Iodide Oxidizing Activity of Bacteria from Subsurface Sediments of the Savannah River Site, SC, U.S.A.

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Abstract—^{129}I is a major by-product of nuclear fission that is of concern because of its extremely long half-life (~16 million yrs), perceived toxicity through bioaccumulation, and the increasing inventory of this radionuclide worldwide. Relatively high concentrations of iodate (27.3%) and organo-iodine (23.9%) are present in a ^{129}I-conatminated aquifer at the Savannah River Site (SRS), SC, U.S.A. To determine if microorganisms could influence ^{129}I speciation in this system, iodide oxidizing activity was evaluated in bacteria isolated from SRS sediments. All strains isolated directly from sediment material ($n = 325$) and from sediment enrichment cultures containing 1 mM iodide ($n = 29$) tested negative for iodide oxidation on R2A agar plates containing iodide and starch. However, tubes from 2/24 enrichment cultures exhibited a distinct yellow coloration, indicative of iodide oxidation to I_2 and/ or volatile organic iodine species, after 22 weeks incubation. Analysis of iodine speciation in the enrichment culture supernatant from these two tubes revealed transformation of iodide to iodate and organo-iodide. Screening of 84 strains for iodide oxidizing activity using a combination of triiodide (I_3^-) formation, radiography and a recently developed, sensitive iodine speciation assay revealed that 44 of these strains were capable of iodide oxidation. These results indicate that iodide oxidation, albeit at very slow rates, can be supported by a variety of terrestrial bacteria.

Keywords: iodide oxidation, ^{129}I, radionuclide, Savannah River Site, organo-iodine

INTRODUCTION

^{129}I is a major by-product of nuclear fission that is of concern because of its mobility in the environment, excessive inventory, long half-life (~16 million

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yrs), and potential toxicity due to bioaccumulation through the food chain and bioconcentration in the thyroid gland. Currently, 146 Ci of ^{129}I is inventoried in soils at two US Department of Energy sites, Hanford Site and Savannah River Site (SRS) (Kaplan *et al.*, 2011; Otosaka *et al.*, 2011). Based on thermodynamic principles, the main iodine species in SRS F-area groundwater should be iodide (I^-), which is thought to have higher subsurface mobility than iodate (IO_3^-) or organo-iodine. Because relatively high concentrations of iodate (27.3%) and organo-iodine (23.9%) have been detected in the SRS subsurface, it is likely that chemical/biological factors, other than pH and Eh, are involved in regulating iodine speciation (Gallard *et al.*, 2009; Li *et al.*, 2011; Xu *et al.*, 2011).

In terrestrial groundwater and sediments where iodide is the thermodynamically favoured form of iodine, it is thought that organo-iodine formation proceeds *after* iodide is transformed into more reactive species, such as I_2, HIO, or I_3^- (Moulin *et al.*, 2001). Because iodide oxidation via a single-step electron transfer is thermodynamically unfavourable, this process requires a strong oxidant (e.g., MnO_2 or H_2O_2) or an enzymatic catalyst (Amachi, 2008; Gallard *et al.*, 2009). Numerous studies have implicated a role for microorganisms in organo-iodine formation in soils and sediments, and growing body of literature has implicated microbial oxidases, perhydrolases and peroxidases in the formation of halogenated organic matter (Ortiz-Bermúdez *et al.*, 2007; Amachi, 2008). Iodide oxidizing bacteria (IOB) have been isolated from environments naturally high in iodine, including marine sediments and natural gas brine waters/sediments (Amachi *et al.*, 2005), and from a marine fish aquarium where I_2 formation was implicated in a fish kill (Gozlan and Margalith, 1974). IOB were also isolated from seawater, however only after enrichment for several months in the presence of 1 mM iodide (Amachi *et al.*, 2005). Using similar approaches, efforts to isolate IOB from terrestrial sources have been unsuccessful.

In a previous study (Li *et al.*, 2011), we isolated bacteria from SRS sediments that were capable of accumulating 0.2 to 2.0% iodide at ambient concentrations (0.1 μM), but based on the relatively low biomass in the subsurface aquifer at F-area and the relatively small amount of iodide associated with the cells, it is unlikely that iodide-accumulating bacteria are responsible for the high fraction of organo-iodine detected at this site. Instead, we hypothesized that IOB may play a more significant role. The goal of this study was to evaluate if IOB are present in F-area subsurface sediments.

MATERIALS AND METHODS

Isolation of bacteria

Bacteria were isolated from F-area sediments as previously described (Li *et al.*, 2011). Sediments from 8 different regions within the contaminated subsurface of F-area were used to start enrichment cultures containing 1 mM KI and either 1) sterile dH$_2$O, 2) 1/10 strength DNB (dilute nutrient broth), or 3) 1/10 DNB with cylcohexamide (50 μg mL^{-1}). This resulted in 24 enrichment cultures. At 2, 9, and 22 weeks, a 50 μL sub-sample from each enrichment culture was used to isolate

bacteria as described in Li *et al*. (2011). Once isolated, all strains were grown on R2A-iodine-starch agar plates (1.2 g L^{-1} KI and 1g L^{-1} soluble starch) to screen for iodide oxidizing activity through the formation of purple I$_2$-starch complexes (Amachi *et al*., 2005).

16S rRNA gene sequencing and phylogenetic analysis

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene was performed as previously described (Li *et al*., 2011). Sequencing of purified PCR products (MinElute PCR Purification Kit, Qiagen) was performed by the Georgia Sequencing Facility at the University of Georgia, Athens. Phylogenetic analysis of 16S rRNA gene sequences was performed using BLAST searches (http://www.ncbi.nlm.nih.gov/Genbank/index.html), the RDP classifier (Cole *et al*., 2009), and maximum likelihood reconstruction of phylogenetic trees with ARB (Wolfgang *et al*., 2004).

Screening for iodide oxidizing strains

Two approaches were applied to identify IOB. The first method examined the formation of triiodide (I$_3^-$) using culture supernatants and crude cell extracts (Amachi *et al*., 2005). To prepare crude cell extracts, cell pellets of individual strains were suspended in 0.1X M9G (M9 minimal medium with 2% glucose), stored on ice for 30 min, then disrupted by 5 cycles of 20 s sonication (Misonix XL2000, 100W) at 20 kHz and 40 s of cooling in an ice bath. Complete lysis of cells was confirmed by microscopy. Cell debris was removed from the crude cell extracts using centrifugation ($3200 \times g$, 20°C, 15 min).

Reaction mixtures for I$_3^-$ formation contained 150 μL crude extract or culture supernatant, 177 mM phosphate buffer (pH 6.0) and 10 mM KI in 300 μL total volume. Sample controls (no KI) and assay controls (no crude extract or culture supernatant) were included. Formation of I$_3^-$ was monitored spectrophotometrically (absorbance 353 nm) at 10 min intervals over 1 hour at 30°C. One unit (U) of iodide-oxidizing activity was defined as the amount of crude extract or culture supernatant capable of oxidizing 1 μmol of iodide min^{-1}.

Autoradiography was the second method used to identify IOB (Li *et al*., 2011). Cell material from certain strains grown in the presence of ^{125}I$^-$ exhibited a ring or "halo" when exposed to radiography film (figure 2 in Li *et al*., 2011), and our preliminary observations led us to believe that this halo pattern could be an indication of iodide oxidation activity.

Size fractionation of the iodide-oxidizing components of IOB supernatants

Supernatant (30 mL) from strains incubated in 1X M9G for 24 h was collected and concentrated by ultra-filtration (AmiconUltra, Millipore). The retentate was washed three times with 1X M9G (300 μL) and suspended to 3 mL in the same medium. Iodide oxidation was measured in the retentate and filtrate using the I$_3^-$ formation assay.
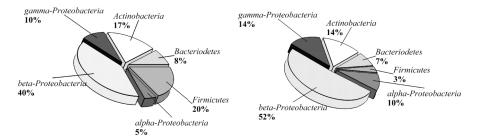
Fig. 1. Phylogenetic affiliation of bacteria isolated directly from 8 F-area sediment samples ($n = 325$, left panel) and from 1 mM KI enrichment cultures ($n = 29$, right panel).
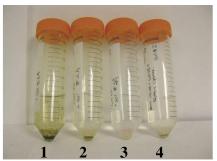
Iodine speciation analysis

Presumptive IOB were incubated in 1X M9G medium containing 10 μM KI for 1 to 30 days with gentle hand shaking twice daily. The supernatant was collected by centrifugation ($3200 \times g$, 20°C, 15 min) for iodine species analysis. 1X M9G medium containing 10 μM KI was used as the background control. Additionally, 1–2 bacterial strains deemed non-IOB based on the I_3^- formation and radiography assays were also included as negative controls. Quantification of inorganic iodine species (iodide and iodate) was performed as described by Zhang *et al*. (2010).

RESULTS AND DISCUSSION

A comprehensive culturing strategy using different combinations of cell dispersion techniques, medium, gelling agents, antibiotics, and incubation times was used to isolate 325 morophologically distinct bacterial strains from F-area sediments. Phylogenetic analysis of 16S rRNA gene fragments revealed that the isolates comprised 6 phyla and 33 bacterial families, common to terrestrial soils and sediments (Fig. 1). No obvious trends were observed relating the phylogeny of the isolates to their environmental source (seep zone versus sand/clay aquifer or depth of the sediment) or the isolation method employed (pyrophosphate versus sonication; gellan gum versus noble agar). Each of the isolates was evaluated for iodide oxidizing ability using R2A or DNB agar plates containing starch and iodide (1 mM KI), where purple coloration is indicative of iodide oxidation to I_2 (Amachi *et al*., 2005). None of the isolates exhibited an iodide oxidation phenotype using this assay.

Enrichment cultures containing high concentrations of iodide have previously been used to isolate IOB from seawater (Amachi *et al*., 2005). Likewise, tubes from 2 of 24 enrichment cultures containing F-area sediments and 1 mM KI exhibited a distinct yellow coloration, indicative of iodide oxidation to I_2 and/or volatile organic iodine species, after 22 weeks of static incubation in the dark (Fig. 2). These two enrichment cultures contained dH_2O and iodide and had been inoculated with organic-rich sediment collected no more than 1.2 m below the
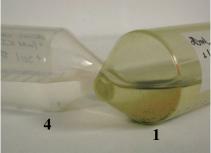
Fig. 2. Volatile iodine formation in enrichment cultures. Yellow discoloration was observed in 2 out of 24 enrichment culture tubes (tubes 1 and 2 shown here).

Table 1. Iodide species distribution in enrichment culture supernatant.

Enrichment culture*	Iodine speciation (%)		
	Iodide	Iodate	Organo-iodide
1A	23.1 ± 0.7**	4.5 ± 0.8	72.4 ± 0.1
1A autoclaved	100	0	0
2A	86.2 ± 3.2	7.2 ± 1.4	6.6 ± 1.9
2A autoclaved	100	0	0

*Enrichment cultures 1A and 2A contained sediment (collected from two separate sites, 0.15 to 1.2 m below the surface in the organic-rich wetland zone of F-area) suspended in sterile dH_2O and were incubated 3 months; the same sediment samples were sterilized by autoclaving for "1A autoclaved" and "2A autoclaved".

**Values represent means from duplicate experiments ± standard deviations.

surface from two locations in the F-area seep zone. All other enrichment cultures (that did not show yellow coloration) were inoculated with organic-poor, sandy sediment collected 13–26 m below the surface from a sandy/clay aquifer or contained DNB medium. A strong iodine smell was detected after removing the cap from each of the 2 yellow colored tubes, suggesting that iodide oxidation had occurred. To confirm that iodide oxidation had occurred and that microorganisms were involved, a second set of iodide enrichment cultures were established in sealed serum vials containing: 1) medium from the original, yellow colored enrichment tubes (as controls autoclaved medium from the enrichment cultures was also used as inoculant), 2) sterile dH_2O, and 3) 1 mM KI (Table 1). After a 2-month incubation period, a yellow color was noted in the two vials that had been inoculated with non-autoclaved medium from the initial enrichment cultures. Analysis of iodine speciation in the medium from these tubes revealed the presence of iodate and organo-iodine, indicating that microbial activity is necessary

Table 2. Summary of iodide-oxidizing activity screening among F-area bacteria.

Description	I_3^- assay[a]	Radiography assay[b]
IOB Screening		
(1) Activity in background control (mU mL^{-1})	0.000 ± 0.009[c]	no halo
(2) # of positive strains	36 (5)[d]	48 (11)
(3) Activity range (mU mL^{-1})	0.006 ± 0.005 to 0.076 ± 0.049[c]	NA
Iodine speciation analysis		
(4) # of strains examined from (2)	28 (5)	46 (11)
(5) # of strains exhibiting >10% I$^-$ oxidation	23 (5)	36 (11)
(6) Range of mean iodide oxidation rates (μM I$^-$ day^{-1})	0.03 to 0.70	0.04 to 0.70
# of confirmed IOB strains	23	36

[a]For the I_3^- production assay 1 U was defined as 1 μmol I_3^- min^{-1}.
[b]For the radiography assay, cell material grown in the presence of ^{125}I that exhibited a "halo" when exposed to radiography film was considered IOB (+) (see Li *et al*., 2011, figure 2, FA-2B-B2* for an example of "halo").
[c]Average values $\pm$ SD for triplicate experiments.
[d]Numbers in parentheses indicated the # of positive strains that were isolated from enrichment cultures.

for oxidation of iodide under these conditions (Table 1).

Bacterial isolates ($n = 29$), obtained from the two yellow colored enrichment cultures using R2A and DNB agar plates were then tested for iodide oxidation by streaking on the same medium amended with starch and iodide. Many purple colored colonies were identified using this approach, however it was determined that the purple coloration was not caused by the formation of I$_2$, but rather the production of the pigmented antibiotic violacein. Interestingly, this pigment was not observed in any of the colonies isolated directly from sediments. Violacein is known to possess strong antioxidant properties and could thus help protect cells from iodination (Konzen *et al*., 2006), yet the reason that violacein-producing strains were preferentially isolated from the 1 mM iodide enrichment cultures is not clear.

From the results presented above, we postulated that either 1) IOB (or fungi) in the enrichment cultures are difficult to isolate on solid surfaces or 2) rates of iodide oxidation catalyzed by microorganisms in the enrichment cultures are relatively low and/or not detectable using the starch-iodide plate technique. To address the second hypothesis, we utilized several different approaches to assess iodide oxidation potential among the bacterial isolates that had been obtained both directly from sediments and from the enrichment cultures.

Crude cell extracts and culture supernatants of 84 F-area bacterial strains, isolated directly from seep zone sediments ($n = 64$) and from enrichment cultures ($n = 20$), were screened to identify iodide oxidizing potential using an I_3^-

formation assay. Rates of I_3^- formation were quite low, and in some cases variable (i.e., triplicate measurements using a single strain sometimes yielded both positive and negative I_3^- formation results). Using this screening assay, 36 of 84 strains exhibited a positive mean value from triplicate analysis, ranging from 0.006 to 0.076 mU mL^{-1} (Table 2).

To confirm iodide oxidizing activity among the strains that tested positive with the I_3^- formation assay, iodide consumption was measured in culture medium (10 μM inital I$^-$) for 28 of the 36 putative IOB following 5–10 day incubation. Total iodide decreased $\geq$10% in culture medium from 23 of these strains (iodide levels remained unchanged in culture medium for the 5 remaining strains and the background control) (Table 2). The iodide oxidation rates for these 23 strains ranged from 0.03 to 0.70 μM I$^-$ day^{-1}.

Autoradiographic analysis was conducted on cell material from 84 F-area strains that had been grown on R2A plates containing ^{125}I$^-$, and a halo pattern was evident with 48 of these strains. The iodide consumption assay was again used to confirm the IOB phenotype. Iodide consumption ($\geq$10%) was measured in cultures of 36 of the 46 strains examined, with rates ranging from 0.04 to 0.70 μM I$^-$ day^{-1} (Table 2). Among these 36 confirmed positive strains, 15 of them also exhibited I_3^--positive phenotypes. Of the strains that produced I_3^-, eight did not exhibit halo patterns with autoradiography (the reason for this discrepancy is unknown).

From the screening and confirmation assays described above, a total of 44 bacterial strains were classified as IOB (84 strains were examined, i.e., 52.4% positive). Phylogenetic analysis of partial 16S rRNA genes (750~900 bp) revealed that these strains were members of the *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, and *Proteobacteria*. Apparent correlations between the IOB phenotype and taxonomic lineage were not noted. A large majority of the IOB isolates ($n = 27$) originated directly from the organic-rich sediments (0.15–1.2 m below surface) or from enrichment cultures that had been initiated with the same organic-rich sediments ($n = 13$). Only 4 IOB strains were obtained from the sandy/clay deep aquifer sediments.

Supernatants from cultures of 4 of the most active IOB were fractionated by size and evaluated for iodide oxidizing activity using the I_3^- formation assay. The extracellular iodide-oxidizing activitiy of three of these strains was identified in the $\geq$30 kDa but $\leq$50 kDa fractions, whereas the $\geq$50 kDa but $\leq$100 kDa fractions were found to be active for the other strain.

CONCLUSIONS

SRS F-area bacteria (44 of 84) were identified as IOB using a combination of I_3^- formation, radiography and iodide consumption. From the I_3^- formation assay or starch-iodide plates alone, it would be difficult to ascertain that any of these strains were IOB. Indeed, it has been noted that haloperoxidase activities are difficult to detect in crude cell extracts because of high detection limits of the existing assays (Leblanc *et al.*, 2006). However, using a newly developed method to detect low levels of iodide, iodate, and organo-iodide (detection limits for

iodide and iodate are 0.34 nM and 1.11 nM, respectively) we measured a decrease ≥10% in cultures (10 μM iodide, initial concentration) of each of the 44 IOB after 5–30 days incubation, whereas there was negligible iodide loss in sterilized controls or with non-IOB strains. Whether this slow, but apparently common, iodide oxidizing activity or that of a highly active IOB strain(s), not yet cultured, was responsible for the bulk of the iodide oxidation observed in the enrichment tubes (Fig. 2) has not been reconciled, but this question is currently being addressed by our group.

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