

Dissimilatory Iodate Reduction by Hanford Microbial Isolates – 15226

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ABSTRACT

Microbial radioactive iodine (^{129}I) redox cycling in saturated soils is not well understood and provides a novel avenue for bioremediation studies. ^{129}I is a major by-product of nuclear fission, and is of environmental concern due to its long half-life (~16 million years), toxicity, and mobility in the environment. The 200 West area of the Hanford Site contains two separate plumes covering 1,500 acres where ^{129}I concentrations are ~3.5 pCi/L in Hanford soils. Speciation analysis shows that iodate comprises 70.6% of the iodine present, and organo-iodide and iodide comprise 25.8% and 3.6% respectively. Microbial redox activity within these iodine plumes affects iodine speciation, consequently impacting iodine mobility within the subsurface and the potential options available for bioremediation. An iodate reducing organism, designated as *Agrobacterium tumefaciens* strain AD35, was isolated from trap material incubated for 50 days in a high ^{129}I concentration plume. Iodate (200 μM) was reduced 47.8% in anaerobic cultures and by 36.3% in transitional cultures in the presence of 10mM nitrate, which was reduced 80.9% and 81.4% respectively. Iodate was also shown to be reduced by 69.2% and 84.0% in anaerobic and transitional growth conditions respectively with steady spiking of nitrate throughout. No iodate reduction was demonstrated without nitrate present. These data indicate there is a coupled reduction of nitrate and iodate by strain AD35. These data provide novel information on microbial iodate reduction in the groundwater at the 200 West Area. These organisms, along with other nitrate/iodate reducing organisms, which have been presently identified and are being characterized, can be employed in an engineered bioremediation approach for the removal of nitrate and iodate in the groundwater. Additionally, applications for stabilizing radioactive iodine in the subsurface are lacking. Results presented allow us to develop an understanding of iodine speciation throughout the Hanford Site, along with providing novel microbial systems for the bioremediation of iodine and also nitrate, which is not well understood to date.

INTRODUCTION

The Hanford Site in Washington (USA) is the most contaminated nuclear site in the United States, where 177 tanks were used to store high-level nuclear waste. The fission of plutonium generates a variety of radionuclides, and of particular environmental interest is radioactive iodine (^{129}I), which has discharged from former disposal cribs in the 200 West Area of the Hanford Site. This discharge is responsible for the majority of ^{129}I contamination found in the groundwater [1]. Models of radionuclide release to the biosphere from disposal cribs show ^{129}I to contribute a large fraction of radionuclides to the population dose [2, 3]. ^{129}I is of environmental concern due to its long half-life (1.6×10^7 years), toxicity, and mobility in the environment [2].

The 200 West Area of the Hanford Site, WA (USA), contains two separate plumes covering 1,500 acres where ^{129}I concentrations are ~3.5 pCi/L (Fig. 1). Speciation analysis shows that iodate comprises 70.6% of the iodine present, and organo-iodide and iodide comprise 25.8% and 3.6% respectively. Iodate, iodine in 5+ oxidation state, is a thermodynamically stable species of iodine. However iodide-iodine in the 1- oxidation state [2], has been shown to be the dominant iodine species in many marine surface waters [4, 5], contrariwise to the findings in the Hanford

Site groundwater [1]. Microbial redox activity within these iodine plumes may affect iodine speciation, consequently impacting iodine mobility within the subsurface and the potential options available for bioremediation strategies. To date, no information is known on the microbial redox interactions on iodine present within the Hanford subsurface.

Few studies have demonstrated the bacterial reduction of iodate. Amachi et al. (2007), demonstrated the dissimilatory iodate reduction by *Pseudomonas* sp. SCT, isolated from marine sediment slurry, in the presence of nitrate under anaerobic conditions. They demonstrated that cells pre-grown without iodate did not reduce it, nor could they reduce iodate aerobically [6]. In another study by Tsunogai and Sase (1969), they reported several marine laboratory strains of nitrate reducing bacteria to reduce iodate aerobically, concluding that iodate is reduced in a coupled mechanism by nitrate reductases [7]. Other studies have shown microbial reduction of iodate with anaerobic cell suspensions of *Desulfovibrio desulfuricans* [2] and marine bacterium *Shewanella putrefaciens* MR-4 [8].

The bacterium used in this study was *Agrobacterium tumefaciens* (currently designated as *Rhizobium radiobacter*), isolated from a trap placed within a groundwater monitoring well in high levels of ^{129}I for 50 days. The ability for *A. tumefaciens* to reduce iodate has not been described in the literature, and provides a novel avenue for understanding the mechanisms for iodine speciation at the 200 West Hanford Site.

MATERIALS AND METHODS

Enrichment of trap material and microbial isolation

Traps constructed from polyvinyl chloride pipe were filled with Ringold sand or control materials (i.e., glass beads) as substrate for microbial attachment, and were incubated for 50 days in a groundwater monitoring well within an iodine plume within the 200 UP-1 Operable unit of the 200 West Area (Fig. 1). The traps were at an interval depth of 217 ft and had an average ^{129}I concentration of 27.25 pCi/L. Substrate from the traps was enriched under aerobic conditions at 25°C (room temperature) in 50 ml 1/2R2A medium supplemented with 7.23 mM potassium iodate at pH 8.0, and shaken at 125 rpm. R2A medium contained (liter⁻¹): enzymatic digest of casein (0.25g), proteose peptone (0.25g), acid hydrolysate of casein (0.5g), yeast extract (0.5g), dextrose (0.5g), soluble starch (0.5g), K₂HPO₄ (0.3g), MgSO₄ · 7H₂O (0.05g), and C₃H₃NaO₃ (0.3g). Cycloheximide was added at a concentration of 90 µg/ml in an effort to inhibit fungal growth on the enrichment media. Enrichments were cultivated for 4 weeks and subsequently streaked for isolation 3 times to assure bacterial isolation.

Cultivation

All incubations were carried out at 25°C in the absence of light throughout this study. 1/2R2A with 200µM iodate was used to subculture AD35. The growth medium used for iodate reduction was a minimal medium and contained (liter⁻¹): KH₂PO₄ (0.14g), MgCl₂ · 6H₂O (0.20g), CaCl₂ · 2H₂O (0.15g), Na₂SO₄ · 10H₂O (0.14), NaHCO₃ (0.5g), ATCC vitamin supplement (1.0 ml), ATCC trace mineral supplement (1.0 ml), bacto-tryptone (1.0g), NaCl (1.5g), 10 mM NaNO₃, and 200 µM KIO₃. The pH was adjusted to 8.0 prior to autoclaving. To detect iodate reducing capabilities, isolates were grown aerobically overnight, harvested during log phase, washed twice with Phosphate Buffered Saline (PBS) and diluted to an OD₆₀₀ of 0.2 (corresponding to 0.14 mg protein ml⁻¹) and inoculated at 1%. Transition cultures were supplemented with 25 mM lactate as electron donor, and the culture headspace was flushed with O₂ free N₂ for 3 min after inoculation

to support transition growth conditions. Anaerobic cultures were supplemented with 10 mM lactate as electron donor and sparged with O₂ free N₂ for 10 min after inoculation to generate anoxic conditions. Under similar conditions, growth studies were conducted where nitrate was consistently spiked into the growth media to maintain 10 mM nitrate once it was depleted approximately 50% to determine if further iodate reduction ensued. Additional experiments were conducted under similar conditions, however with 50 mM lactate and 20 mM lactate for transition and 10 mM and 25 mM lactate anaerobic conditions respectively were investigated to determine if excess carbon would drive further reduction of iodate.

Growth characterization

Temperature and pH growth optima was conducted by inoculating 1% mid-log phase growth into ½ R2A aerobically over a temperature range of 4-37 °C and a pH range of 3-11. Growth was determined spectrophotometrically by determining the optical density at a wavelength of 600 nm (OD₆₀₀). Substrate spectrum was analyzed by transferring 1.0% mid-log phase growth into DIR medium containing lactate (10 mM), acetate (10 mM), glucose (1%), H₂ (10% H₂: 10% CO₂:90% N₂), succinate (10 mM), cellobiose (0.2%), cellulose (0.2%), xylan (0.75%), xylose (1%), and ethanol (10 mM). All substrate growth experiments contained 10 mM nitrate. The ability of AD35 to respire on a variety of electron acceptors was analyzed by transferring 1.0% mid-log phase growth into SAG medium. To determine AD35 ability to respire on certain compounds, 25 mM lactate was used throughout as the electron donor. Nitrate (10 μM), nitrate:iodate (10 mM:200 μM), iodate (200 μM), Iron(III), fumarate (10 mM), chromium (10 μM), and sulfate (10 μM) were all analyzed. All experiments were conducted anaerobically and media with no electron acceptor added was used as the negative control. Due to the varying absorbances in studies with different electron donors and acceptors, growth was determined using the bicinchoninic acid-copper (BCA) reduction method described below.

Protein determination

Protein concentration was determined using the BCA method as previously described [9]. Briefly, 1 mL of working reagent (bicinchoninic acid/copper sulfate mixed at a 50:1 ratio) was added to 100 μL of cell lysate or standards, and the absorbance was measured at 562 nm after incubating at 37°C for 30 minutes. Different concentrations of bovine serum albumin (BSA) were used as a protein standard: 0, 0.4, 0.8, 1.2, 1.6, and 2.0 mg/mL. One mL of reagent was added to 100 μL BSA standard and incubated at 37°C for 30 minutes, and the absorbance was measured at 562 nm to establish a standard curve [10].

Sequencing of 16S rRNA and phylogenetic analysis

16S rRNA was amplified by colony PCR using the bacterial consensus primers 8F and 1492R. Colony PCR was performed with an initial denaturation at 95°C for 5 min (to lyse cells and extract DNA) followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, followed by a final 10 min extension at 72°C. The PCR mixture, generated using *Taq* PCR Core Kit (www.qiagen.com), contained a small amount of bacterial colony material (template), 1 μL of each primer (25mM), 5 μL of 10x PCR buffer, 1 μL of BSA (15mg/mL), 1 μL of dNTP's (10mM each), 0.25 μL *Taq* DNA polymerase (5U/ μL), in a final reaction volume of 50 μL. PCR products were purified with Qiagen's QIAquick PCR Purification Kit according to the manufactures instructions and sequenced by a 3130XL DNA sequencer using primers 8F, 341F, 907R, and 1492R, and assembled accordingly using BioEdit. The obtained 16S rRNA sequence was subjected to BLAST search to determine 16S rRNA similarities with sequences deposited into GenBank. The retrieved sequences were aligned by using the ClustalW function within MEGA 6. A phylogenetic tree was constructed based on the

distance matrix data obtained with the Nearest-Neighbor-Interchange heuristic method. Robustness of the tree topology was evaluated by bootstrap resampling analysis with 1000 bootstraps and applying maximum-likelihood analysis using MEGA 6 [11].

Analytical Techniques

Iodate concentrations were determined colorimetrically according to the method by Amachi et al. (2007). Briefly, 40 μ l of 2% (wt/vol) sulfamic acid and 20 μ l of 2N HCl were added to 400 μ l of clarified supernatant, mixed by vortexing, and incubated for 5 min at room temperature. Subsequently, 400 μ l of 300mM potassium iodide was added to yield triiodide (I_3^-), followed by 400 μ l of 0.1% (wt/vol) soluble starch to yield a purple iodine-starch complex, which was immediately measured at 525 nm [6]. Nitrate concentrations were determined by measuring the OD₂₂₀ of clarified cellular supernatant as previously described [12, 13].

Nucleotide sequence accession numbers

The 16S rRNA gene sequence determined in this study has been deposited in the GenBank database under accession number KM272990.

RESULTS

Isolation and identification of an iodate reducing bacterium

Traps containing Ringold formation sediments were incubated in a monitoring well in ¹²⁹I-impacted groundwater for 50 days. Due to the relatively high ¹²⁹I concentrations (~20 pCi/L) in the groundwater, bacteria with the ability to transform iodine may be present. Since the sediments were autoclaved prior to placement in the monitoring wells, bacteria attached to the sediment likely originated from the groundwater. Sediment material from traps was used to set up enrichment cultures for isolation of bacteria with the ability to reduce iodate. From the enriched trap material, 23 morphologically distinct colony types were isolated on 1/2R2A with 90 μ g/ml cycloheximide and 7.23mM potassium iodate agar plates. Following sequencing, analysis showed that 6 of those 23 (26%) were phylogenetically and functionally identical related to iodate reducing potential (data not shown). Isolate AD35 was selected as representative of these 6 isolates and used for further testing. Analysis of pure culture DNA did not show multiple 16S rRNA gene sequences, indicating successful isolation of the identified strain. Isolate AD35 was phylogenetically associated with *Agrobacterium tumefaciens* isolate Y36 (KF730752.1) and *Rhizobium* sp. C2C sequence (KF170820.1) with 99% identity based on partial 16S rRNA sequences (Fig. 2). These organisms belong to the class *Alphaproteobacteria* and family *Rhizobiales*.

General characterization

Strain AD35 is a facultative anaerobe and a gram-negative bacilliform. On solid media, it formed smooth, opaque, glossy, and circular colonies, and grew well on lactate with nitrate-iodate as electron acceptors. Additionally, it was able to grow on acetate, glucose, hydrogen, succinate, cellobiose, cellulose, xylose, xylan, and ethanol as sole carbon sources. AD35 can reduce nitrate, nitrate and iodate, Fe(III), and fumarate, but was not able to reduce Cr(VI), or SO₄²⁻ (Fig. 3). It was able to grow at a pH range of 6-9 with pH 7 being optimal (Fig. 4A), and could thrive at a temperature range of 15-37 °C, and grew optimally at 25 °C (Fig. 4B and Table 1).

Batch iodate reduction

Batch studies with 200 μM iodate were conducted during transition from microaerobic to anaerobic growth or anaerobically under denitrifying conditions using washed cell suspensions as inoculum. In transition growth experiments iodate was reduced from 200 μM to 125 μM after 114 hrs along with nitrate being reduced from 10 mM to 1.91 mM (Fig. 5B). During growth under anaerobic conditions, Fig. 5C shows that iodate was reduced to 113 μM and nitrate was reduced to 1.86 mM. The ability for AD35 to reduce iodate more rapidly in transitional growth is perhaps associated to a more rapid growth rate observed as compared to the anaerobic growth rate (Fig. 5A). Iodate reduction productivity for strain AD35 was 0.141 mg/L h^{-1} and 0.163 mg/L h^{-1} for batch transition and anaerobic growth respectively. Minimal reduction of iodate was further observed after 63 hrs and 114 hrs of incubation in transition and anaerobic batch studies which appear to correlate when stationary phase was succeeded (Fig. 5). Additionally, testing varying lactate concentrations in the growth medium in similar batch studies, where 50 mM and 20 mM lactate was compared in transition and 25 mM and 10 mM lactate was compared in anaerobic growth conditions, did not yield further reduction of iodate or nitrate by AD35. No iodate reduction was demonstrated in the absence of nitrate in both culture conditions, nor was there growth present anaerobically.

Iodate reduction with nitrate spiking

A second set of iodate reduction experiments were performed in which AD35 was grown in similar conditions as above, however the cultures were spiked with additional nitrate multiple times throughout cultivation. Growth experiments demonstrated greater initial cell density in transition growth when compared to anaerobic growth (Fig. 6A). Additional nitrate was added to the growth media at 44 hrs and 72 hrs for the transition culture, and at 72 hrs and 114 hrs for the anaerobic culture. In transition growth, iodate was reduced from 200 μM to 60 μM at 160 hrs with an iodate reduction productivity of 0.187 mg/L h^{-1} . In anaerobic growth experiments with nitrate spiking, iodate was reduced down to 32 μM , at 184 hrs (Fig. 6) with a reduction productivity of 0.195 mg/L h^{-1} .

DISCUSSION

Here we described the first analysis of an *A. tumefaciens* species capable of reducing iodate in the presence of nitrate. Phylogenetic analysis revealed AD35 to be most closely related to *A. tumefaciens* (99% similar) based on partial 16S rRNA gene sequencing. General phenotypic characterization of AD35 was very similar to a study of *A. tumefaciens* by Kado et al (1972), where it formed smooth, opaque, glossy, circular colonies on solid media, and was a gram-negative rod morphology [14]. The optimum growth temperature was 25 °C with optimal growth pH being 7.5, similar to previous findings [15, 16]. The electron donor tests (Table 1), showed *A. tumefaciens* to grow on a variety of sole carbon sources such as lactate, acetate, glucose, hydrogen, succinate, cellobiose, cellulose, xylose, xylan, and ethanol as electron donors. These characteristics are typical compared to other studies on *A. tumefaciens* [16]. However, there are no reports mentioned on *A. tumefaciens*' ability to oxidize acetate, hydrogen, fumarate, cellulose, xylan, and ethanol in the refereed literature. AD35 was able to grow when nitrate, nitrate and iodate, and Fe(III) were supplied as electron acceptors, but not with Cr(VI), or SO_4^{2-} . The ability of *A. tumefaciens* to grow using iron as an electron acceptor has not been documented in the literature.

A. tumefaciens has also been described for its ability to reduce nitrate [14, 17-20], however no literature to date has described its ability to reduce iodate. There are few reports describing the bacterial reduction of iodate. Anaerobic reduction of iodate by *S. onedensis* MR-4 [8], and washed cells of *D. desulfuricans* and *S. putrefaciens* [2] have been previously described. Tsunogai and Sase (1969) initially described the importance of nitrate reducing bacteria in iodate reduction, and originally discovered multiple aerobic bacteria that could reduce iodate in the presence of nitrate. Here, a groundwater bacterium, *A. tumefaciens* strain AD35, was isolated and found to reduce iodate under transition and anaerobic conditions in the presence of nitrate.

The results from this study indicate that iodate reducing organisms are present in the groundwater at the 200 West Area in Hanford. Amachi et al. (2007) described an iodate-reductase in the periplasm of *Pseudomonas stutzeri* SCT, which was induced by the presence of iodate. However, our isolate only reduced iodate in the presence of nitrate, indicating that nitrate reductase may be responsible for the reduction of iodate. Minimal differences are seen between transition and anaerobic growth studies presented here, largely due to *A. tumefaciens* ability to perform balanced transitions from aerobic and anaerobic respiration [19]. It is hypothesized that *A. tumefaciens* has the ability to reduce iodate in a coupled-redox reaction where nitrate reductase appears to systematically reduce iodate. The reduction potential (*Eh*) for nitrate and iodate are very similar [21], suggesting a possible tendency for the coupled reduction of iodate-nitrate. Denitrification is an important function in the purification of contaminated groundwater, wastewater treatment, water purification, and in the biogeochemical cycling of nitrogen [19]. With an overlap of iodate and nitrate contamination in the groundwater at the 200 West Area at Hanford (Fig. 1), the ability to couple the reduction of both contaminants could be significant.

CONCLUSIONS

This study demonstrated the ability of an environmental isolate to reduce iodate in the presence of nitrate. Iodate (200 μ m) was reduced 47.8% in anaerobic cultures and by 36.3% in transitional cultures in the presence of 10mM nitrate, which was reduced 80.9% and 81.4% respectively. Iodate was also shown to be reduced by 69.2% and 84.0% in anaerobic and transitional growth conditions respectively with steady spiking of nitrate throughout. No iodate reduction was demonstrated without nitrate present. These data indicate there is a coupled reduction of nitrate and iodate by strain AD35. Additionally, these data provide novel information on microbial iodate reduction in the groundwater at the 200 West Area. This isolate was able to grow on a variety of electron donors and electron acceptors, and showed mesophilic as well as neutro- to alkaliphilic growth ranges. Strain AD35, along with other nitrate/iodate reducing organisms, which are presently being identified, could be employed in an engineered bioremediation approach for the removal of nitrate and iodate in the groundwater.

Table 1. General characterization of *Agrobacterium tumefaciens* AD35.

<i>Agrobacterium tumefaciens</i>	
AD35	
Morphology	Bacilli
Optimal growth temperature	25 °C
Temperature growth range	15-37 °C
Optimal growth pH	7.5
pH growth range	6.0-9.0
Aerotolerance	Facultative
Growth on:	
Electron donors	
Lactate	+
Acetate	+
Hydrogen	+
Succinate	+
Glucose	+
Cellobiose	+
Cellulose	+
Xylan	+
Xylose	+
Ethanol	+
Electron acceptors	
Oxygen	+
Nitrate	+
Nitrate/Iodate	+
Iodate	-
Fumarate	+
Chromium	-
Sulfate	-
Iron(III)	+

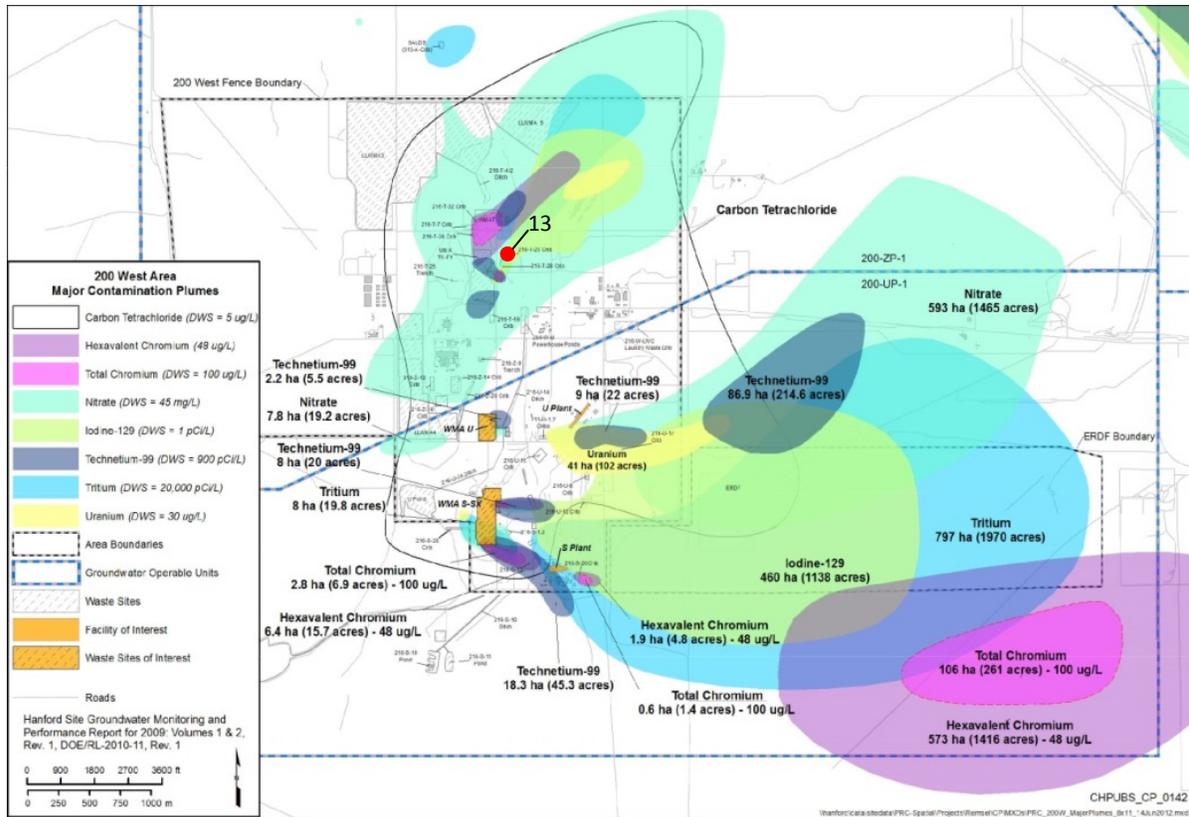


Figure 1. 200 West groundwater plume map showing the various contaminants present. The location of where AD35 was isolated is shown in red (●). Modified from (http://www.hanford.gov/files.cfm/CAL_Proposed_Plan_200-UP-1.pdf).

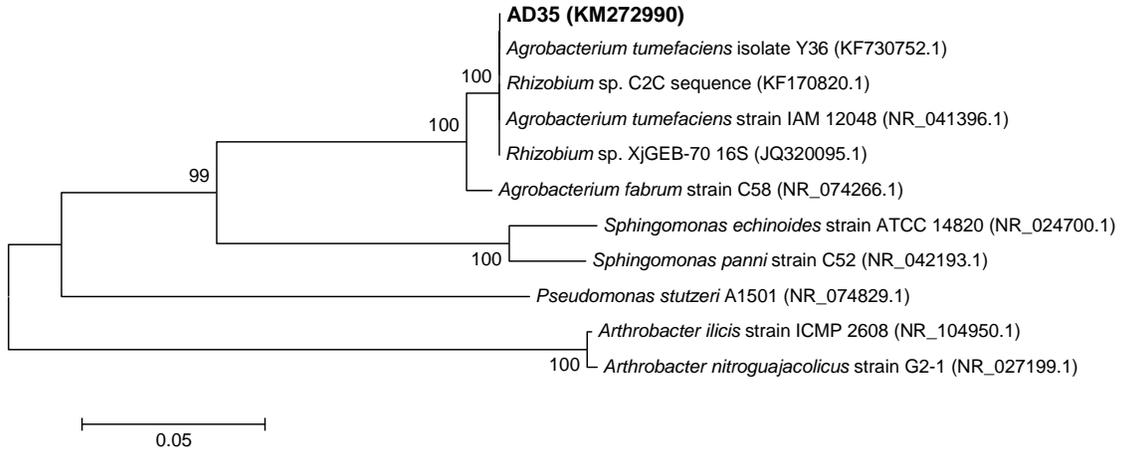


Figure 2. Phylogenetic tree illustrating the evolutionary position of isolates obtained from the 50 day deployment traps based on partial 16S rRNA sequencing. Scale bar corresponds to 5 substitutions per 100 nucleotides. Accession numbers are shown in parentheses.

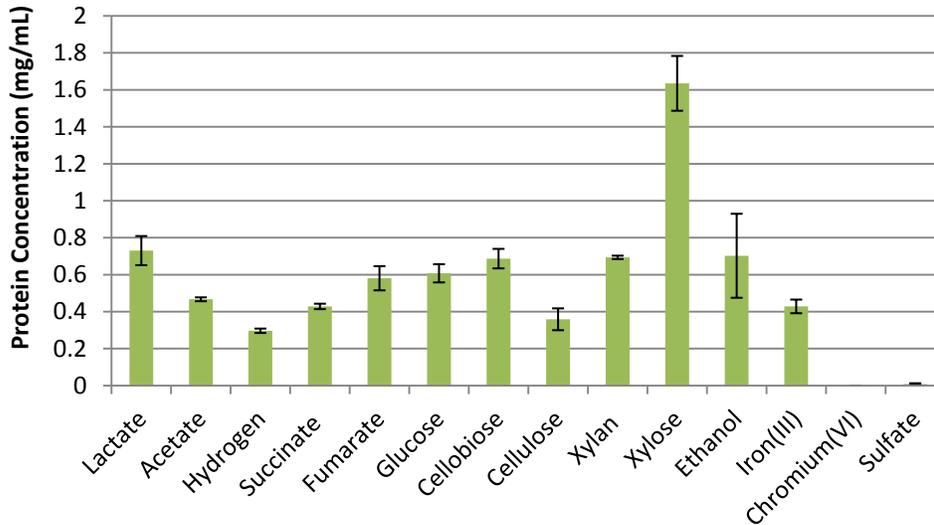


Figure 3. Total protein production by AD35 on a variety of electron donors and electron acceptors.

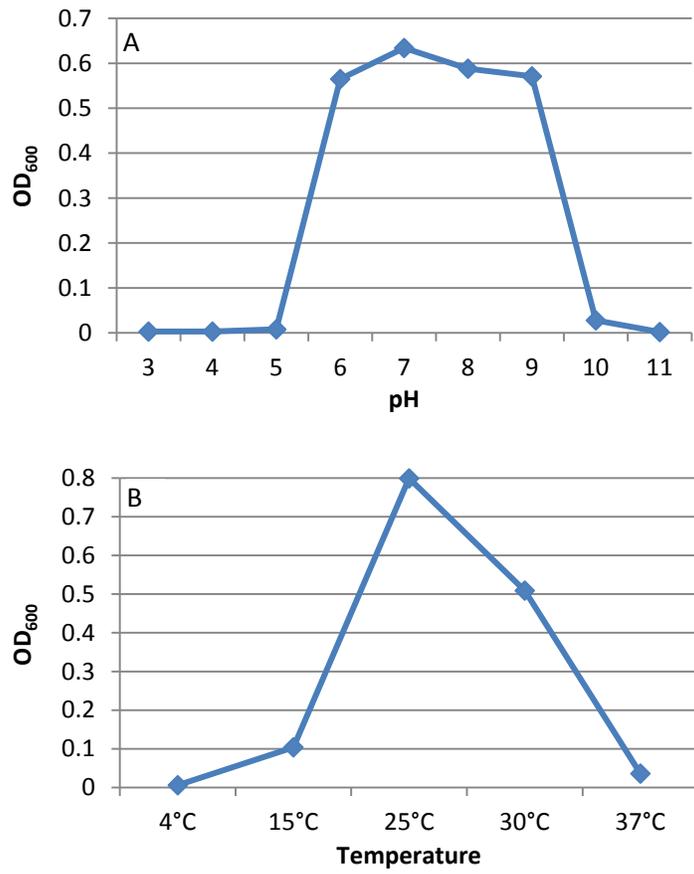


Figure 4. A) pH and B) temperature growth characterization of strain AD35.

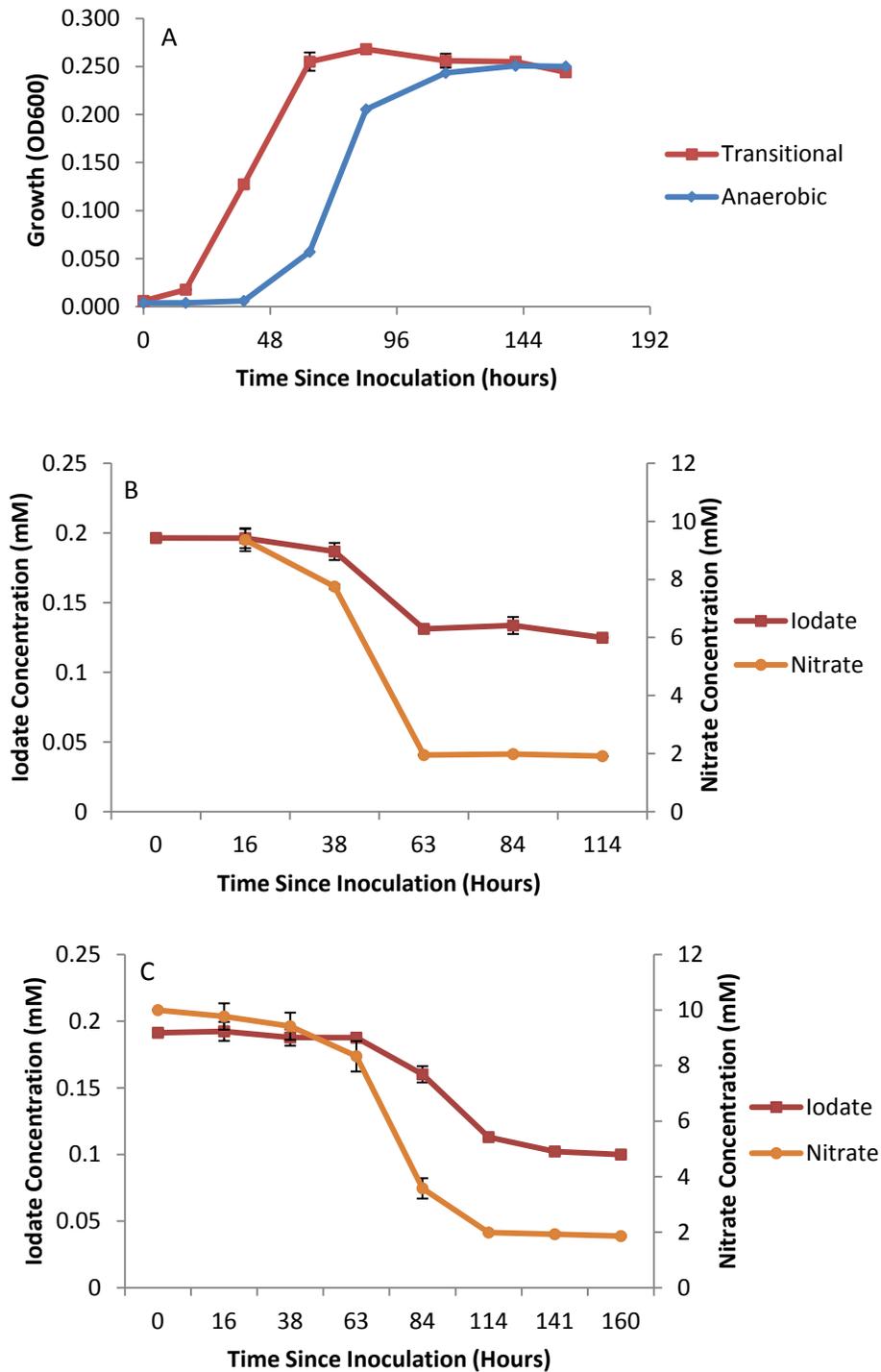


Figure 5. A) Anaerobic and transitional growth curves of AD35 over time. B) Iodate and nitrate reduction by AD35 over time in transitional growth. C) Iodate and nitrate reduction by isolate AD35 over time in strict anaerobic conditions.

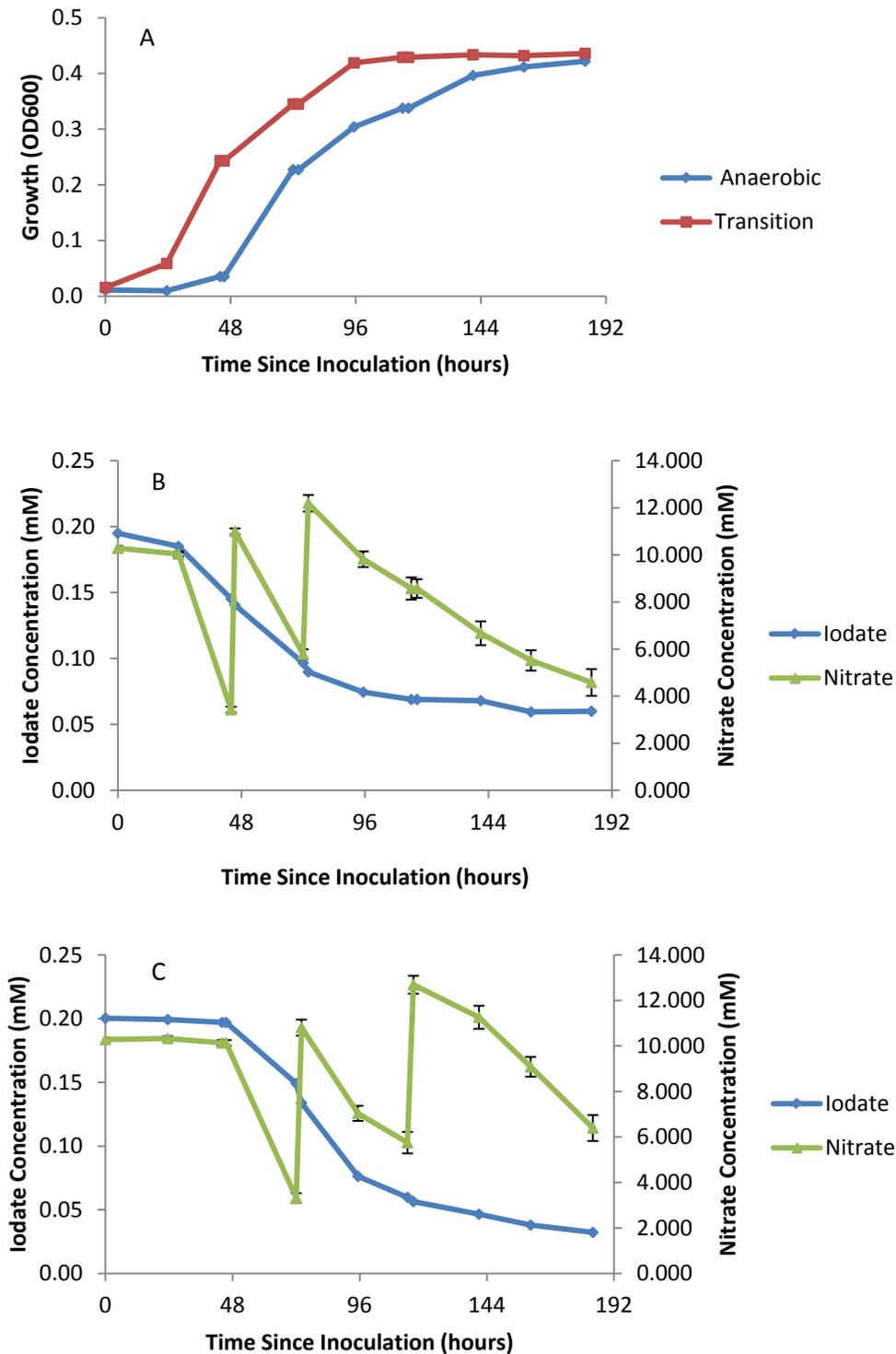


Figure 6. A) Anaerobic and transitional growth curves of AD35 in DIR media over time spiked with nitrate. B) Iodate and nitrate reduction by AD35 over time in transitional growth conditions spiked with nitrate. C) Iodate and nitrate reduction by isolate AD35 in anaerobic conditions spiked with nitrate.

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