*, which confirms that it is a new species.

Bacterium isolated from the surface of the aquatic macrophyte *Potamogeton pectinatus* in Hot Creek, California. The strain type is AOL15—deposited in the American Type Culture Collection (ATCC BAA-24).

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