

**ROLES OF NATURALLY OCCURRING BACTERIA IN CONTROLLING
IODINE-129 MOBILITY IN SUBSURFACE SOILS**

A Dissertation

by

HSIU-PING LI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Marine Biology

Roles of Naturally Occurring Bacteria in Controlling Iodine-129 Mobility in Subsurface
Soils

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ABSTRACT

Roles of Naturally Occurring Bacteria in Controlling Iodine-129 Mobility in Subsurface
Soils. (August 2012)

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^{129}I is of major concern because of its biophilic nature, excessive inventory, long half-life (~16 million yrs), and high mobility in the natural environment that depends on its chemical speciation. Iodide (I^-) has the highest mobility than iodate (IO_3^-) and is the predominant species in the terrestrial environment due to prevailing pH and Eh conditions. In order to transform I^- to less mobile organo-iodine (OI), strong oxidants are necessary to activate the first electron transfer step from I^- to reactive intermediates. The aim of this study was to determine the influence of naturally occurring aerobic bacteria isolated from an ^{129}I contaminated aquifer (F-area of the Savannah River Site, SC) on I^- oxidation and OI formation.

It was demonstrated that 3 of 136 strains accumulated I^- (0.2~2%) in the presence of H_2O_2 , when incubated in the presence of an environmentally relevant concentration of I^- (0.1 μM). The accumulation was likely through electrophilic substitution resulting in the iodination of cellular constituents. The results indicated that culturable I^- -

accumulating bacteria are not directly responsible for the high fraction of oxidized iodine species (IO_3^- and OI , >50% of total I) present in the SRS F-area.

Several bacterial strains were found to be capable of stimulating I^- oxidation through excretion of oxidants and enzymes. Organic acids in spent liquid medium from 27 of 84 aerobic bacterial cultures enhanced H_2O_2 -dependent I^- oxidation 2-10 fold. Organic acids enhanced I^- oxidation by (1) lowering the pH of the spent medium and (2) reacting with H_2O_2 to form peroxy carboxylic acids, which are strong oxidizing agents.

In the absence of H_2O_2 , spent medium from 44 of 84 bacteria cultures showed I^- oxidizing capacities. One I^- oxidizing bacterium was studied to characterize its extracellular I^- oxidizing component(s). The I^- oxidizing capability from the spent medium was inactive by treatments with heat and H_2O_2 and absent under anaerobic conditions. Conversely, NADH, NADPH and FMN additions stimulated I^- oxidation in the spent medium. These results indicate an oxidase(s) catalyzed I^- oxidation. Understanding the bacterial activities involved with I^- oxidation and OI formation is expected to help reduce ^{129}I mobility in water-soil systems.

DEDICATION

To the lovely Lord

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I would like to express my greatest appreciation to my committee chairs, Dr. Robin Brinkmeyer and Dr. Peter Santschi, not only for their generosity and support, but also for allowing me to be part of the family in Laboratory of Oceanographic and Environmental Research. Their guidance, encouragement, and philosophy in both research and life have proven to be invaluable to my career. I am also deeply grateful to Dr. Chris Yeager, who was always ready to discuss and answer my questions. My greatest thanks also go out to my committees, Dr. Rainer Amon and Dr. Robin Autenrieth, for their guidance throughout the course of this research.

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Finally, thanks to my husband, Kung-Jen (Calvin) Liu, family and family-in-law for their selfless love and support all the time.

NOMENCLATURE

| | |
|------------------------|---|
| ^{127}I | Stable iodine |
| ^{129}I | Radioactive iodine-129 |
| ^{238}U | Uranium-238 |
| ABTS | 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid |
| ATP | Adenosine-5'-triphosphate |
| Br^- | Bromide |
| Cl^- | Chloride |
| CoA | Coenzyme A |
| DOE | Department of Energy |
| DOE-EM | Department of Energy's Office of Environmental Management |
| EDTA | Ethylenediaminetetraacetic acid |
| Eh | Redox potential |
| EPA | Environmental Protection Agency |
| FAD | Flavin adenine dinucleotide |
| FMN | Riboflavin-5'-phosphate (flavin mononucleotide) |
| GC-MS | Gas chromatography- Mass spectrometry |
| H_2O_2 | Hydrogen peroxide |
| HLRW | High-level radioactive waste |
| HOI | Hypoiodous acid |
| I^- | Iodide |

| | |
|----------|--|
| I_2 | Elemental iodine |
| I_3^- | Triiodide |
| IAB | Iodide-accumulating bacteria |
| IMB | Iodide-methylating bacteria |
| IO_3^- | Iodate |
| IOB | Iodide-oxidizing bacteria |
| K_d | Distribution coefficient |
| LLRW | Low-level radioactive waste |
| MALDI | Matrix-assisted laser desorption ionization |
| MCL | Maximum Contaminant Level |
| NAD^+ | Nicotinamide adenine dinucleotide |
| NADH | Reduced nicotinamide adenine dinucleotide |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| NaN_3 | Sodium azide |
| NMR | Nuclear magnetic resonance |
| NRC | Nuclear Regulatory Commission |
| OI | Organo-iodine |
| PCR | Polymerase chain reaction |
| Pu | Plutonium |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrlamide gel electrophoresis |
| SGZ | Syringaldazine |

| | |
|--------|----------------------------------|
| SRS | Savannah River Site |
| TOF MS | Time-of-flight mass spectrometry |
| Xe | Xenon |

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CHAPTER I

INTRODUCTION

Iodine has 25 isotopes, including 1 long-lived radioisotope (^{129}I , $t_{1/2} = 15.7 \times 10^6$ yrs), 23 short-lived radioisotopes, and 1 stable isotope (^{127}I). Iodine radioisotopes are naturally produced through cosmic ray spallation of xenon (Xe) in the atmosphere and neutron-induced fission of natural uranium-238 (^{238}U) and plutonium (Pu) in the Earth's crust, but these natural sources account for a small fraction of the global inventory (2, 103). The primary source of iodine radioisotopes is from the fission reactions in nuclear reactors, production of weapons-grade Pu, and detonation of nuclear weapons (2, 103). Among the radioisotopes of iodine, ^{129}I is of concern to human health and environmental integrity due to the longevity, large inventory (e.g., at the Department of Energy (DOE) storage facilities), high perceived mobility in water-soil systems, and bioaccumulation in thyroid glands and breast tissues (32, 115). Thus, ^{129}I has recently been recognized by the U.S. Department of Energy's Office of Environmental Management (DOE-EM) as one of the key risk radionuclides in the groundwater at the Hanford Site and the Savannah River Site (SRS). Moreover, due to the biophilic properties of iodine, the Environmental Protection Agency (EPA) has regulated the Maximum Contaminant Level (MCL) of ^{129}I in drinking water at the lowest level (1 pCi/L) of all radioisotopes.

This dissertation follows the style of Applied and Environmental Microbiology.

1.1 Inventory of anthropogenic ^{129}I in environment

In nuclear reactors ^{129}I occurs as a gaseous product of fission that forms in fuel rods. In a typical boiling water reactor, the fuel rods consist of small (1 cm \times 1 cm) ceramic uranium oxide ‘fuel’ pellets sealed inside a protective tube of ‘Zircaloy’ that is stable up to 1,200 °C. Hundreds of rods are then assembled to make a reactor core, which is then sealed in a high-pressure vessel filled with water that is subsequently heated by the fission chain reaction (285 °C) to produce steam that drives a turbine to generate electricity. Under normal reactor operations, the fission chain reactions are maintained at temperatures below 1,200 °C, whereby the heat is absorbed by boron and cooling water. In the critical state, the ^{129}I is contained under pressure within the spent fuel pellets and Zircaloy tube as a liquid and/or solid phase. If the reactor chemistry and temperature are not carefully controlled, the rods can corrode and eventually crack, releasing ^{129}I into the surrounding cooling water that can be discharged to the regional environment. Nuclear accidents such as those at Chernobyl in Ukraine (1986) and Fukushima Daiichi in Japan (2011) demonstrate how the failure of controlling the critical state can result in the release of large quantities of ^{129}I into the water surrounding the core and ultimately into the environment in just a few hours.

In addition to discharges from nuclear accidents, large quantities of ^{129}I have been emitted to the environment from the reprocessing facilities, as well as leakage to soils from spent fuel wastes (2, 54, 103). For example, up to 1998, a total of 2,360 kg of ^{129}I was discharged to the marine environment by two European facilities at La Hague in

France and Sellafield in England, an amount that is 50 times the total release from nuclear weapon tests (53). To date, these two European facilities are still operating of which more ^{129}I is expected to be discharged into environments.

1.1.1 ^{129}I inventories and waste disposal from USA Department of Energy (DOE) facilities

The highest inventories of ^{129}I in the USA are at two DOE storage facilities (Fig. 1.1), Hanford Site (120 Ci ^{129}I) in the state of Washington and the SRS (26 Ci ^{129}I) in South Carolina (32). Hanford Site and SRS housed the primary facilities for the production and processing of weapons-grade Pu (i.e. ^{238}Pu & ^{239}Pu) and tritium during the Cold War. The Hanford Site was selected in 1944 to produce the first weapons-grade Pu for the Manhattan Project, and 54.5 metric tons (MT) of fissile material was produced until 1987 (61). At the SRS, approximately 36 MT of Pu were produced from 1953 to 1988. Nuclear weapons design, component fabrication, assembly, and final testing took place at the other twelve US defense facilities spread across a dozen states (Fig. 1.1) (88).



FIG. 1.1. Locations of DOE defense nuclear weapon facilities in the US consisting of the following active/closure project sites and their primary functions before 1992: Hanford Site (plutonium production for WWII & Cold War operations), Fernald Closure project (former uranium processing plant), Idaho National Laboratory (INL, research and development, waste retrieval and remediation activities), Los Alamos National Laboratory (LANL, stockpile stewardship and management to ensure the safety, security, and reliability of the nation's nuclear deterrent), Lawrence Livermore National Laboratory (LLNL, nuclear weapons research and development), Miamisburg Environmental Management Project (Mound, former manufacturing facility, which underwent environmental remediation), Nevada National Security Site (NNSS, outdoor laboratory and national experimental center for the preparation of nuclear waste deposition, training for emergency response, and waste management), Y-12 National Security Complex/Oak Ridge National Laboratory (Y-12/ORNL, uranium component manufacturing and storage), Pantex Plant (stockpile of nation's nuclear weapons), Rocky Flats Environmental Technology Site (former manufacturer of components for nuclear weapons; the environmental remediation process was complete in 2005), Sandia National Laboratories (SNL), Savannah River Site (SRS, production of basic materials for nuclear weapons before Cold War), Waste Isolation Pilot Plant (WIPP, safe disposal site for Transuranic waste), West Valley Demonstration Project (WVDP, commercial storing site for mixed nuclear waste from State of New York) (<http://www.dnfsb.gov/about/where-we-work/doe-defense-nuclear-facilities>).

Production of nuclear defense materials ended in 1992 and the DOE's mission shifted to the dismantlement of nuclear weapons, the disposal of fissionable materials, and decontamination of the production, testing, and storage facilities (88). Currently, about 60% (35 megacuries) of high-level radioactive waste (HLRW) is stored at the Hanford Site. The remainder is stored at the SRS and Idaho National Laboratory (2, 61).

Plans to transfer most of DOE's HLRW to the Yucca Mountain Nuclear Waste Repository in Nevada were terminated by President Obama in 2009. In March 2010 the DOE withdrew its license application for permanent storage of HLRW at Yucca Mountain. While the debate over a permanent storage site by the US Congress continues (47, 127), HLRW remains at Hanford, SRS, and Idaho facilities in shallow waste pits or 'cribs' and steel tanks, many of which have cracked after chemicals were added in an attempt to neutralize the most radioactive materials (113). Furthermore, in order to conserve tank space, low-level radioactive waste (LLRW) or partly decontaminated waste was injected into the subsurface with the reasoning that waste would be trapped in the capillary pores of unsaturated materials, such as sand lying above the water table (31). In the early years of disposal (late 1940s to early 1950s), the movement of liquids in the unsaturated (i.e. vadose) zone above the water table was not well-understood, thus, researchers were unable to accurately assess the storage capacity in soils and migration of radioactive wastes (31). Hydrologists estimated that it would take 175-180 years for Pu-contaminated groundwater at the Hanford Site to reach the Columbia River; however, Pu was detected in the river only 11 years after it was first produced (113). Liquid HLRW (and LLRW) from pits and steel tanks at Hanford Site and the SRS have contaminated aquifers, which contain ^{129}I at concentrations well above (10 ~1000 times) the MCL levels regulated by EPA (2, 58, 90). These radionuclides such as ^{129}I have a high potential to seep into the Columbia River that borders the Hanford Site and the Savannah Rivers which borders the SRS.

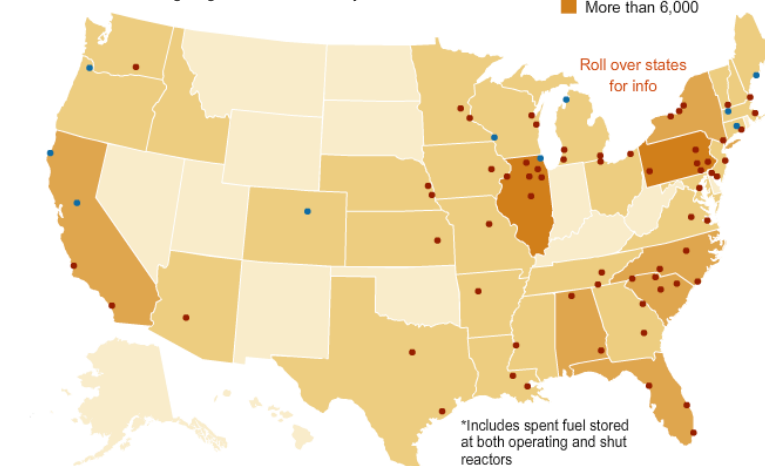
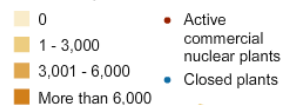
1.1.2 Disposal of ^{129}I wastes from commercial nuclear reactors

Environmental contamination with HLRW from commercial nuclear power plants is of equal concern. By 1991, 20% of the electricity in the US was generated by 104 nuclear reactors at 65 commercial nuclear power plants (Fig. 1.2) (134). To date, no permanent disposal facilities have been designated in the US for the ~63,000 tons of spent fuels produced by these commercial reactors. The Yucca Mountain repository was also intended as a permanent storage facility for HLRW from commercial power reactors. As a temporary solution, the Nuclear Regulatory Commission (NRC) has sanctioned an increase in the allowable numbers of rods that can be 're-racked' in plant pools. For example, the pool at Reactor 3 of the Millstone Nuclear Power Plant in Waterford, Connecticut was originally licensed to hold 756 rods but now holds 1,040 and has recently been licensed by the NRC to hold 1,860 (30). Considering the dire problems at the DOE facilities, it is not difficult to imagine similar HLRW contamination of surface water and aquifers from 'long term' storage at commercial nuclear power plants. Figure 1.2 illustrates the proximity of commercial nuclear power plant accumulated HLRW to major aquifers in the US.

(A)

The nuclear crisis in Japan is a reminder that the U.S. has no long-term storage solution to 71,862 tons of radioactive nuclear waste that sits, and continues to build, at its nuclear reactors. There are currently 54,696 tons of waste stored in cooling pools like those at the stricken Fukushima Dai-ichi nuclear complex. Another 17,166 tons of waste sit in garage-sized outdoor dry casks.

Total spent nuclear fuel stored, in tons*



(B)



FIG. 1.2. (A) Locations of commercial nuclear power plants in the US and accumulated HLRW (134) and (B) principal aquifers in the US (86). Color used in pink indicates minor aquifers.

There are now over 440 commercial nuclear power reactors in the world today with 377 gigawatts of total capacity (135). Given that ^{129}I is produced by nuclear fission

at a rate of 1 Ci per gigawatt of electricity produced and assuming a reactor efficiency of 98% (32), 385 Ci of ^{129}I is currently generated per year. In order to respond to global warming, 11 applications to the NRC are proposed to construct 18 new nuclear reactors, increasing the total number of reactors in the USA from 104 to 122 (134). China alone has 17 reactors under construction and anticipates a six-fold increase in nuclear power capacity to at least 50 gigawatts, with more large units than in the U.S. by 2020 (136). If only a fraction of the “Nuclear Renaissance” is realized, a significant increase in worldwide ^{129}I waste will be created by the nuclear power industry.

1.2 Mobility of ^{129}I in soil-water systems

Iodine can exist in multiple oxidation states ranging from -1 to +7 depending on the environmental conditions. Thus, one of the key factors that controls ^{129}I mobility is its chemical speciation. For example, one can predict the predominant iodine species in aqueous solution based on a Pourbaix diagram, which determines iodine speciation based on pH and Eh conditions (94). As shown in Figure 1.3, iodate (IO_3^- , oxidation state +5) is expected to be stable under oxic conditions across the pH values that are typically found in the natural environment. Elemental iodine (I_2 , oxidation state 0) is expected to be present under moderately oxidized and acidic ($\text{pH} \leq 4.0$) conditions; whereas, iodide (I^- , oxidation state -1) would be expected when the environment becomes more reducing. Among these inorganic iodine species, I^- would be expected to be the dominant species in most groundwaters due to its stability under a wide range of natural pH and Eh conditions (Fig. 1.3). This is important because I^- has been recognized

to have lower soil affinity (i.e. high mobility), with a distribution coefficient (K_d) of $\sim 1 \text{ L}^3 \text{ kg}^{-1}$, than that of IO_3^- ($K_d \sim 1000 \text{ L}^3 \text{ kg}^{-1}$) (13, 54, 65, 103, 107), which would explain the relatively high mobility of ^{129}I that has been observed in Hanford Site and SRS. However, several field investigations of water-soil systems have observed that I^- and IO_3^- coexist in different proportions and that another iodine species, organo-iodine (OI), is often present (52-53, 90, 107, 142). These observations signify that biogeochemical factors other than pH and Eh control iodine speciation in natural water-soil systems.

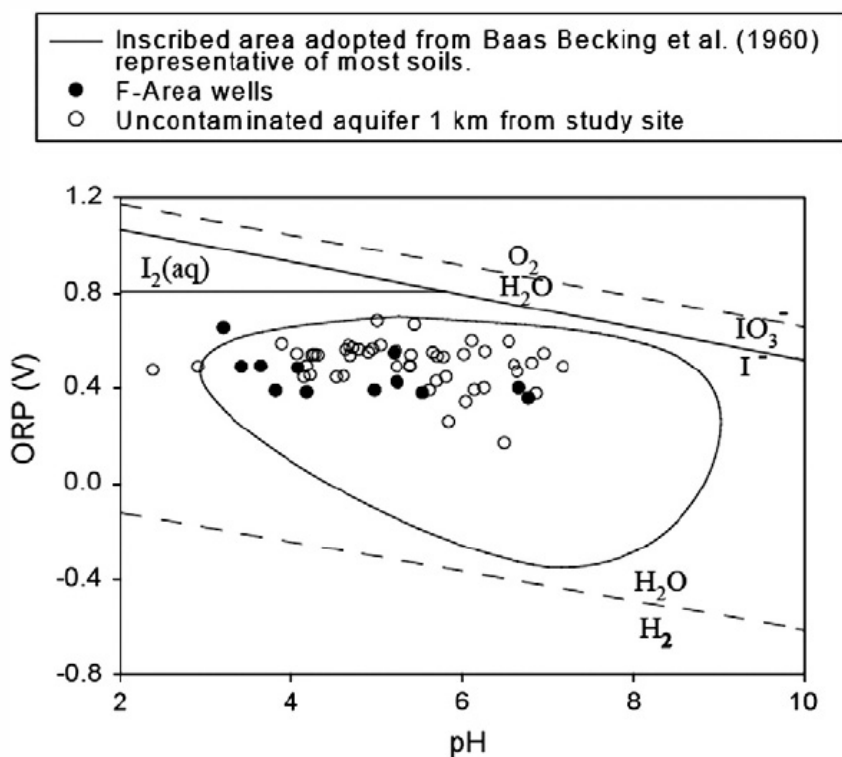


FIG. 1.3. Pourbaix plot illustrates the theoretical aqueous iodine speciation based on the pH and Eh values (90). Solid and hollow dots indicated the groundwater pH and Eh values of the SRS F-area, indicating that I^- should be the predominant iodine species (90).

Formation of OI occurs through a complex, poorly characterized process. First, a reactive intermediate iodine species, such as I_2 , hypiodous acid (HOI, oxidation state +1) or triiodide (I_3^-), needs to be formed from I^- oxidation or IO_3^- reduction, as shown in Fig. 1.4. Next, the reactive iodine species is thought to attack organic matter, forming OI through electrophilic substitution on aromatic moieties (25, 82, 97, 104, 139, 141). Thus, organic-rich soils are considered to have a higher tendency to serve as an iodine sink, whereby soil organic matter acts as a barrier to decrease ^{129}I mobility. Indeed, Hu *et al.* (53-54) and Xu *et al.* (138) have successfully demonstrated that soils with high organic matter content are capable of taking up >80% of iodine as OI, whereas inorganic iodine became dominant (~50%) in soils with low organic content. However, several studies reported that a small fraction of soil OI can be released to groundwater (90, 107, 138, 142). Recent observations have pointed out that the solubility and molecular weight of OI species can play a crucial role whereby high molecular weight OI would tend to become a sink for ^{129}I , whereas very low molecular weight or truly dissolved OI species can leach out and migrate into groundwater (39, 107, 137-138).

Regardless of the leaching potential of OI, it is clear that the initial iodine species and the presence of organic matter in a water-soil system play important roles in ^{129}I mobility. As mentioned earlier, I^- is expected to be the dominant species in most water-soil systems due to its stability under natural groundwater Eh and pH conditions. Since I^- is the most reduced chemical form of all iodine species, an oxidation process is the most plausible pathway to generate reactive iodine species or IO_3^- (Fig 1.4). However, the first electron transfer from I^- to an electron acceptor such as oxygen is thermodynamically

unfavorable unless catalyzed by strong oxidants (77). Abiotic oxidants present in soil, such as manganese oxide (MnO_2 , major component in pyrolusite) and ferric oxide (Fe_2O_3 , major component in hematite), are capable of oxidizing I^- ; however, this reaction is favorable only under acidic conditions ($\text{pH} < 5.0$) (36, 42, 137).

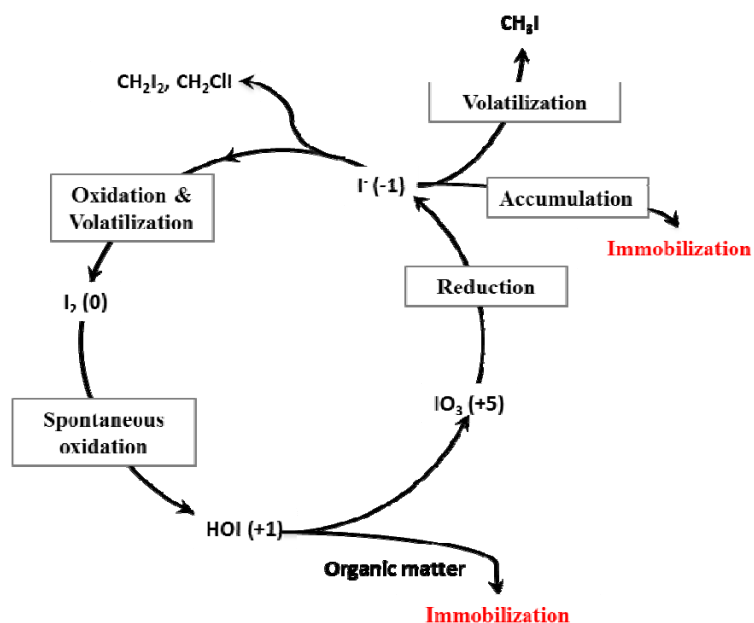


FIG 1.4. Redox cycling of iodine species mediated by biotic and abiotic processes (after Amachi et al. (4)).

1.3 Potential means by which soil bacteria could decrease ^{129}I mobility

It is hypothesized that microbes can influence I^- oxidation and OI formation due to iodine's biophilic nature, strong affinity for organic matter and redox sensitivity (3, 32). Moreover, given the high abundance of microbes occurring in water-soil systems, especially in organic-rich soils (e.g. 10^8 - 10^9 cells per gram soil) (118, 129), it is reasonable to assume that microbes could play an important role in the mobility of ^{129}I in

terrestrial systems. This assumption is supported by recent field observations and laboratory experiments (3-4, 6, 83-84, 107, 112). These laboratory studies have used soils/sediments treated with heat, chloroform, γ -irradiation, or prokaryotic antibiotics, to demonstrate that microbial activity is required for significant iodine binding capacity onto soils (6, 83-85). Moreover, inoculation of sterilized soil with fresh soil or viable microorganisms can restore the iodine-organic matter binding potential (84). Contradictory results, however, have also been observed, whereby only a minor increase in Γ sorption had been observed in an organic-rich soil that had first been sterilized by autoclaving and then enriched with viable microbes (110). These contradictory results led to debates of the importance of microbes on ^{129}I mobility, especially given the fact that the potential for Γ oxidation by metal oxides could be high at most nuclear waste storage sites where groundwater is acidic (110-111, 141).

A growing body of literature has implicated microbial enzymes, such as oxidases, laccases, perhydrolases and peroxidases, in the formation of halogenated organic matter compounds in soils (6, 20, 48, 67, 84-85, 89, 95, 98, 112, 140). Furthermore, a series of recent studies on marine bacteria have identified a number of species that can influence iodine speciation/mobility via Γ accumulation in bacterial cells, Γ oxidation in exudates (e.g. extracellular enzymes), and Γ -methylation (3, 5-7, 9-10, 12, 117). To date, little research has been devoted to identifying terrestrial bacteria associated with the transformation of iodine species. If terrestrial bacteria also have similar capabilities for Γ accumulation and Γ oxidation that has been found in marine bacteria, bacteria could have a profound effect on ^{129}I mobility in contaminated aquifers.

1.3.1 Cellular iodide accumulation

Iodine is an essential trace element for mammals because of its role as a constituent of thyroid hormones (i.e. thyroxine and triiodothyronine). In the thyroid gland, I^- is taken up with sodium ions by a sodium/iodide symporter utilizing an electrochemical potential gradient, where it is then incorporated into hormones via an iodination process. For centuries it has been known that marine macroalgae can also concentrate iodine, by a mechanism distinct from that used by mammals (67). For example, the brown algae *Laminaria digitata* utilizes an unidentified haloperoxidase associated with the cell wall to oxidize I^- extracellularly to HOI, which can penetrate algal cell walls by facilitated diffusion (67). The maximum amount of accumulated I^- in *L. digitata* can reach up to $50,000 \mu\text{g g}^{-1}$ of dry weight, whereas terrestrial plants accumulate, on average, just $0.2 - 0.5 \mu\text{g g}^{-1}$ dry weight (24, 45, 66, 92). Inside the *L. digitata* cell HOI is reduced and stored as I^- which acts as an inorganic antioxidant, scavenging reactive oxygen species (66). X-ray absorption spectroscopy experiments have revealed that while inside *L. digitata* tissue, I^- is surrounded by organic molecules such as phenols, carbohydrates, and proteins, without forming chemical bonds with these organic compounds (66).

To date, information concerning I^- accumulation by bacteria has been obtained primarily through the pioneering efforts of Seigo Amachi and his research group at Chiba University, Japan (7, 9, 11). I^- accumulating bacteria (IAB), isolated from marine sediments, are classified phylogenically within only one family, *Flavobacteriaceae*,

within the phylum *Bacteroidetes* (9). The maximum I^- content observed in IAB cells was $30 \mu\text{g g}^{-1}$ dry weight, with a cellular concentration factor of 5.5×10^3 , when I^- was provided at an environmentally relevant concentration ($0.1 \mu\text{M}$) (7, 9). By comparison, *L. digitata* has been found to have an I^- concentration factor of 1.5×10^5 (66-67). Results indicate that I^- uptake by marine bacteria mechanisms are facilitated by membrane-bound enzymes. Furthermore, I^- uptake was enhanced in the presence of glucose/oxygen or H_2O_2 (3, 7). Amachi proposed a model whereby extracellular H_2O_2 , generated by glucose oxidase, is used to oxidize I^- to I_2 or HOI via an unidentified haloperoxidase. HOI is then transported across the cell membrane via a facilitated diffusion-type mechanism (3, 7) which is similar to that observed in *L. digitata*. Once inside the cell, HOI would be reduced to I^- or form OI species (3, 7).

Terrestrial bacteria with I^- -accumulating abilities were also examined by Amachi's group (8). Bacterial strains were isolated from enrichments of anoxic natural gas formation waters that contained exceptionally high concentrations of total iodine ($\sim 120 \text{ mg L}^{-1}$, with I^- as the major species), and a set of 16 "anaerobic" terrestrial bacterial strains were obtained from culture collections that included sulfate and iron reducers, denitrifiers and methanogens (8). Results indicated very limited adsorption/accumulation of iodide ($0.7\text{-}2.0 \mu\text{g g}^{-1}$ dry weight) by these anaerobic terrestrial bacteria.

Cellular surface adsorption could also be considered as a mechanism for I^- accumulation by bacterial cells. For example, MacLean et al. (78) used cells of *Bacillus subtilis* to model I^- -bacteria adsorption through electrostatic interactions of I^- and the

positively charged functional groups (e.g. amine groups) on the bacterial surface. They found that Γ^- was readily adsorbed to the surface of *B. subtilis* at pH values < 4 , but that Γ^- could also be quickly desorbed (~ 2 hours) by raising the pH to 7.0.

1.3.2 Iodide oxidation through extracellular reactions

In the 1960's a fish kill related to the liberation of I_2 in a saltwater aquarium led to the discovery of the first iodide oxidizing bacterium. *Pseudomonas iodooxidans* sp. nov. (γ -Proteobacteria) was isolated from the aquarium and found to be capable of oxidizing Γ^- to I_2 by an extracellular or membrane bound peroxidase (45). Moreover, this bacterium exhibited high tolerance to I_2 toxicity, and its I_2 production could only be detected in the presence of 8 mM Γ^- , but not of 0.8 mM (45). Unfortunately, this bacterium was not deposited in any culture collections; thus, the characteristics and identity of the functional peroxidase, as well as its ecological role, remain unclear.

Since that time, two studies involving Γ^- oxidizing bacteria (IOB) have been conducted. Fuse et al. (40) isolated two strains of marine bacteria capable of producing I_2 and volatile OI, such as methyl iodine, in spent medium containing Γ^- and H_2O_2 indicating that a peroxidase-like enzyme was involved in Γ^- oxidation. The 16S rRNA gene sequences of these two marine bacteria were phylogenetically related to *Roseovarius tolerans* (phyla α -Proteobacteria). The other study was conducted by Amachi's research group (10, 12, 117), who initially attempted to "directly isolate" IOB from three environmental sources, including terrestrial soils, seawater, and natural gas brine water, using Γ^- -starch agar plates. The attempt was, however, only successful with

natural gas brine water samples which contained high concentrations of I^- (0.06~1.20 mM). They later incubated seawater and terrestrial samples in the presence of high concentrations of I^- (1-5 mM) and detected a yellow coloration and an I_2 smell from only the seawater samples. IOB were subsequently isolated from the high I^- seawater enrichments. The IOB isolated from brine water and seawater were phylogenetically classified within two genera, *Rhodothalassium* spp and *Roseovarius* spp, both within the α -Proteobacteria sub-phylum (10). It was concluded that these IOB utilized the produced I_2 as an antimicrobial to give them a competitive advantage in the high I^- environment (12). A laccase-like enzyme (i.e. one of the multi-copper oxidases) was then identified and characterized from one of the brine water IOB that carry out I^- oxidation (117).

Together with implications from other studies that microbial oxidases, perhydrolases, and peroxidases could be involved in the halogenation of soil organic matter (6, 20, 48, 67, 84-85, 89, 95, 98, 112, 140), microbial enzymes associated with I^- oxidation can be classified into two major types, 'oxidase-like' and 'peroxidase-like' enzymes which utilize oxygen and H_2O_2 as electron acceptors, respectively. In studies conducted thus far, 'oxidase-like' enzymes capable of transforming I^- to a reactive intermediate iodine species (e.g. I_2) are limited to a blue laccase secreted by the fungus *Myceliophthora thermophila* (140) and the laccase-like enzyme secreted by brine water IOB (*Roseovarius* spp) (117). Laccases are multi-copper phenolytic oxidases that are widely secreted by plants, fungi and bacteria that assist in lignin transformations and can utilize multiple, disparate substrates (e.g. Mn^{2+}) (109). In recent field studies of ^{129}I

mobility, laccase activities were determined from the top ~20 cm of surface soils and correlated with Γ oxidation and OI formation (112, 141). Whether laccases, or other oxidase-like enzymes capable of carrying out Γ oxidation, exist in terrestrial bacteria still needs to be clarified.

The best-known peroxidase-like enzymes associated with halide oxidation are the haloperoxidases. These enzymes are widely distributed among different biota, including humans, bacteria, fungi, plants, and algae (50, 120, 122). Haloperoxidases commonly exhibit broad-substrate specificity and are classified based on the most electronegative halide that they can catalyze – chloroperoxidases use chloride (Cl^-), bromide (Br^-) and Γ , bromoperoxidases utilize Br^- and Γ , and iodoperoxidases are limited to Γ as the halogen source. They generate hypohalides via the direct H_2O_2 -dependent oxidation of halides, and thus are capable of halogenating a wide variety of organic moieties. Interestingly, the involvement of haem (i.e. iron) as a cofactor for electron transfer is only observed in eukaryotic haloperoxidases whereas prokaryotic haloperoxidases require no haem (51). Among soil bacteria, these enzymes have been primarily identified in *Actinobacteria*, *Firmicutes*, and several groups of *Proteobacteria* (51). Given the wide distribution of haloperoxidases, it seems reasonable to find the existence of haloperoxidases in terrestrial bacteria. However, studies conducted thus far, none have reported any direct evidence for the existence of haloperoxidase action associated with iodinated organic matter in natural water-soil systems.

1.4 Research hypotheses and objectives

In groundwater from F-area of DOE's SRS, a nuclear waste disposal site in South Carolina, I^- was expected to be the predominant iodine species based on the prevailing pH (3.2 ~ 6.8) and Eh (360 ~ 654 mV) (Fig. 1.3) (90). However, field investigations in the SRS F-area have quantified appreciable amounts of IO_3^- (~30 %) and OI (~35%) in groundwater (90, 107, 142), indicating that I^- oxidation has occurred. As mentioned earlier, abiotic oxidants such as MnO_2 and Fe_2O_3 capable of oxidizing iodide are limited to acidic conditions (pH < 5.0) (36, 42, 137). Thus, it was hypothesized that bacteria occurring in the SRS F-area are capable of carrying out I^- oxidation and OI formation. In order to better understand the impact that terrestrial bacteria exert on ^{129}I mobility in water-soil systems via I^- oxidation and OI formation, this study utilized naturally occurring bacteria isolated from the ^{129}I -contaminated, oxic plume in F-area. Two hypotheses were proposed in this study and are listed below with the specific objectives.

Hypothesis I:

Certain types of terrestrial, aerobic bacteria can accumulate I^- through internalization or binding onto their cell surface, thus, temporarily or permanently retarding the transport of ^{129}I in a water-soil system.

Objectives:

- a) Isolate and characterize aerobic I^- -accumulating bacteria (IAB) from the SRS F-area sediments;

- b) Identify mechanisms of bacterial accumulation of I^- (i.e. adsorption onto cell membrane or incorporation into cells);
- c) Determine the abundance of IAB at the site;
- d) Estimate the importance IAB to retardation of ^{129}I mobility from microbial accumulation rates determined at environmental relevant I^- concentrations.

Hypothesis II:

There are aerobic bacteria present in water-soil systems that can produce enzymes to oxidize I^- and control the mobility of iodine.

Objectives

- a) Identify aerobic I^- -oxidizing bacteria (IOB) isolated from the SRS F-area;
- b) Identify IOB produced enzymes that participate in iodide oxidation;
- c) Evaluate the environmental importance of IOB and their associated enzymatic activities that relate to the transformation of I^- to OI in a natural water-soil system.

CHAPTER II

IODIDE ACCUMULATION BY AEROBIC BACTERIA ISOLATED FROM SUBSURFACE SEDIMENTS OF A ^{129}I -CONTAMINATED AQUIFER AT THE SAVANNAH RIVER SITE, SOUTH CAROLINA*

2.1 Overview

^{129}I is of major concern because of its mobility in the environment, excessive inventory, toxicity (it accumulates in the thyroid), and long half-life (~16 million years). The aim of this study was to determine if bacteria from a ^{129}I -contaminated oxic aquifer at the F-area of the U.S. Department of Energy's Savannah River Site, South Carolina, could accumulate I^- at environmentally relevant concentrations (0.1 μM I^-). I^- accumulation capability was found in 3 out of 136 aerobic bacterial strains isolated from the F-area that were closely related to *Streptomyces/Kitasatospora* spp., *Bacillus mycoides*, and *Ralstonia/Cupriavidus* spp. Two previously described iodide-accumulating marine strains, a *Flexibacter aggregans* strain and an *Arenibacter troitsensis* strain, accumulated 2 to 50% total I^- (0.1 μM), whereas the F-area strains accumulated just 0.2 to 2.0%. I^- accumulation by FA-30 was stimulated by the addition of H_2O_2 , was not inhibited by chloride ions (27 mM), did not exhibit substrate saturation

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kinetics with regard to I^- concentration (up to $10 \mu M I^-$), and increased at pH values of <6 . Overall, the data indicate that I^- accumulation likely results from electrophilic substitution of cellular organic molecules. This study demonstrates that readily culturable, aerobic bacteria of the F-area aquifer do not accumulate significant amounts of I^- ; however, this mechanism may contribute to the long-term fate and transport of ^{129}I and to the biogeochemical cycling of iodine over geologic time.

2.2 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 years), and potential toxicity due to bioaccumulation through the food chain and bioconcentration in the thyroid gland (32, 38, 52). Currently, 146 Ci of ^{129}I is inventoried in soils at two U.S. Department of Energy (DOE) sites, the Hanford Site and the Savannah River Site (SRS), where it has been identified as a key risk driver in contaminated soils and groundwater (49, 62). Furthermore, the global inventory of ^{129}I will increase significantly if just a fraction of the expected “Nuclear Renaissance” is realized (e.g., between China and India alone, 50 to 60 new nuclear reactors are expected to come online by the year 2020) (32, 133). Thus, it is critical to understand the environmental behavior of ^{129}I in order to rigorously assess storage and disposal options for current and future stockpiles of ^{129}I .

In general, little information is available about the chemical properties and mobility of iodine in subsurface aquifers, particularly its tendency to form organo-iodine or its mobility as organo-iodine. However, our own measurements of groundwater from

several of the F-area aquifer bore holes found that organo-iodine could account for up to 25% of total iodine (107, 142). The various isotopes of iodine can be strongly bound to macromolecular organic matter, which can significantly decrease or increase its transport, bioavailability, and transfer to humans, depending on the molecular weight and physicochemical properties of the resulting iodine-organic matter species (28, 43, 105-107, 132).

Microbial activity has been linked to the production of organo-iodine and sorption of iodine to soil, and a small but growing body of literature has implicated microbial oxidases, perhydrolases, and particularly peroxidases in the halogenations of soil organic matter (6, 20, 48, 56, 67, 84-85, 89, 96, 98). Yet, details concerning the mechanisms and bacterial species or groups involved are lacking. The most-recent advances in research concerning microbial-iodine interactions have been contributed by Amachi et al. (3, 5-11, 40). In a series of papers, Amachi's research group has isolated (i) Γ -accumulating bacteria (IAB) from marine sediments that concentrate Γ by a factor of 6×10^3 (9), (ii) Γ -oxidizing bacteria from seawater and natural gas brine water that transform Γ into I_2 and volatile organo-iodine species (10), and (iii) Γ -methylating bacteria from a variety of soil and seawater samples (5-6). For the marine IAB, Amachi et al. (7) proposed a model whereby extracellular H_2O_2 , generated by glucose oxidase, oxidizes Γ to I_2 or hypoiodous acid (HOI) via an unidentified haloperoxidase. HOI is then transported across the cell membrane via a facilitated diffusion-type mechanism. Once inside the cell, HOI either is reduced to Γ or forms organo-iodine.

Notably, I^- -oxidizing and I^- -accumulating strains were readily obtained from marine or brine waters but not from surface soils (22 samples from a rice paddy, upland field, and forest soils) (9, 11). The only study to date examining the potential for microorganisms from subsurface aquifers to associate with I^- was performed using enrichments from anoxic natural gas formation waters that contain exceedingly high concentrations of iodine ($\sim 120 \text{ mg liter}^{-1}$) and a set of 16 anaerobic bacterial strains obtained from culture collections that included sulfate and iron reducers, denitrifiers, and methanogens (8). The results indicated very limited adsorption or accumulation of I^- by anaerobic microorganisms.

Overall, the research conducted thus far demonstrates that microbial activity is an important factor in iodine biogeochemical cycling (particularly in enhancing iodine binding to high molecular-weight organic matter) and leads us to hypothesize that select soil and sediment bacteria could be capable of influencing the chemical behavior of I^- (the most common form of ^{129}I found in groundwater) via accumulation, volatilization, and oxidation under aerobic conditions. In particular, we wish to develop an understanding of the role that microorganisms play in the formation of organo-iodine in the subsurface and how they influence iodine mobility. As an initial step toward this overarching goal, the aim of this study was to determine if naturally occurring bacteria from a ^{129}I -contaminated oxic aquifer at the Savannah River Site, SC, could accumulate iodide at environmentally relevant concentrations.

2.3 Materials and methods

2.3.1 Isolation of bacteria

Sediment samples were collected from three locations and multiple depths within the F-area plume zone (10 to 100 pCi/liter ^{129}I) at the Savannah River Site, SC (Fig. 2.1). Sediments from the first two locations, FAW-4 and FAW-1, were collected from a sandy/clay aquifer 13.1 to 20.7 m and 24.1 to 25.9 m below the surface, respectively. The other sampling location, FSP-07, was an organic-rich wetland zone (0.15 to 1.2 m below the surface). Two approaches, sonication or pyrophosphate based, were used to isolate microbes directly from each of the subsurface soils. For the sonication method, soil samples (1 g) were suspended in 100 ml sterile distilled water (dH_2O) and stirred for 15 min. A portion (1 ml) of this soil slurry was sonicated for 15 s to generate a 10^{-2} dilution of the original soil slurry. For the pyrophosphate method, soil samples (1 g) were suspended in 100 ml sterile phosphate-buffered saline (pH 7.0) containing 25 mM pyrophosphate and stirred for 30 min and then allowed to settle for 30 min to generate a 10^{-2} dilution of the original soil slurry.

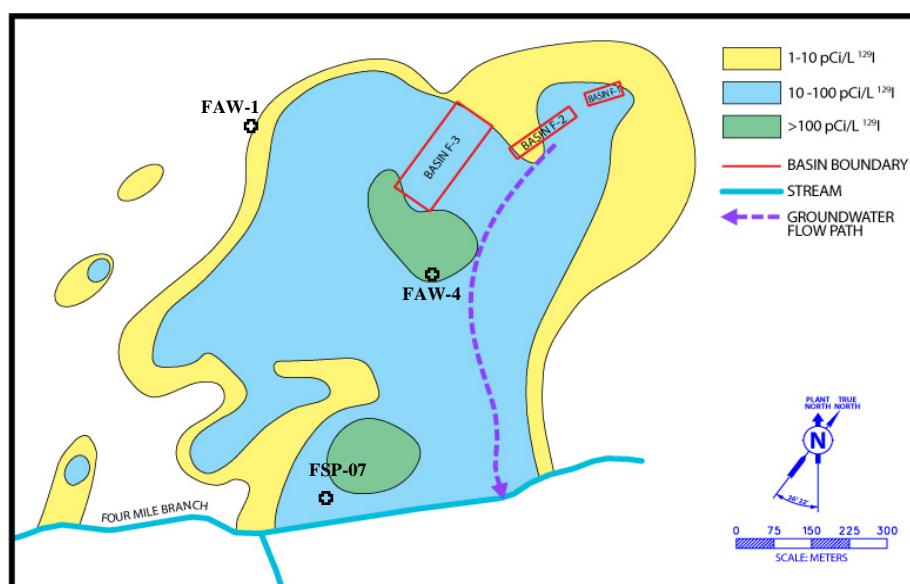


FIG. 2.1. Map of the F-area at the Savannah River Site with ^{129}I concentrations in groundwater. Mixed radioactive wastes originating from three storage basins (red rectangles) have seeped into the groundwater and have, over time, created a groundwater plume that flows toward Four Mile Branch Creek, a tributary to the Savannah River. Sediment samples for this study were collected at FAW-4 (13.1 to 20.7 m below the surface) and FAW-1 (20.7 to 25.9 m below the surface) in a sandy/clay aquifer and at FSP-07 (0.15 to 1.2 m below the surface) in an organic-rich wetland zone.

Subsequent dilutions (10^{-3} to 10^{-6}) were prepared from the sonication- and pyrophosphate-generated soil slurries in both R2A (1/10 strength; Difco) and DNB (Oxoid) medium. A portion (100 μl) of each dilution (10^{-2} to 10^{-6}) series prepared in R2A was spread onto R2A (1/10 strength) plates solidified with either noble agar (Difco) or gellum gum (Sigma Aldrich), with or without cycloheximide ($100 \mu\text{g ml}^{-1}$; Sigma Aldrich). A portion (100 μl) of each dilution (10^{-2} to 10^{-6}) series prepared in DNB was spread onto DNB agar plates, with or without cycloheximide ($100 \mu\text{g ml}^{-1}$). Plates were incubated at room temperature in the dark and monitored for growth over a period of 1 week to 3 months. As colonies with unique morphologies appeared on the plates, they

were transferred to fresh R2A (1/10 strength) or DNB plates. In this manner, 325 distinct morphotypes were isolated and stored (agar plates at 4°C and glycerol stocks at -80°C) for further analysis.

Enrichment cultures containing 1 mM iodide were also prepared with sediments collected from the aquifer and wetland zones as means to isolate IAB. Enrichments were generated by suspending sediments (0.05 g) in 50 ml conical tubes containing (i) 20 ml dH₂O, (ii) 20 ml 10% nutrient broth (Oxoid), or (iii) 20 ml 10% nutrient broth plus 50 µg ml⁻¹ cycloheximide. I was added (as KI; Sigma) to give a final concentration of 1 mM. This resulted in a total of 24 enrichment cultures (8 sediment samples, with 3 treatments for each sediment sample). The enrichments were incubated in the dark at room temperature and agitated by tube inversion once each day. At 2, 9, and 22 weeks, 50 µl from each tube was transferred to sterile tubes containing 5 ml R2A (1/10 strength) to generate a 10⁻² dilution. Dilutions of 10⁻⁴ and 10⁻⁶ were then prepared in R2A (1/10 strength), and 25 µl of each dilution was spread on both 25% DNB-IS (IS consists of 1.2 g liter⁻¹ KI and 1g liter⁻¹ soluble starch) and 25% R2A-IS agar plates. In this manner, 29 distinct morphotypes were isolated and stored (agar plates at 4°C and glycerol stocks at -80°C) for further analysis.

2.3.2 16S rRNA gene sequencing and phylogenetic analysis

To determine the taxonomic identity of the isolates, a portion of the 16S rRNA gene was amplified and sequenced. Crude bacterial lysate was collected by placing a

small amount of isolated colonies into 10 μl Tris-EDTA (TE) buffer (pH 7.5) with sterile pipette tips. Amplification was performed using 1 μl of the crude lysate as a template in 50- μl reaction mixtures containing 5 μl 10 \times buffer, MgCl_2 (2.5 mM), the 27f primer (5'-GAGTTTGATCMTGGCTCAG-3') (10 pmol), the 1492r primer (5'-GGTTACCTTGTTACGACTT-3') (10 pmol), deoxynucleoside triphosphates (200 μM each), and 1.25 U of HotStar *Taq* DNA polymerase (Qiagen). Reactions were amplified in a PTC-100 thermocycler (MJ Research, Inc.) as follows: 95°C for 10 min and 32 cycles of 95°C for 1 min, 45°C for 45 s, and 72°C for 1 min, followed by a 7-min elongation step. PCRs were visualized on a 1.2% agarose gel to ensure amplification of a single product of the expected size. The 16S rRNA gene amplicon was purified with a MinElute PCR purification kit (Qiagen), and the concentration of the purified product was adjusted to $\sim 15 \text{ ng } \mu\text{l}^{-1}$ in dH_2O using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Sequencing of the purified PCR products was performed by the Georgia Sequencing Facility at the University of Georgia, Athens, GA.

Phylogenetic similarity of 16S rRNA sequences was first determined with BLAST searches of the GenBank (<http://www.ncbi.nlm.nih.gov/genbank/index.html>) and the RDP classifier (26), followed by maximum-likelihood reconstruction of phylogenetic trees with ARB (131).

2.3.3 Culture conditions

Two marine I-accumulating bacterial (IAB) strains, *Flexibacter aggregans* NBRC15975 and *Arenibacter troitsensis* JCM11736, were purchased from the NITE

Biological Research Center, Chibin, Japan, and used as reference strains for the I⁻ accumulation assay. The two reference strains and all microbial isolates from the F-area were grown on marine agar 2216 (Difco) and 1/4-strength R2A agar medium (EMD), respectively.

2.3.4 Iodide accumulation screening

Two methods using ¹²⁵I⁻ as a tracer were combined to determine the I⁻ accumulating ability from F-area microbes. The first used autoradiography, similar to the procedure of Amachi et al. (11). A mixture of I⁻, 0.016 μM stable I⁻ (as NaI; Sigma), and 0.80 kBq ml⁻¹ ¹²⁵I⁻ was applied evenly to R2A agar plates. Microbial isolates from the F-area were inoculated onto the plates using sterile toothpicks and incubated for 3 days in the dark at room temperature (~27°C). A small portion of microbial colonies, ~0.25 mm in diameter, was transferred with sterile toothpicks into 7-ml glass vials containing 4 ml of scintillation cocktail (Ecolume). After the colonies were vortexed for 1 min, the radioactivity of accumulated ¹²⁵I was determined by a liquid scintillation counter (Beckman Coulter LS6500) for 10 min as a preliminary screen. Colonies testing positive for ¹²⁵I accumulation were then transferred from the agar plates via a traditional plate lift technique onto a nitrocellulose membrane filter (82-mm diameter; Whatman Optitran BA-S 85). As a control for the background, liquid on the agar surface was also transferred onto the nitrocellulose membrane. The membranes were dried at room temperature for 30 min and then exposed to a maximum-sensitivity autoradiography film

(Kodak BioMax) at -20°C for 14 days in the dark. Film was processed with developer, a deionized-water stop bath, and fixer according the manufacturer's instructions (Kodak).

For the second method for assessment of iodide accumulation, microbial isolates from the F-area and the two reference marine IAB strains (the *F. aggregans* and *A. troitsensis* strains) were cultivated in 50-ml conical tubes containing 5 ml of nutrient broth and marine broth, respectively. Stable I^{-} was added to the cultures at a concentration ($0.1 \mu\text{M}$) that reflected the ambient concentration of $^{127}\text{I}^{-}$ (stable iodine) in the F-area. $^{125}\text{I}^{-}$ ($1.33 \text{ kBq liter}^{-1}$) was added to the cultures as the tracer. Incubations were performed at room temperature in the dark with continuous shaking (150 rpm). After a 24-h incubation period, a subsample ($100 \mu\text{l}$) was collected and was immediately transferred into a scintillation vial. Radioactivity of ^{125}I was determined by liquid scintillation counting for 10 min and referred to as “ ^{125}I activity in the microbial culture.” To investigate the effect of hydrogen peroxide on cellular iodide accumulation, H_2O_2 (final concentration, 5 mM) was added to a portion (4 ml) of the cell-iodide mixture after the initial 24-h incubation period, and the incubation was continued for an additional 4 to 24 h. Portions of the cell suspension ($900 \mu\text{l}$) were collected immediately after the initial 24 h of incubation (before H_2O_2 addition) or 4 or 24 h after H_2O_2 addition. The collected cell suspension was centrifuged at $16,000 \times g$ for 20 min at 4°C . The supernatant was discarded, and the cell pellet was washed 3 times and suspended in $900 \mu\text{l}$ of fresh medium (without added stable I^{-} and $^{125}\text{I}^{-}$). To determine “cell-associated ^{125}I ,” a portion ($100 \mu\text{l}$) of the washed cell suspension was measured by scintillation counting for 10 min (count errors were consistently below 20%). The I^{-} accumulating

ability (percent accumulation) of microbial cells was calculated as the ratio of “cell-associated ^{125}I ” versus “ ^{125}I activity in the microbial culture.” Cell densities in aliquots were measured at 600 nm with a spectrophotometer (Turner SP8001). Correlations of bacterial dry weight with optical density at 600 nm (OD600) for each target bacterium were obtained during different periods of exponential growth through linear regression, resulting in an r^2 value higher than 0.98 for each strain.

2.3.5 Effect of chloride on iodide-accumulating abilities

Bacteria were grown in two types of aqueous minimal media, M9 and Cl⁻-deficient M9 (M9X). M9 contained 47.75 mM Na₂HPO₄, 22.04 mM KH₂PO₄, 8.56 mM NaCl, 18.70 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 22.22 mM glucose, and 0.5% yeast extract. M9X was prepared by replacing NaCl, NH₄Cl, and CaCl₂ with the same concentrations of Na₂SO₄, (NH₄)₂SO₄, and Ca(OH)₂, respectively. Cells grown in M9 and M9X media were exposed to 2 different concentrations of stable I⁻ (0.1 and 10 μM) while maintaining the same concentration of $^{125}\text{I}^-$ (1.33 kBq liter⁻¹). Incubations were performed in the dark at room temperature with continuous shaking at 150 rpm for 24 h. H₂O₂ (final concentration, 5 mM) was then added to the microbial culture. After an additional 24-h incubation, aliquots were collected for the determination of cellular ^{125}I activity and cell density as described above.

2.3.6 Effect of pH and sodium azide on iodide-accumulating abilities

To evaluate Γ accumulation as a function of pH, IAB from the F area were grown in the M9 medium containing 0.5% yeast extract at room temperature in the dark with shaking (150 rpm). When the optical density (600 nm) of the cultures reached ~ 0.8 , cells were harvested by centrifugation ($3,500 \times g$ at 20°C for 15 min) and washed 2 times with pH-adjusted minimal medium (pH 4 to 9). The minimal medium contained 8.6 mM NaCl, 18.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2 , 22.2 mM glucose, and 50 mM KH_2PO_4 . Acetate buffer, phosphate buffer, and borate buffer were used to adjust the pH from 4 to 5, from 6 to 8, and to 9, respectively. To avoid bursting or shrinking of cells by transferring them from M9 to pH-adjusted medium, the ionic strength of pH-adjusted medium was maintained at 177 mM, which was the same ionic strength as in the M9 medium. Cell pellets were suspended in pH-adjusted minimal medium containing stable Γ (0.1 μM), ^{125}I (1.33 kBq liter $^{-1}$) and H_2O_2 (5 mM).

In order to investigate the role of heme-containing enzymes or other active cell processes on iodide accumulation, a parallel assay was performed with the addition of sodium azide (NaN_3). NaN_3 (10 mM) was added to the cell suspension to inhibit enzymatic activities before addition of Γ and H_2O_2 . Cell suspensions were then incubated at room temperature in the dark for 24 h, after which aliquots were collected for the determination of ^{125}I activity and cell density as described above.

2.3.7 Iodide desorption

A procedure similar to that of MacLean et al. (78), with slight modifications, was performed to determine if iodine desorption could occur after accumulation. After I⁻ accumulation at pH 4 to 9 (described above), cells were collected via centrifugation (16,000 × *g* for 20 min at 4°C), suspended in M9 medium (pH 7), and incubated with end-over-end shaking (150 rpm) for 2 h. Aliquots were then collected for the determination of ¹²⁵I activity and cell density as described above.

2.3.8 Statistical analyses

Analyses of variance (ANOVA) were performed to evaluate the significant differences in I⁻-accumulating abilities as a function of I⁻ concentrations, as well as the presence versus absence of chloride ions. Tukey's *post hoc* tests were used to compare means of values for I⁻-accumulating abilities from significant ($P < 0.05$) ANOVA test results. The statistical analyses were performed using SPSS 16.0 (SPSS Institute, Inc.).

2.4 Results

2.4.1 Isolation and phylogenetic analysis of F-area soil bacteria

A total of 325 aerobic microbes were directly isolated from subsurface sediments of the F-area at the Savannah River Site (Fig. 2.1). Of these isolates, 32% were cultured from sandy/clay sediments of an aquifer (sites FAW-1 and FAW-4, 13.1 to 25.9 m below the surface) and 29% and 39% were cultured from the dark, organic rich sediments of the seep zone (site FSP-07), 0.15 to 1 m and 1 to 1.2 m below the surface,

respectively. Analysis of the 16S rRNA gene revealed that the isolates were members of four phyla within the domain *Bacteria*. The majority of isolates (55%) were classified as *Proteobacteria*, followed by *Firmicutes* (20%), *Actinobacteria* (17%), and *Bacteroidetes* (8%). No obvious trends 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 or gellan gum versus noble agar) were observed (data not shown).

A yellow coloration was noted on the tubes of 2/24 enrichment cultures (8 sediment sources, with 3 enrichment conditions for each culture (see Materials and Methods) after 22 weeks of incubation in the dark. A strong I₂ smell was also detected after the caps of the discolored conical tubes were opened, indicating the transformation of I⁻ to I₂ (8). The two enrichment cultures that exhibited yellow coloration were derived from the seep zone (FSP-07) sediments collected 0.15 to 1 and 1 to 1.2 m below the surface, and both were incubated in dH₂O with 1 mM I⁻. Isolation efforts from the 24 enrichment cultures yielded 29 distinct morphotypes. Analysis of 16S rRNA gene sequences revealed that these 29 strains were members of the same 4 phyla, as the isolates recovered directly from sediments. The majority of isolates from the enrichment cultures (76%) were classified as *Proteobacteria*, followed by *Actinobacteria* (14%), *Bacteroidetes* (7%), and *Firmicutes* (3%).

2.4.2 Screening for iodide-accumulating strains

Forty-two strains, representing the phylogenetic diversity of isolates from F-area soils and enrichment cultures, were screened for their ability to accumulate $^{125}\text{I}^-$ during growth on R2A agar. Cell material from 14 strains exhibited detectable radioactivity by liquid scintillation counting following growth in the presence of $^{125}\text{I}^-$. Cell mass from colonies of these 14 isolates and liquid from the surface of the $^{125}\text{I}^-$ R2A plates was transferred to a nitrocellulose membrane, which was exposed to film for 14 days. An obvious solid black circle was observed where the film had been exposed to cell material from strain FA-30, indicating that this strain accumulated iodide relatively strongly (Fig. 2.2). Autoradiographic impressions from four of the isolates, FA-5, FA-16, FA-17B, and FA-18, showed a slightly darker region in the center of each image, whereas impressions from FA-15, FA-2B-NB, and FA-2B-B* showed a dark halo around the outer border of each of their respective images (Fig. 2.2). These distinct, strain-specific autoradiographic patterns were repeatable, suggesting that the strains accumulate or interact with $^{125}\text{I}^-$ differently.

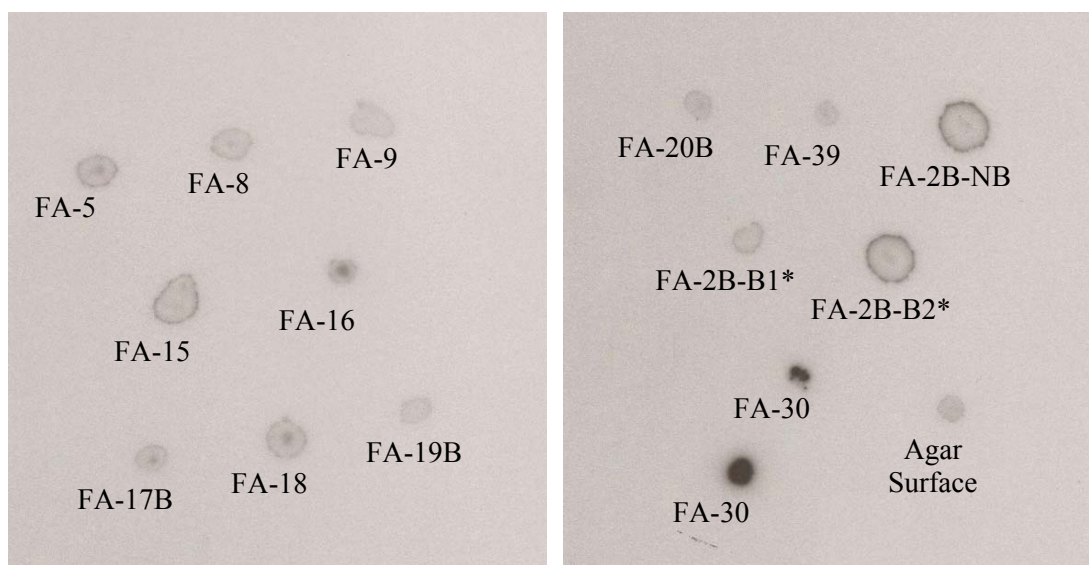


FIG. 2.2. Autoradiographic image of cell material from 14 F-area isolates (FA-30 was blotted in duplicate) that had been grown on R2A plates containing ^{125}I . The “Agar Surface” dot shows the background radioactivity from the surface of an R2A plate containing ^{125}I .

It has been reported that H_2O_2 plays a role in iodide accumulation and oxidation by marine bacteria (9, 40); thus, I^- accumulation was assessed for the same 139 strains and 2 marine IAB strains in liquid cultures as described above, except that H_2O_2 was added to the cultures either 4 or 24 h prior to harvesting of the cells. H_2O_2 addition resulted in greater I^- accumulation for FA-30 and the two marine strains and enabled the identification of two additional iodide-accumulating strains, FA-2C-B* and FA-191 (Table 2.1). In each of these strains, I^- accumulation was greater in cells that had been exposed to H_2O_2 for a longer period of time (24 versus 4 h). However, the three IAB strains isolated from the F-area, FA-30, FA-2C-B*, and FA-191, exhibited an I^- -specific accumulation that was 2 orders of magnitude less than that observed for the marine IAB strains, the *F. aggregans* and *A. troitsensis* strains (Table 2.1). Among the F-area isolates,

the only strain that exhibited an I⁻ accumulation phenotype with both the plate and the liquid culture assays was FA-30 (among the F-area isolates, FA-30 also exhibited the strongest ¹²⁵I⁻ accumulation phenotype with the use of both assays).

Table 2.1 I⁻ accumulation by F-area bacterial isolates and two known IAB isolates

| Bacterial Isolate | (-) H ₂ O ₂ | | (+) H ₂ O ₂ , 4 hrs | | (+) H ₂ O ₂ , 24 hrs | |
|------------------------------------|-----------------------------------|-------------------------------|---|------------------|--|------------------|
| | Cell activity (cpm) ^a | Accumulation (%) ^b | Cell activity (Bq/mL) | Accumulation (%) | Cell activity (Bq/mL) | Accumulation (%) |
| <i>F. aggregans</i> ^c | 726 | 2.410 | 637 | 2.096 | 2097 | 7.246 |
| <i>A. troitsensis</i> ^c | 10170 | 25.482 | 10365 | 25.609 | 17635 | 41.489 |
| FA-30 | 105 | 0.383 | 120 | 0.428 | 315 | 1.100 |
| FA-2C-B* | 33 | 0.157 | 61 | 0.296 | 121 | 0.593 |
| FA-191 | 27 | 0.099 | 40 | 0.154 | 67 | 0.251 |
| Other strains ^d | 31 (±18) | 0.143 (±0.087) | 26 (±13) | 0.125 (±0.066) | 28 (±17) | 0.134 (±0.082) |

^a Activity associated with cells washed 3 times following incubation of cells in the presence of ¹²⁵I for 24, 28, or 56 h. H₂O₂ was added after 24 h for the 28- and 56-h incubations.

^b (Number of cpm ¹²⁵I in cell pellet after incubation/number of cpm ¹²⁵I in supernatant after incubation) × 100.

^c Marine strains previously identified as IAB (9).

^d Average values ± SD for the 133 strains that did not consistently accumulate iodide.

Phylogenetic analysis of the partial 16S rRNA genes (750 to 900 bp) from FA-30, FA-2C-B*, and FA-191 revealed that these strains were members of the *Actinobacteria*, *Firmicutes*, and *Proteobacteria* phyla, respectively. FA-30 was most closely related to various *Streptomyces* and *Kitasatospora* species (16S rRNA gene similarity, ~94%), FA-2C-B* was closely related to *Bacillus mycoides* L2S8 (EU221418; 98% similarity), and FA-191 was most closely related to various *Ralstonia* and *Cupriavidus* species (97 to 98% similarity). FA-191 and FA-30 were cultured from seep zone (FSP-07) sediments, 0.15 to 1 and 1 to 1.2 m below the surface, respectively, whereas strain FA-2C-B* was

isolated from one of the I^- enrichment cultures. No IAB were found among the isolates obtained from the deeper, oligotrophic sandy/clay aquifer material.

2.4.3 Impact of chloride on iodide accumulation

To evaluate whether Cl^- present in M9 medium could inhibit iodide accumulation by the F-area IAB, cells of FA-30, FA-2C-B*, and FA-191 were incubated in medium with and without added chloride (27 mM total Cl^- concentration, added as 8.56 mM NaCl, 18.70 mM NH_4Cl , and 0.1 mM CaCl_2). Strains FA-30 and FA-2C-B* accumulated more iodide in the presence of chloride ($P > 0.05$), whereas chloride did not have a significant effect on I^- accumulation in strain FA-191 (Fig. 2.3).

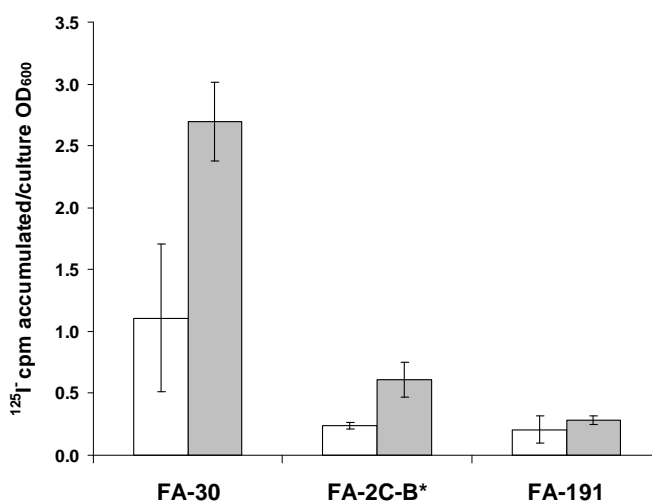


FIG. 2.3. I^- accumulation by FA-30, FA-2C-B*, and FA-191 in M9 medium containing 27 mM chloride ions (gray bars) versus that in medium without chloride salts (white bars). The experiment was performed using $0.1 \mu\text{M}$ I^- . Bars represent average amounts of I^- accumulated per cell culture biomass (OD600), and error bars show standard deviations (SD) ($n = 3$).

2.4.4 Impact of pH and sodium azide on iodide accumulation

The I⁻-accumulating ability of strains FA-30, FA-2C-B*, and FA-191 in liquid cultures grown in pH-adjusted medium (pH 4 to 9) containing iodide (0.1 μM) was evaluated. Between pH 4 and pH 6, there were significant decreases in cellular iodine content in strains FA-30 and FA-191 as the pH increased (Fig. 2.4). Above pH 6, the cellular iodine content was low, <0.1 μg/g dry cell weight, and either gradually decreased with increasing pH (FA-30) or remained at a constant low level with increasing pH (FA-191). A relationship between pH and I⁻ accumulation was not observed for FA-2C-B* (data not shown).

Strains FA-30 and FA-191 were tested in a parallel pH assay where sodium azide (NaN₃; 10 mM) was included. Both strains exhibited lower I⁻ accumulation when incubated with NaN₃, particularly below pH 6.0 (Fig. 2.4). At pH 4.0, cells of FA-191 and FA-30 accumulated 40% and 90% less I⁻, respectively, in the presence of NaN₃. At pH levels of >6.0, I⁻ accumulation was essentially unaffected by NaN₃ (it should be noted, however, that the cellular iodine content of cells incubated in medium with a pH value of >6.0 was barely above background levels).

2.4.5 Desorption of iodine from IAB

Desorption experiments were conducted with strains FA-30 and FA-191 to evaluate the nature of the interaction between I⁻ and the cells. Desorption of iodine as a function of pH was not observed in cells of FA-30 or FA-191 (only ±10% variation in

the cellular iodine content of the cells before and after the desorption step) (data not shown).

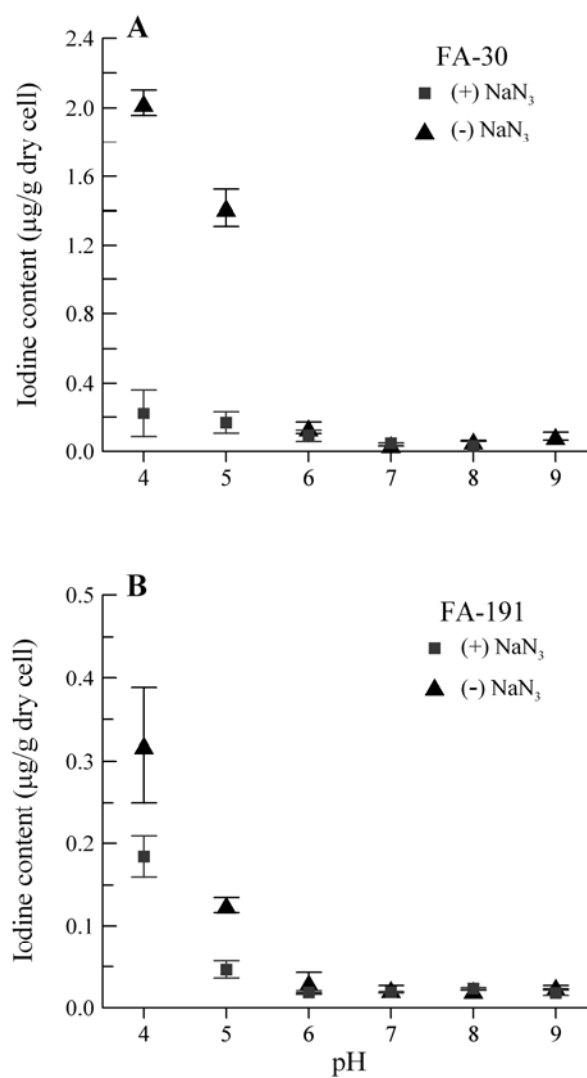


FIG. 2.4. Correlation between pH and iodide accumulation by FA-30 (A) and FA-191 (B) in the presence (squares) or absence (triangles) of sodium azide. Symbols represent average amounts of I^- accumulated per cell culture biomass ($\mu\text{g g dry cell weight}^{-1}$), and error bars show standard deviations ($n = 3$).

2.5 Discussion

Mobility of radioactive iodine in the subsurface environment is affected by the iodine's chemical speciation and interactions with soil constituents, including minerals, organic matter, and microorganisms. Based on thermodynamic principles, the main iodine species in SRS F-area groundwater and sediments should be I^- , which is thought to have the highest subsurface mobility, i.e., to be least sorbed or taken up by sediments, compared to IO_3^- and organo-iodine (84, 103, 107). However, organo-iodine has been found to contribute a significant fraction (up to 25%) of total iodine in groundwater from the F-area (107, 142). The extent to which I^- binds to or is incorporated within bacterial cells in oxic subsurface aquifers has not previously been investigated; thus, we tested the I^- accumulating ability of 139 phylogenetically distinct bacterial isolates from F-area sediments.

Three aerobic bacterial strains from the F area, FA-30, FA-2C-B*, and FA-191, were shown to accumulate I^- . I^- accumulation by these three strains was significantly different from the background value observed for 136 other strains evaluated (consistently 2 to 10 times higher), and in the case of FA-30, this result was validated by two different approaches, (i) autoradiography of cell material grown on $^{125}I^-$ agar plates and (ii) liquid scintillation analysis of washed cell material that had been grown in liquid medium containing $^{125}I^-$. Compared to that of IAB isolated from marine sources, however, the I^- -accumulating capacity of the F-area strains was quite small. Amachi et al. (9) reported accumulation of 80 to 90% of total I^- by various marine strains, including *F. aggregans* NBRC15975 and *A. troitsensis* JCM11736, when these strains were

incubated in the presence of 0.1 μM Γ . Under our experimental conditions (i.e., much more rigorous cell washing and higher pH [7.0 versus 6.0] than those used by Amachi et al.), *F. aggregans* and *A. troitsensis* accumulated 2 to 50% total Γ (0.1 μM), whereas F-area strains FA-30, FA-2C-B*, and FA-191 accumulated 0.2 to 1.5% of the total Γ (0.1 μM).

Another difference between the IAB isolated from F-area sediments and the IAB previously identified from marine sediments (9) is that the latter microorganisms were classified exclusively within the *Flavobacteriaceae* family of the *Bacteroidetes*, whereas the F-area IAB represented three phyla, *Actinobacteria* (FA-30, most closely related to *Streptomyces* and *Kitasatospora* spp.), *Firmicutes* (FA-2C-B*, a putative *Bacillus* sp.), and *Betaproteobacteria* (FA-191, closely related to *Ralstonia/Cupriavidus* spp.). As these three phyla are often dominant members of terrestrial soil microbial communities (they comprise >90% of the isolates obtained from F-area sediments) whereas *Bacteroidetes* spp. are more common in marine environments, it appears that Γ accumulation is not restricted to a distinct phylogenetic lineage(s) but rather is manifested in select bacterial taxa adapted to their respective environments.

There are two mechanisms that provide the most parsimonious explanation for Γ accumulation by bacteria. One is electrostatic adsorption of Γ by positively charged functional groups, such as amines from proteins and peptides, present on the surface of the cell. For example, MacLean et al. (78) used *Bacillus subtilis* to examine Γ -bacterium adsorption through electrostatic interaction and found that it readily adsorbed Γ at pH values of <4 and that Γ could then be quickly desorbed (~2 h) by raising the pH to 7.0. In

that study, an aqueous solution of diluted HNO_3 was used as the experimental wash solution, precluding other halide ions from competing with I^- for electrostatic interaction with bacterial cells. In the present study, the three terrestrial IAB strains were incubated and maintained in a M9 minimal medium solution that contained ~ 27 mM chloride ions, which is $\sim 2.7 \times 10^5$ times higher than the iodide concentration ($0.1 \mu\text{M}$) present in the experimental assays. I^- accumulation by FA-30, FA-2CB*, and FA-191 was not inhibited by chloride ions (27 mM) (Fig. 2.3). Indeed, at low I^- concentrations ($0.1 \mu\text{M}$), I^- accumulation by FA-30 and FA-2C-B* was greater in M9 medium than in chloride-deficient M9 medium (we note that there was no difference in ionic strength between the standard M9 medium and chloride-deficient M9 medium used in this experiment). Furthermore, pH-dependent desorption tests failed to reveal reversibility of the interaction between I^- and cells of FA-30 and FA-191. These results indicate that I^- accumulation by the F-area terrestrial strains was not due to electrostatic surface adsorption when pH levels were ≥ 4.0 .

The second possible mechanism for adsorption between I^- and cellular constituents is electrophilic substitution resulting in iodination of organic molecules. Strong electrophiles such as $\text{HOI}/\text{I}_2/\text{I}_3^-$ produced by biotic or abiotic processes from I^- could attack aromatic rings or other organic moieties of the cell and replace $-\text{H}$ with $-\text{I}$ to form a stable organoiodine bond (25, 82, 114). Given the high concentration of organic matter in a bacterial culture, it is reasonable to expect that the oxidized I species, i.e., $\text{HOI}/\text{I}_2/\text{I}_3^-$, could react with it. Several observations signify that the I^- accumulation phenotype exhibited by F-area strains FA-30, FA-2C-B*, and FA-191 likely proceeds

via this mechanism. First, the Γ -accumulating abilities from FA-30, FA-2C-B*, FA-191 were all facilitated by the addition of H_2O_2 . Since H_2O_2 is a strong oxidant, Γ is readily oxidized to I_2/I_3^- without enzymatic catalysis, especially under acidic conditions. However, at the near-neutral pH values (pH 6 to 7) that were used during the screening portion of our study, H_2O_2 consistently stimulated Γ accumulation in just 5 of 141 strains tested (including the two marine strains). Thus, it is unlikely that H_2O_2 , itself acting as an oxidizing agent, transformed Γ into a highly reactive species capable of binding nonspecifically to cell material (though at lower pH values [<4 to 5], this mechanism could be more relevant). Alternatively, haloperoxidases, which are found in animals, plants (including algae), fungi, and bacteria, utilize H_2O_2 as a cosubstrate and are considered the primary enzyme system responsible for nonspecific halogenations of organic substrates in nature (22, 121, 124). NaN_3 significantly inhibited Γ accumulation in the F-area strains, implicating the involvement of an enzymatic driven process, such as an active transport system or heme haloperoxidases, which are NaN_3 sensitive (121). The F-area strains also exhibited increased Γ accumulation with decreasing pH, characteristic of many haloperoxidases, which exhibit optimal activity under acidic conditions (27, 93, 98). Although our data support electrophilic substitution or internalization rather than electrostatic adsorption to the cell surface, the precise nature and location of bacterially bound Γ in these terrestrial strains and the accumulation mechanism remain to be determined.

Background concentrations of stable I in F-area groundwater range from 10 to 100 nM, and plume concentrations of ^{129}I are typically ~ 60 pCi/liter (2.4 nM) at “hot

spots” but can reach levels of >900 pCi/liter in the organic-rich seep zone. Our results demonstrate that the majority (98%) of aerobic bacteria isolated from F-area sediments do not accumulate Γ^- (<0.2% accumulation) at ambient Γ^- concentrations (0.1 μM), and the three IAB strains that were identified accumulate less than 2% Γ^- under environmental conditions (aerobic; pH 4 to 9; 0.1 μM total Γ^-) associated with most of the F-area plume (at the center of the plume, pH values as low as 3.2 have been documented, where electrostatic adsorption of Γ^- by bacterial cells as demonstrated by MacLean et al. (78) could play a role in Γ^- transport). Our experiments were conducted with dense cell cultures ($\geq 1 \times 10^9$ cells ml^{-1}), whereas cell numbers in groundwater from the sandy/clay aquifer of the F-area are lower than 1×10^4 cells ml^{-1} (data not shown). At these cell concentrations, cellular accumulation of Γ^- would be exceedingly low. Furthermore, each of the IAB strains identified in this study was isolated from the seep zone sediments, not the sandy/clay aquifer material. These results indicate that IAB are most likely not responsible for the high fraction of organo-iodine (up to 25% of total iodine) that has been measured in groundwater of the F-area subsurface aquifer above the seep zone (107, 142). However, our ongoing experiments with Γ^- -oxidizing bacteria from F-area soils thus far indicate that this pathway for organo-iodine formation is more significant.

Our multifaceted, carefully controlled approach allowed us to definitively identify an IAB phenotype that was 1 to 2 orders of magnitude less, in terms of specific Γ^- accumulation activity, than that previously established for bacteria from very different environments (i.e., brines). This is important for several reasons. ^{129}I has an extremely

long half-life (~16 million years), and its production is increasing each year. The DOE and other entities are tasked with modeling the long-term (centuries to thousands of years) fate and transport of ^{129}I . Over decades or centuries, I^- accumulated by bacterial cells and covalently attached to cellular constituents could conceivably make its way to the organo-iodine pool through cell lysis and possible incorporation into more-refractory organic soil material (e.g., humic or fulvic acids). Even when bacteria, whose biomass typically accounts for 1% or less of sedimentary organic matter (such as the F-area seep zone sediments) (63), incorporate less than 2% of iodine into their cells, this process could contribute appreciably to the organo-iodine pool over the long term. Similar mechanisms have been proposed to explain chloride retention in forest soils and peat bogs over decades to centuries (20). Carefully controlled, long-term column studies are needed to examine the extent that IAB, such as those identified in this study, affect ^{129}I speciation and mobility in F-area seep zone sediments. Finally, uncultivated bacterial species yet to be discovered from the F-area or fungi may be capable of much higher levels of iodide accumulation (19). We are currently examining that possibility through a microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) approach (74).

CHAPTER III
**BACTERIAL PRODUCTION OF ORGANIC ACIDS ENHANCES H₂O₂-
DEPENDENT IODIDE OXIDATION***

3.1 Overview

In order to develop an understanding of the role that microorganisms play in the transport of ¹²⁹I in soil-water systems, bacteria isolated from subsurface sediments were assessed for iodide oxidizing activity. Spent liquid medium from 27/84 bacterial cultures enhanced iodide oxidation 2-10 fold in the presence of H₂O₂. Organic acids secreted by the bacteria were found to enhance iodide oxidation by 1) lowering the pH of the spent medium, and 2) reacting with H₂O₂ to form peroxy carboxylic acids, which are extremely strong oxidizing agents. H₂O₂-dependent iodide oxidation increased exponentially from 8.4 to 825.9 μM with decreasing pH from 9 to 4. Organic acids, with ≥2 carboxy groups, enhanced H₂O₂-dependent iodide oxidation (1.5 – 15 fold) as a function of increasing pH above pH 6.0, but had no effect at pH ≤ 5.0. The results indicate that, as pH decreases (≤ 5.0), increasing H₂O₂ hydrolysis is the driving force behind iodide oxidation. However, ≥ pH 6.0, spontaneous decomposition of peroxy carboxylic acids, generated from H₂O₂ and organic acids, contributes significantly to iodide oxidation. The results reveal an indirect microbial mechanism, organic acid

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secretion coupled to H_2O_2 production, that could enhance iodide oxidation and organo-iodine formation in soils and sediments.

3.2 Introduction

With the development of nuclear power, large inventories of fission products have been produced and, in some cases, released into the environment. ^{129}I , one of the major fission products, has been given relatively little attention despite its potential toxicity to man, high perceived mobility, radioactive longevity ($t_{1/2} = 1.6 \times 10^7$ yrs), and growing inventories in engineered disposal facilities and in the environment. For example, 146 Ci of ^{129}I was deposited in soils at two US Department of Energy (DOE) sites, the Hanford Site and Savannah River Site (SRS), and has been detected in mixed waste plumes at these sites well above (10 ~1000x) the federally regulated Maximum Contaminant Level (MCL) of 1 pCi L^{-1} (58, 90). As a consequence, ^{129}I has been identified as a key risk driver at both sites. It is therefore critical to understand the environmental behavior of ^{129}I in order to rigorously assess its storage and disposal options.

The mobility of ^{129}I in water-soil systems is affected by its chemical speciation and interactions with soil constituents, including minerals and organic matter. Major iodine species in water-soil systems includes iodide (I^-), iodate (IO_3^-) and organo-iodine (OI). Iodide has a lower soil affinity, with a distribution coefficient (K_d) of $\sim 1 \text{ L}^3 \text{ kg}^{-1}$, than that of iodate ($K_d \sim 1000 \text{ L}^3 \text{ kg}^{-1}$) under oxic conditions (13, 39, 54, 59, 107). Both iodide oxidation and iodate reduction can result in the production of several reactive

intermediates, including iodine (I_2), hypiodous acid (HOI) and triiodide (I_3^-). These intermediates in turn can be taken up by soil organic matter to form OI through iodination of aromatic moieties (82, 104, 139), adsorbed onto mineral surfaces (58-59), and volatilized into the atmosphere (6). All these processes would influence the mobility of ^{129}I . To date, several studies have demonstrated that organic-rich soils have the tendency to serve as a ^{129}I sink, i.e. immobilize ^{129}I , mainly through the iodination process (84, 138, 141). In addition, the molecular weight of OI in soils can play a major role in the mobility of iodine, whereby the higher molecular weight OI would tend to become a sink for ^{129}I , whereas lower molecular weight colloidal or truly dissolved OI species can leach out and migrate in groundwater (39, 107, 137-138, 142).

In theory, iodide is expected to be the dominant species in most water-soil systems due to its stability over the typical range of Eh and pH found in these environments (94). Oxidation of iodide via a single-step electron transfer is thermodynamically unfavorable, unless a strong oxidant is available (77). Abiotic oxidants (e.g. MnO_2 , Fe_2O_3) have been demonstrated to facilitate iodide oxidation; however, the importance of their reactions is limited under acidic conditions ($pH \leq 5.0$) (36-37, 42, 137). Microbial activity has long been linked to iodide oxidation and soil OI formation (6, 84-85). These studies have demonstrated that soils or sediments treated with heat, γ -irradiation, or prokaryotic antibiotics exhibit a considerable reduction in iodine binding capacity onto soils (6, 85), whereas inoculation of sterilized soil with fresh soil can restore the iodine-organic matter binding potential (84). Mechanisms of microbial activities that could immobilize ^{129}I include bacterial accumulation and

extracellular oxidation, which would also lead to iodination of soil organic matter. Indeed, a growing body of literature has implicated microbial oxidases, perhydrolases and peroxidases in the formation of halogenated organic matter in soils (6-7, 10, 20, 84-85, 89). Both iodide oxidation and accumulation activity have been identified in marine and brine water bacteria; however, using similar approaches, efforts to demonstrate iodine immobilization by cellular uptake or oxidation by terrestrial bacteria was not successful (7, 10).

In soils and sediments of the SRS F-area, based on the observed ranges of pH (3.2 ~ 6.8) and Eh (360 ~ 654 mV), iodide is expected to be the predominant iodine species (90). However, $\geq 50\%$ of iodine species is present in an oxidized form (i.e. IO_3^- and OI) in the groundwater of the SRS F-area, indicating that iodide oxidation occurred (90, 107, 142). We previously demonstrated that bacteria isolated from SRS F-area subsurface sediments irreversibly bound iodide, but only a small fraction (0.2~2%) was accumulated at ambient iodine concentrations (0.1 μM) (72). It was concluded that bacterial iodide accumulation was not responsible for the high fraction of OI detected in F-area groundwater. Interestingly, enhanced iodide oxidation was observed in the spent medium from a number of SRS F-area bacterial isolates in the presence of H_2O_2 . In water-soil systems, H_2O_2 can originate from common metabolic processes of fungi and bacteria under aerobic conditions and freely diffuse across the cell membrane into the surrounding milieu where the concentration can reach up to 2~15 μM (44, 46). Moreover, studies have demonstrated that H_2O_2 produced by photochemical or biological reactions in surface soils could be transported down-gradient to the subsurface

environment (29, 125). This result leads us to believe that a number of terrestrial bacteria are capable of influencing the chemical behavior of ^{129}I via extracellular H_2O_2 -dependent reactions. In this study, we report that organic acids secreted by SRS F-area bacteria can enhance H_2O_2 -dependent iodide oxidation.

3.3 Materials and methods

3.3.1 Culture conditions and preparation of spent medium and crude cell extracts

Bacteria isolated from soils in the F-area of the SRS (72) were cultured aerobically by shaking (150 rpm) in liquid 1X M9GY medium (M9 minimal salt medium enriched with 0.4% glucose and 0.4% yeast extract, pH 7.4 ± 0.2) at 30 °C in the dark. When the optical density (OD_{600}) of the cultures reached ~ 1.2 (late exponential phase), bacterial cells were harvested by centrifugation ($3200 \times g$, 20 °C, 15 min), then washed and suspended in fresh 1X M9G medium. After 24 h incubation at 30 °C in the dark with shaking (150 rpm), spent M9G medium and cell pellets were separated by centrifugation ($3200 \times g$, 20 °C, 15 min). The spent medium was then directly used for the assays detailed below. To obtain crude cell extracts, cell pellets were suspended with fresh 0.1X M9G medium and then disrupted by 5 cycles of 20 s sonication (Misonix XL2000, 100W) at 20 kHz and 40 s cooling in an ice bath. Complete lysis of bacterial cells was confirmed through microscopy. Crude cell extracts were centrifuged to remove cell debris.

3.3.2 Determination of H₂O₂-dependent iodide oxidizing activity

A method for monitoring triiodide (I₃⁻) formation (10) was applied to determine iodide oxidation rates. Reaction mixtures for the determination of I₃⁻ formation were prepared in 96-well plates (Polystyrene, Greiner UV-Star) and consisted of: 150 µL spent media or crude cell extract, 100 mM phosphate buffer (pH 7.2), 10 mM KI and 5 mM H₂O₂ in 300 µL total volume. Controls (i.e., reactions without KI or spent medium/crude cell extract) were also carried out to differentiate abiotic from biotic reactions. Formation of I₃⁻ was monitored spectrophotometrically at 353 nm after incubation at 30 °C for 20 min. The increase in absorbance at 353 nm was converted to the concentration of I₃⁻ by applying Beer-Lambert's law with a molar extinction coefficient (ϵ) of 25.5 mM⁻¹ cm⁻¹ (10). In this study, the amount of I₃⁻ formed, in µM, from 150 µl of spent medium or crude cell extract was used to express the iodide oxidizing capacity.

3.3.3 16S rRNA gene sequencing and phylogenetic analysis

For phylogenetic analysis of SRS bacteria capable of oxidizing iodide extracellularly, 16S rRNA gene was sequenced as previously described (72). Briefly, genomic DNA was extracted by Tris-EDTA buffer (pH 7.5). Amplification by polymerase chain reaction (PCR) of the 16S rRNA was performed by using bacterial 27f primer (5'-GAGTTTGATCMTGGCTCAG-3') and the 1492r primer (5'-GGTTACCTTGTTACGACTT-3') and followed with a purification procedure (MinElute PCR purification kit, Qiagen). Sequencing of purified PCR products 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 (26), and maximum likelihood reconstruction of phylogenetic trees with ARB (131).

3.3.4 Correlation of peroxidase and iodide oxidation activities

To evaluate the correlation of H₂O₂-dependent iodide oxidation and peroxidase activities, 1 – 5 mL of spent medium was fractionated by centrifugal ultrafiltration with molecular weight cut-offs of 3, 10, 30, 50 and 100 kDa (AmiconUltra, Millipore). Polysulfonate standards (40 and 100 kDa, Sigma) were used as ultrafiltration controls. The retentate was washed with 300 µL of fresh 1X M9G at least 3 times and suspended to 1 mL with 1X M9G. The retentates and filtrates of each fraction were analyzed to determine H₂O₂-dependent iodide oxidation rates using the I₃⁻ formation method. The fraction with the highest iodide oxidizing activity was also analyzed for general peroxidase activity using 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS, Sigma) as the substrate. The assay mixture contained 1.3 mM ABTS, 150 µL retentate or filtrate sample and 5 mM H₂O₂ in 100 mM phosphate buffer (pH 7.2). Oxidized ABTS was monitored spectrophotometrically at 420 nm at 30 °C over 30 min intervals for 2 h. The increase in absorbance at 420 nm was converted to the concentration of oxidized ABTS by applying Beer-Lambert's law with ϵ of 36 mM⁻¹ cm⁻¹ (92).

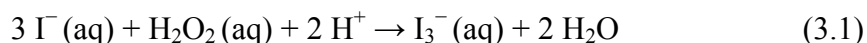
3.3.5 Quantification of organic acid concentration

Spent medium was passed through a 0.22 μm syringe filter (cellulose acetate, VWR) and injected (50 μL) into an ion chromatograph equipped with an IonPac AS11-HC column set (analytical column, 4 \times 250 mm; guard column, 4 \times 50 mm) and a conductivity detector from Dionex. The anionic components in the samples were eluted with a gradient program of NaOH flowing at 1.5 mL min^{-1} . The gradient program of NaOH was set as follows: 1 mM NaOH for 6 min, NaOH to 15 mM over 7 min, NaOH to 30 mM over 15 min, and then NaOH was maintained at 30 mM for 7 min. The eluted anionic components were quantified based on the standard curve of the following organic acids: lactic, pyruvic, succinic, maleic, oxalic, and citric acids.

3.4 Results and discussion

3.4.1 Bacterial enhancement of H_2O_2 -dependent iodide oxidation

Iodide can be oxidized directly by reacting with H_2O_2 (Equation 3.1).



In this study, increased H_2O_2 -dependent iodide oxidation was observed in liquid cultures of numerous bacterial strains isolated from F-area of the SRS. Analysis of spent medium and crude cell extracts localized the activity to the extracellular medium (Table 3.1). It should be noted that components of cell extracts could hamper H_2O_2 -dependent iodide oxidation (e.g. intracellular catalases and/or peroxidases could rapidly deplete H_2O_2). Spent medium from 84 SRS bacteria was subsequently screened for iodide oxidizing

activity in the presence of H_2O_2 , and enhancement occurred with 27 of the strains. These 27 positive strains were members of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla. No obvious trends relating the phylogenic affiliation of these bacteria to their capacity for H_2O_2 -dependent iodide oxidation were observed (data not shown). In M9G medium without cells, $38 \mu\text{M I}_3^-$ was formed from 10 mM iodide and 5 mM H_2O_2 over a 20 min incubation period. In contrast, $342 \pm 4 \mu\text{M I}_3^-$ accumulated in spent medium from strain FA-130 (phyla *Firmicutes*) containing the same concentrations of iodide and H_2O_2 (Table 3.1). These results indicate that a component(s) commonly secreted by SRS bacteria can accelerate iodide oxidation by reacting with H_2O_2 or using H_2O_2 as an electron acceptor. To characterize these reactive component(s), 10 SRS strains exhibiting enhanced H_2O_2 -dependent iodide oxidation capacity, $142 \sim 342 \mu\text{M I}_3^-$ produced from 10 mM iodide and 5mM H_2O_2 over 20 min, were selected for further experiments (Table 3.1).

Initially, we hypothesized that the reactive component(s) responsible for enhanced iodide oxidation was a haloperoxidase or other, non-specific peroxidase. Peroxidases are commonly found in soil bacteria (92), and haloperoxidases have been implicated in halogenation of natural organic matter (20, 89). However, using molecular-size fractionation, the reactive component(s) secreted by SRS bacteria was found to be smaller than 3 kDa (data not shown), and peroxidase-like activity (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid oxidation assay, (92)) was not detected (data not shown). Furthermore, the spent medium of three different strains exhibited the same level of enhanced triiodide formation before and after boiling (15 min). These results

indicated that enzymatic activity was not directly responsible for the enhanced H₂O₂-dependent iodide oxidation detected in spent medium from SRS bacteria.

Table 3.1. Enhancement of H₂O₂-dependent iodide oxidation by spent medium and crude cell extracts of various bacterial cultures and the pH values and carboxylic acid concentrations in the spent medium

| Bacteria | Phylogenetic affiliation | | I ₃ ⁻ formation (μM) ^a | | pH | Carboxylic anions (mM) ^b | | | | | |
|------------------|--------------------------|--|---|--------------------|------|-------------------------------------|------|------|-----|------|-----|
| | Accession no. | Most closely related organism in GenBank database (% similarity) | Spent medium | Crude cell extract | | Lac/Ace | Pyr | Suc | Mal | Oxa | Cit |
| M9G ^c | | | 38 ± 0 | 38 ± 0 | 7.45 | > 5.55 | ND | ND | ND | ND | ND |
| FA-86 | JQ765450 | <i>Paenibacillus</i> sp. HGF7 (83%) | 250 ± 2 | <DL | 6.24 | > 5.55 | 0.18 | 1.68 | ND | 0.06 | ND |
| FA-121 | JQ765444 | <i>Bacillus cereus</i> Rock3-44 (99%) | 180 ± 6 | <DL | 6.43 | > 5.55 | 0.11 | 2.13 | ND | 0.00 | ND |
| FA-130 | JQ765447 | <i>Bacillus anthracis</i> CNEVA-9066 (95%) | 342 ± 4 | <DL | 6.04 | > 5.55 | 0.11 | 1.81 | ND | 0.34 | ND |
| FA-132 | JQ765446 | <i>Bacillus thuringiensis</i> IBL 4222 (91%) | 269 ± 11 | <DL | 6.28 | > 5.55 | 0.08 | 1.85 | ND | 0.11 | ND |
| FA-139 | JQ765445 | <i>Burkholderia phytofirmans</i> PsJN (98%) | 196 ± 2 | <DL | 6.42 | > 5.55 | 0.14 | 1.40 | ND | 0.06 | ND |
| FA-149 | JQ765451 | <i>Bacillus cereus</i> G9842 (83%) | 247 ± 5 | <DL | 6.28 | > 5.55 | 0.07 | 1.72 | ND | 0.25 | ND |
| FA-151 | JQ765449 | <i>Brevibacillus brevis</i> NBRC 100599 (94%) | 337 ± 27 | <DL | 6.11 | > 5.55 | 0.08 | 2.34 | ND | 0.46 | ND |
| FA-152 | JQ765448 | <i>Bacillus cereus</i> 03BB108 (83%) | 306 ± 8 | <DL | 6.13 | > 5.55 | 0.09 | 2.12 | ND | 0.17 | ND |
| FA-183B | JQ765442 | <i>Bacillus thuringiensis</i> IBL 4222 (98%) | 173 ± 5 | <DL | 6.60 | > 5.55 | 0.29 | 1.25 | ND | 0.06 | ND |
| FA-2CB* | JQ765443 | <i>Bacillus cereus</i> Rock3-44 (98%) | 143 ± 13 | <DL | 6.50 | > 5.55 | 0.42 | 1.32 | ND | 0.04 | ND |

^aThe standard deviation is derived from triplicate assays. <DL, below detection limit.

^bAbbreviations of organic acids: Lac/Ace, lactate/acetate; Pyr, pyruvate; Suc, succinate; Mal, maleate; Oxa, oxalate; Cit, citrate. ND, not detected.

^cM9G medium without cells.

3.4.2. Influence of pH on H₂O₂-dependent iodide oxidation

Changes in pH can also influence H₂O₂-dependent iodide oxidation according to the reaction shown in Equation (3.1). To evaluate the effect of pH on H₂O₂-dependent iodide oxidation in M9G medium, I₃⁻ formation was measured as a function of pH. The

degree of I_3^- formation exponentially increased from 8 ± 2 to $826 \pm 73 \mu\text{M}$ with decreasing pH from 9 to 4 (Fig 3.1A). The pH values of the spent M9G medium from the SRS bacterial cultures ranged from 6.04-6.60, which is substantially lower than the pH (7.45) of fresh M9G medium (Table 3.1). A theoretical iodide oxidation rate, factoring in the effect of pH, was estimated for each SRS spent medium listed in Table 1 using the equation shown in Fig 1A. These calculations indicate that the bacterial-mediated decrease in pH value would have provided an appreciable enhancement of H_2O_2 -dependent iodide oxidation (Fig 3.1B). However, the estimated rates of iodide oxidation were still significantly lower (31~58 %) than the experimentally determined values (Fig 3.1B), indicating additional factors were involved.

3.4.3 Organic acids enhance H_2O_2 -dependent iodide oxidation

Four anions of organic acids, including lactate, pyruvate, succinate and oxalate anions, were commonly detected in the <3 kDa fraction of the spent medium from SRS cultures (Table 3.1). In each case, high concentrations of lactate (> 5.55 mM) were observed, which could contribute substantially to the pH decrease of the bacterial culture medium. The high concentration of lactate interfered with the quantification of acetate; thus, lactate and acetate concentrations were shown as a single value using lactate as the standard.

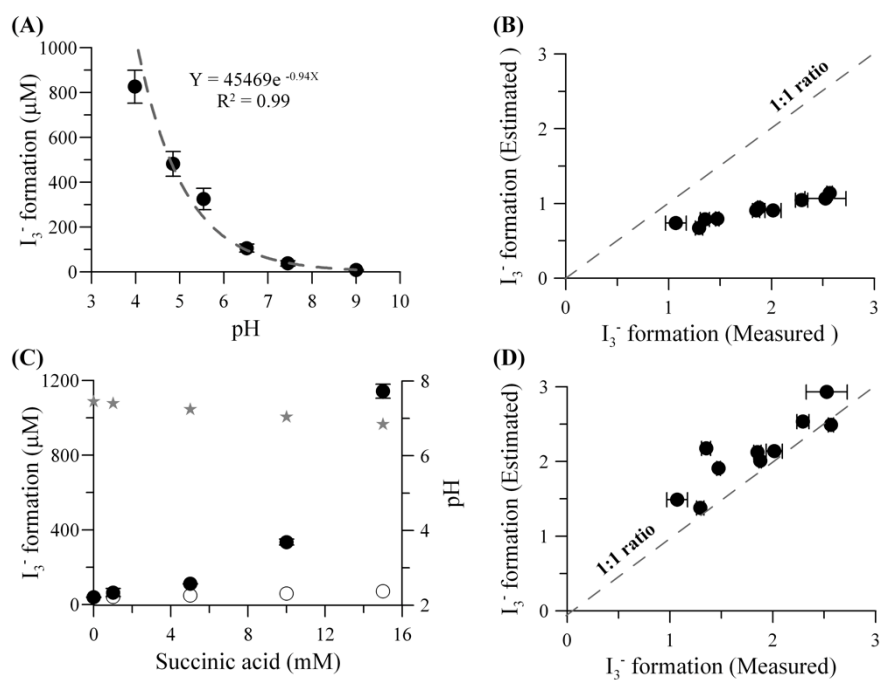
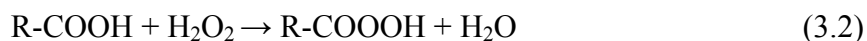


FIG. 3.1. (A) Relationship between pH and H_2O_2 -dependent iodide oxidation in M9G medium. (B) Correlation between the measured rate of H_2O_2 -dependent iodide oxidation and that estimated from pH effects alone in spent medium from 10 SRS bacterial isolates. (C) Difference in the measured rate (solid circles) of succinic acid enhanced H_2O_2 -dependent iodide oxidation and the rate estimated to be due to pH effects alone (hollow circles). The pH values of the succinic acid-amended M9G medium are indicated with stars. (D) Correlation between the measured rate of H_2O_2 -dependent iodide oxidation and the rate estimated from the combined action of pH (equation from Fig. 1A) and organic acids (equations from Figure 2). Error bars in each figure represent one standard deviation ($n = 3$).

Organic acids can acidify aqueous solutions by dissociation of the hydrogen ion from the carboxyl group. In order to differentiate pH-related effects from other mechanisms that could be associated with the observed enhancement of iodide oxidation, rates of I_3^- formation were measured in M9G medium containing pure succinic acid (the predominant dicarboxylic acid detected in the bacterial cultures; Table 3.1) ranging in concentration from 0-15 mM, then compared to the expected rates based on pH change alone using the equation shown in Fig 3.1A. As shown in Fig 3.1C, when the succinic acid concentration increased from 0 to 15 mM, the pH value of the M9G

medium decreased from 7.45 to 6.65. This discrepancy in pH (0.8 units) was estimated to enhance I_3^- accumulation from 38 μM in M9G medium to 61 μM , an increase of <2 fold over the time course of the experiment. Yet, I_3^- concentration in the presence of 15 mM succinic acid was measured as $323 \pm 19 \mu\text{M}$, an increase of ~ 10 fold (Fig 3.1C). These results further demonstrate that organic acid enhanced H_2O_2 -dependent iodide oxidation is not simply a function of pH change.

Next, rates of I_3^- formation were measured in reaction mixtures buffered at a constant pH (pH 6.0) containing M9G medium and 0 – 20 mM of select organic acids (Fig 3.2). Enhanced H_2O_2 -dependent iodide oxidation was detected in the reaction mixtures containing citric, maleic, oxalic or succinic acid, but not acetic, lactic or pyruvic acid (Fig 3.2). Moreover, the H_2O_2 -dependent iodide oxidation capacity exhibited a “second order correlation” with respect to the concentration of citric, succinic, maleic or oxalic acid ($R^2 \geq 0.995$). The most plausible mechanism that can explain the enhanced capacity of iodide oxidation by organic acids in the presence of H_2O_2 is the formation of peroxy carboxylic acid (Equation 3.2).



Peroxy carboxylic acids are powerful oxidants that could readily oxidize I^- . Similar to H_2O_2 , their instability in aqueous solution leads to a spontaneous decomposition, whereby 2 electrons are consumed by each peroxy carboxyl group ($-\text{COOOH}$) (Equation 3.3) (16, 18, 35).



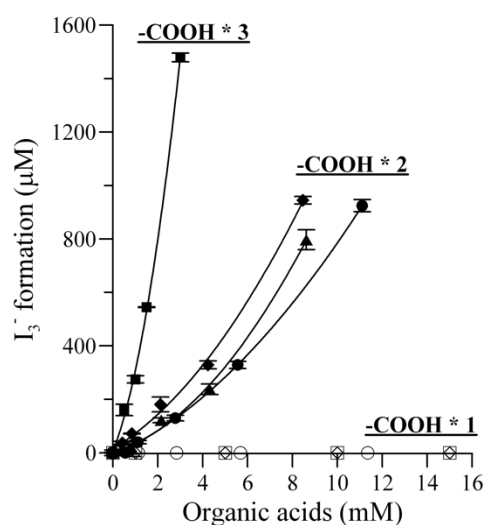


FIG 3.2 Correlation between I_3^- formation and organic acid concentration. Reactions were buffered at pH 6.0 and contained citric (solid rectangles), succinic (solid diamonds), oxalic (solid circles), maleic (solid triangles), acetic (hollow diamonds), lactic (hollow rectangles), or pyruvic (hollow circles) acid in M9G medium with 5 mM H_2O_2 . Each line expressed a second order correlation between triiodide formation and organic acid concentration with $R^2 \geq 0.995$ (citric acid, $Y=95.1X^2+205.6X+7.3$; succinic acid, $Y=7.0X^2+50.0X+15.8$; maleic acid, $Y=7.8X^2+25.0X+2.6$, oxalic acid, $Y=4.2X^2+37.5X+8.9$). Error bars represent one standard deviation ($n = 3$).

The data also revealed a correlation between the degree of enhanced H_2O_2 -dependent iodide oxidization and the number of carboxyl groups (-COOH) on a given organic acid (Fig 3.2). The highest iodide oxidizing capacity ($479 \pm 16 \mu M I_3^-$ produced) was observed in reaction mixtures with citric acid, which contains 3 carboxyl groups. Reactions containing succinic, maleic or oxalic acid, all dicarboxylic acids, produced 224, 151, and 139 $\mu M I_3^-$, respectively. H_2O_2 -dependent iodide oxidation was not enhanced in reactions containing monovalent organic acids (acetic, lactic or pyruvic acid; I_3^- formation in M9G medium, with or without these monocarboxylic acids, was 38 μM). These results imply that the H_2O_2 -dependent iodide oxidation enhancement potential of a given organic acid is dependent on the number of available sites for peroxy

carboxyl group formation. The lack of H₂O₂-dependent iodide oxidation enhancement by monovalent organic acids, which could only produce one peroxy carboxyl group, does not imply that no iodide oxidation occurred. It is more likely related to the fact that the spontaneous decomposition of one peroxy carboxyl group consumed 2 electrons, the same electrons required for the hydrolysis of one H₂O₂.

As discussed earlier, 31 to 58 % of the H₂O₂-dependent iodide oxidation capacity measured in the spent medium of SRS bacterial cultures was not accounted for when pH change was the only variable considered (Fig 3.1B). To determine if this differential could be explained by the action of peroxy carboxylic acids, the estimated H₂O₂-dependent iodide oxidation capacities were recalculated by summing (i) the influence of peroxy carboxylic acids, using the regressions derived from Fig 3.2, and (ii) the influence of pH change, using the equation from Fig 3.1A. For example, the estimated I₃⁻ formation for strain FA-86, which secreted 1.68 mM succinate and 0.06 mM oxalate and lowered the pH value from 7.45 to 6.24 (Table 3.1), was 248 μM using the following equations:

$$1) 7.00 \times (1.68)^2 + 49.99 \times (1.68) + 15.79 = 119.87 \mu\text{M from succinate (Fig 3.2)}$$

$$2) 4.19 \times (0.06)^2 + 37.51 \times (0.06) - 8.93 = 2.34 \mu\text{M from oxalate (Fig 3.2)}$$

$$3) 45469 \times e^{(-0.94 \times 6.24)} = 125.74 \mu\text{M from the decrease in pH value (Fig 3.1A)}$$

After performing this normalization, the ratio of the estimated H₂O₂-dependent iodide oxidation capacities to the measured values approximated 1 (Fig 3.1D). This result demonstrates that the enhanced H₂O₂-dependent iodide oxidation from SRS bacterial

strains could be fully accounted for by considering pH and the concentration of dicarboxylic acids in the mixture.

3.4.4 Relevance of organic acids influencing ^{129}I mobility in natural water-soil systems

Several reasons lead us to believe that iodide oxidation by peroxy carboxylic acids may be relevant in the F-area subsurface. First, although organic acid concentrations in SRS F-area groundwater or sediments have not been measured, evidence from other studies suggests that organic acids are ubiquitous and abundant in soil-water systems. Numerous field observations place organic acid concentrations in pore waters and stream waters in the μM range (1, 23, 60, 119). Organic acids in soils are mainly derived from biotic processes including microbial decomposition and exudation from plant roots, bacteria, and fungi, and appreciable quantities (μM ~ mM) of organic acids can be found in the surrounding milieu of these organisms (57, 80). Although the range of organic acid concentrations evaluated in our experiments (400 μM to 10 mM) exceeded that found in the bulk phase of typical soil porewater, it was well within the range of concentrations expected for microenvironments surrounding bacterial biofilms, plant roots, and fungal hyphae (75, 80). Finally, much of the low level radioactive waste inventoried in soils and sediments at DOE sites, including F-area, contain abundant chelating agents that were used in the decontamination processes (90), of which organic acids, including citric and oxalic acid, were often principal components (99, 108).

In addition to organic acids, enhancement of iodide oxidation via the formation of peroxy carboxylic acids described in this paper requires H_2O_2 . As addressed earlier, H_2O_2 in water-soil system mainly originates from metabolic processes of fungi and bacteria whereby porewater concentration can reach up to 2-15 μM and possibly much higher at micro- or nano-scale near the microorganisms (44, 46). To determine if environmentally relevant concentrations of H_2O_2 could interact with organic acids to enhance iodide oxidation, I_3^- formation was measured in the presence of citric, succinic, or oxalic acid as a function of H_2O_2 concentration (Fig 3.3). Iodide oxidation was enhanced by organic acids in the presence of H_2O_2 at all concentrations tested. However, the degree of enhancement (I_3^- formation with organic acid versus I_3^- formation without organic acid) decreased from 2.5 – 5.7x enhancement to 1.5 – 3.0x enhancement as H_2O_2 concentrations decreased from 5 mM to 5 μM .

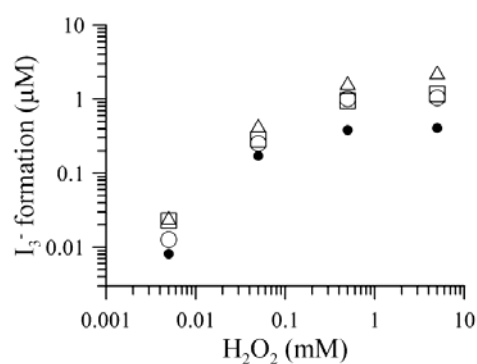


FIG. 3.3. I_3^- formation as a function of H_2O_2 concentration with 3 mM citric acid (hollow triangles), succinic acid (hollow squares), and oxalic acid (hollow circles), and without organic acids (solid circles). Organic acid standards were prepared in M9G medium (pH 7.45).

Our results implicate groundwater pH as a primary control on H₂O₂-dependent iodide oxidation (Fig 3.1A). In the SRS subsurface, bacterial activity is not likely to affect the pH of bulk soils or porewater, because the alkalinity of soil or porewater is in the mM range. However, microniches surrounding bacterial biofilms, fungi, and plant roots are often more acidic than the surrounding bulk phase due to respiratory activity and organic acid secretion (75, 80). Coupled to H₂O₂ production, these acidic microniches could serve as hotspots for iodide oxidation in water-soil systems.

Theoretically, the production rate of peroxy carboxylic acids is directly related to the availability of protons (18, 33, 35, 143). The pH of the contaminated groundwater within F-area of SRS ranges from 3.0 to 9.0 due to the acidic nature of the mixed waste plume and ongoing base injection remediation efforts (90). To determine if environmentally relevant pH ranges could influence the organic acid enhancement of H₂O₂-dependent iodide oxidation, I₃⁻ formation was measured in the presence of citric, succinic or oxalic acid as a function of pH. Overall, the rate of H₂O₂-dependent iodide oxidation decreased with increasing pH from 3.0 to 8.0 (Fig 3.4A), similar to the pattern illustrated in Fig 3.1A. However, as presented in Fig 3.4B, the contribution of peroxy carboxylic acids to H₂O₂-dependent iodide oxidation, as calculated in equation (3.4), prevails only at pH ≥ 6.0.

$$I_3^- \text{ ratio} = \frac{I_3^- \text{ produced from peroxy carboxylic acids and H}_2\text{O}_2}{I_3^- \text{ produced from H}_2\text{O}_2 \text{ hydrolysis only}} \quad (3.4)$$

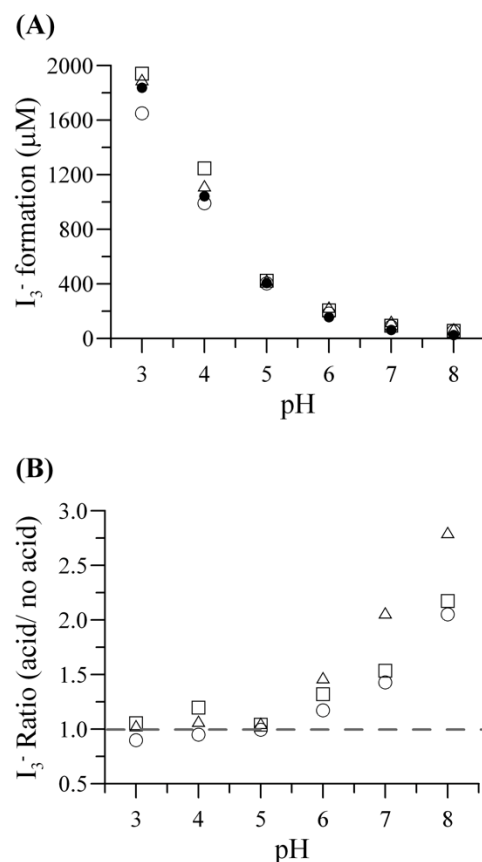


FIG. 3.4. (A) I_3^- formation as a function of pH with 3 mM citric acid (hollow triangles), succinic acid (hollow squares), and oxalic acid (hollow circles), and without organic acids (solid circles). (B) Relative I_3^- formation (i.e. ratio of I_3^- formed with the presence of 3 mM organic acid to I_3^- formed without the presence of organic acid) as a function of pH. Citric acid (hollow triangles), succinic acid (hollow squares), and oxalic acid (hollow circles) were prepared in the pH-adjusted M9G medium individually.

It is quite possible that iodide oxidation at $\text{pH} < 6.0$ can be carried out concomitantly via H_2O_2 hydrolysis and spontaneous decomposition of peroxy carboxylic acids. However, our results indicate that at low pH values, particularly $\leq \text{pH} 5.0$, increased H_2O_2 hydrolysis is the driving force behind iodide oxidation; whereas, above $\text{pH} 5.0$, spontaneous decomposition of peroxy carboxylic acids, generated from H_2O_2 and organic acids, can contribute significantly to iodide oxidation. Indeed, the dissociation constants of citric ($\text{pK}_{\text{a}3}=6.40$), succinic ($\text{pK}_{\text{a}2}=5.60$), and oxalic acids

($pK_{a2}=4.14$) are such that at higher pH values (i.e. $pH > 7.0$) full dissociation of the carboxyl groups is more likely, thus increasing the number of reactive sites to form peroxy carboxylic groups (18).

In summary, organic acids produced by terrestrial bacteria can enhance iodide oxidation in the presence of H_2O_2 . As demonstrated in this study, the magnitude of this effect can be controlled by several environmental factors such as pH, the composition and redox properties of the organic acids and the concentration of H_2O_2 . In terms of the long-term fate and transport of ^{129}I , H_2O_2 - and organic acid-dependent iodination reactions could be a relevant mechanism for immobilizing ^{129}I in the terrestrial environment.

CHAPTER IV

**IODIDE OXIDIZING ACTIVITY OF BACTERIA FROM SUBSURFACE
SEDIMENTS OF THE SAVANNAH RIVER SITE, SC, USA***

4.1 Overview

^{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 -contaminated aquifer at the Savannah River Site, SC, USA. 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

* Reproduced with permission from “Li, H.-P., R. Brinkmeyer, W. L. Jones, S. Zhang, C. Xu, Y.-F. Ho, K. A. Schwehr, D. I. Kaplan, P. H. Santschi, and C. M. Yeager. 2012. Iodide Oxidizing Activity of Bacteria from Subsurface Sediments of the Savannah River Site, SC, USA, p. 89-97. *In* M. Kawaguchi, K. Misaki, H. Sato, T. Yokokawa, T. Itai, T. M. Nguyen, J. Ono, and S. Tanabe (ed.), *Interdisciplinary Studies on Environmental Chemistry Vol. 6 - Environmental Pollution and Ecotoxicology*. Terra Scientific Publishing Company Tokyo.” Copyright © 2012 TERRAPUB.

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.

4.2 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 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) (58, 90). 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 (42, 72, 137).

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^- (82). 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 (3, 42). Numerous studies have implicated a role for microorganisms in organo-iodine formation in soils and sediments, and growing body of literature has implicated microbial oxidase, perhydrolases and peroxidases in the formation of halogenated organic matter (3, 89). Iodide oxidizing bacteria (IOB) have been isolated from environments naturally high in iodine, including marine sediments and natural gas brine waters/sediments (10), and from a marine fish aquarium where I_2 formation was implicated in a fish kill (45). IOB were also isolated from seawater, however only after enrichment for several months in the presence of 1 mM iodide (10). Using similar approaches, efforts to isolate IOB from terrestrial sources have been unsuccessful.

In a previous study (72), we isolated bacteria from SRS sediments that were capable of accumulating 0.2 to 2.0% iodide at ambient concentrations ($0.1 \mu\text{M}$), 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 organ-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.

4.3 Materials and methods

4.3.1 Isolation of bacteria

Bacteria were isolated from F-area sediments as previously described (72). 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 dH₂O, 2) 1/10 strength DNB (dilute nutrient broth), or 3) 1/10 DNB with cyclohexamide (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 above. 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 I₂-starch complexes (10).

4.3.2 16S rRNA gene sequencing and phylogenetic analysis

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene was performed as previously described (72). 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 (26), and maximum likelihood reconstruction of phylogenetic trees with ARB (131).

4.3.3 Screening for iodide oxidizing strains

Two approaches were applied to identify IOB. The first method examined the formation of triiodide (I_3^-) (10) using culture supernatants and crude cell extracts. Culture supernatants and crude cell extracts were examined. 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×g, 20°C, 15 min).

Reaction mixtures for I_3^- formation contained 150 μ L crude extract or supernatant, 177 mM phosphate buffer (pH 6.0) and 10 mM KI in 300 μ L total volume. Sample controls (no KI) and assay controls (no crude extract or 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 supernatant capable of oxidizing 1 μ mol of iodide min^{-1} .

Autoradiography was the second method used to identify IOB (72). 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.*(72)), and our preliminary observations led us to believe that this halo pattern could be an indication of iodide oxidation activity.

4.3.4 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 μ L) and suspended to 3 mL in the same medium. Iodide oxidation was measured in the retentate and filtrate using the I_3^- formation assay.

4.3.5 Iodine speciation analysis

Presumptive IOB were incubated in 1X M9G medium containing 10 μ M 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 μ M 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.* (142).

4.4 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 morphologically 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 4.1). 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 (10). None of the isolates exhibited an iodide oxidation phenotype using this assay.

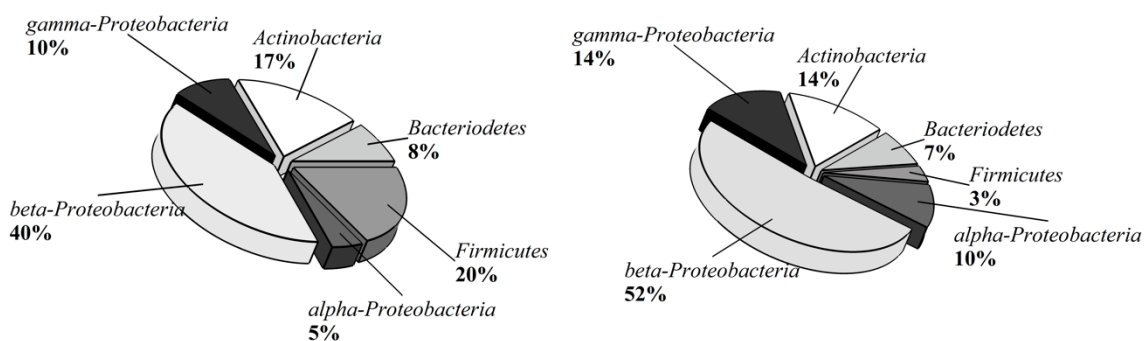


FIG. 4.1. Phylogenetic affiliation of bacteria isolated directly from 8 F-area sediment samples (n=325, left panel) and from 1 mM KI enrichment cultures (n=29, right panel).

Enrichment cultures containing high concentrations of iodide have previously been used to isolate IOB from seawater (10). Likewise, tubes from 2 of 24 cultures 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. 4.2). These two enrichment cultures contained dH₂O and iodide and had been inoculated with organic-rich sediment collected no more than 1.2 m below the 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 dH₂O, and 3) 1 mM KI (Table 4.1). 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 for oxidation of iodide under these conditions (Table 4.1).

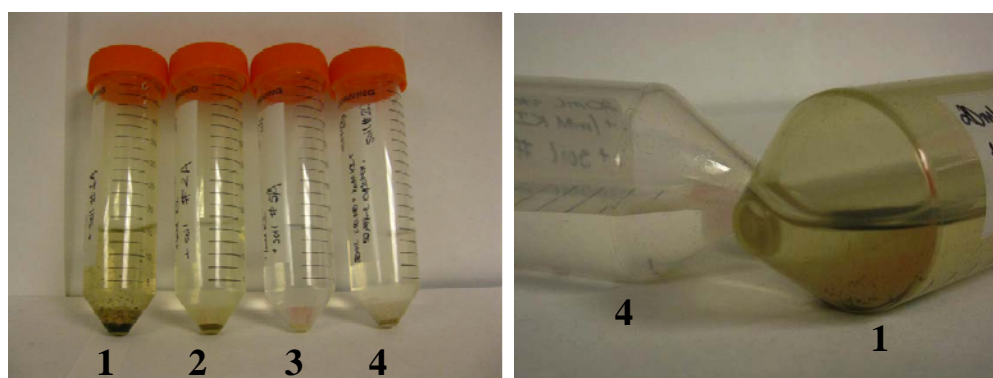


FIG. 4.2. Volatile iodine formation in enrichment cultures. Yellow discoloration was observed in 2 out of 24 enrichment culture tubes (tubes 1 and 2 shown here).

Table 4.1. Iodine species distribution in enrichment culture supernatant

| Enrichment Culture ^a | Iodine Speciation (%) | | |
|---------------------------------|-------------------------|-----------|---------------|
| | Iodide | Iodate | Organo-iodine |
| 1A | 23.1 ± 0.7 ^b | 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 |

^aEnrichment 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, dH₂O and were incubated 3 months; the same sediment samples were sterilized by autoclaving for “1A autoclaved” and “2A autoclaved”.

^b Values represent means from duplicate experiments ± standard deviations.

Bacterial isolates (n=29) were 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 I₂, 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 (64), 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 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 4.2).

Table 4.2. Summary of iodide-oxidizing activity screening among F-area bacteria

| Description | I_3^- assay ^a | Radiography assay ^b |
|--|--|--------------------------------|
| <u>IOB Screening</u> | | |
| (1) Activity in background control (mU mL ⁻¹) | 0.000 ± 0.009 ^c | no halo |
| (2) # of positive strains | 36 (5) ^d | 48 (11) ^d |
| (3) Activity range (mU mL ⁻¹) | 0.006 ± 0.005 to 0.076 ± 0.049 ^c | NA |
| <u>Iodine speciation analysis</u> | | |
| (4) # of strains examined from (2) | 28 (5) ^d | 46 (11) ^d |
| (5) # of strains exhibiting >10% I^- oxidation | 23 (5) ^d | 36 (11) ^d |
| (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 |

^aFor the I_3^- production assay 1 U was defined as 1 μmol I_3^- min⁻¹

^bFor the radiography assay, cell material grown in the presence of ¹²⁵I that exhibited a “halo” when exposed to radiography film was considered IOB (+) (see Li *et al.* 2011, Fig. 2, FA-2B-B2* for the example of “halo”)

^cAverage values ± SD for triplicate experiments

^dNumbers in parentheses indicated the # of positive strains were isolated from enrichment cultures

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

initial 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 4.1). The iodide oxidation rates for these 23 strains ranged from 0.03 to 0.70 $\mu M I^- \text{ day}^{-1}$.

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 $\mu M I^- \text{ day}^{-1}$ (Table 4.1). Among these 36 confirmed positive strains, 15 of them also exhibited I_3^- -positive phenotypes. 8 bacterial strains exhibited I_3^- -positive phenotype but no halo patterns in autoradiography. Coincidentally, it was noticed that these 8 strains exhibited no moisture in their colonies while growing on the $^{125}I^-$ -enriched R2A agar plate which might result the difficulty to exam the halo formation in autoradiography.

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*, *Bacteroidetes*, *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 (13 strains). 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 activity of three of these strains was identified in the ≥ 30 kDa but ≤ 50 kDa fractions, whereas the ≥ 50 kDa but ≤ 100 kDa fractions were found to be active for the other strain.

In conclusion, 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 (70). 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) we measured a decrease $\geq 10\%$ in cultures (10 μ M 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. 4.2) has not been reconciled, but this question is currently being addressed by our group.

CHAPTER V

**CHARACTERIZATION AND IDENTIFICATION OF AN IODIDE OXIDIZING
ENZYME FROM A *BACILLUS Sp.* ISOLATED FROM ¹²⁹I-CONTAMINATED
SUBSURFACE SOILS OF SRS**

5.1 Overview

Iodide (I⁻) oxidizing activity was observed in spent medium of 44/84 aerobic bacteria that were isolated from ¹²⁹I-contaminated subsurface soils of the Savannah River Site (SRS) in South Carolina. The aim of this study was to characterize and identify the reactive compound(s) secreted from one of these I⁻ oxidizing strains, FA-2CB* (16S rRNA gene was >98% similar to that of *Bacillus cereus*). An oxidase-like enzyme(s) was suspected to be the reactive I⁻ oxidizing compound(s) because I⁻ oxidation was inactivated by treatments with heat, H₂O₂ and anaerobic incubation. Results from substrate utilization assays, copper amendments and size exclusion experiments largely precluded the possibility that the reactive enzyme was a multicopper oxidase (laccase). In the presence of cofactors NADH, NADPH or FMN the I⁻ oxidizing capability of FA-2CB* spent medium was enhanced 1.3, 2.7 and 3.3 times, respectively. Overall, the results led us to conclude that an oxidase-type enzyme, but not of the multicopper oxidase family, was responsible for the I⁻ oxidizing activity observed in the FA-2CB* spent medium. Additional assays are underway to identify the reactive enzyme, including: 1) substrate (NADPH and NADPH+FMN) kinetics for I⁻ oxidation, 2) the production of superoxide anions and H₂O₂, and 3) peptide identification using

HPLC-MS.

5.2 Introduction

^{129}I is one of the major byproducts of nuclear fission reactions. Due to its biophilic nature, excessive inventory, high perceived mobility in water-soil systems and radioactive longevity ($t_{1/2} = 1.6 \times 10^7$ yrs) ^{129}I has been recognized as a key risk drivers at Department of Energy (DOE) nuclear disposal facilities, e.g., the Hanford and Savannah River Sites (SRS), where contaminant plumes were detected containing ^{129}I concentrations 10 ~1000 times above the federal regulated Maximum Contaminant Level of 1 pCi/L (58, 90). Thus, it is critical to understand the environmental behavior of ^{129}I in order to rigorously assess its storage, disposal and remediation options.

The mobility of ^{129}I is mainly determined by its chemical speciation and interaction with natural organic matter (NOM). In most groundwater and aquifers, iodide (I^-) is expected to be the dominant chemical species based on the prevailing Eh and pH conditions and has a lower soil affinity (i.e., high mobility), with a distribution coefficient (K_d) of $\sim 1 \text{ L}^3 \text{ kg}^{-1}$, than that of iodate (IO_3^- , $K_d \sim 1000 \text{ L}^3 \text{ kg}^{-1}$) (13, 54, 65, 103, 107). Soil with a high NOM content could serve as a sink for ^{129}I due to the formation of organo-iodine (OI) through halogenation processes, i.e. electrophilic substitution of reactive iodine species (e.g. elemental iodine (I_2), triiodide (I_3^-) or hypoiodous acid (HOI)) on aromatic moieties (54, 138-139). Since I^- is the most reduced chemical form (oxidation state, -1) of all iodine species, oxidation is required to obtain a reactive iodine species (of higher oxidation state, 0/+1) or IO_3^- (oxidation state, +5).

However, the first electron transfer from I^- to an electron acceptor such as oxygen is thermodynamically unfavorable unless catalyzed by strong oxidants (e.g. catalytic enzymes or metal oxides) (36, 42, 77, 137).

From several field observations and laboratory experiments, it was concluded that microbial activities can mediate I^- oxidation and OI formation in water-soil systems (3-4, 6, 83-84, 107, 112). For bacteria, their particular roles in I^- transformation have been classified as 1) cellular accumulation (7-9, 72), 2) extracellular iodide oxidation (10, 73), and 3) methylation (5-6). Among these roles, extracellular I^- oxidation might lead to the greatest impediment of ^{129}I mobility in soils by promoting the iodination of soil aromatic moieties. A growing body of literature has implicated microbial oxidases, perhydrolases and/or peroxidases in the halogenation of soil organic matter (6, 20, 48, 67, 84-85, 89, 95, 98). However, little research has been devoted to identifying iodide-oxidizing enzymes and their mechanism(s) of action.

In the studies conducted thus far, two types of oxidoreductases, haloperoxidases and laccases, have been demonstrated to directly extract one electron from I^- , forming reactive intermediate iodine species (50, 116, 121, 123, 130, 140). Haloperoxidases are widely distributed among different biota, including humans, bacteria, fungi, plants, and algae (50, 120, 122) and commonly exhibit broad-substrate specificity. These enzymes have thus been classified based on the most electronegative halide that they can catalyze – chloroperoxidases use chloride (Cl^-), bromide (Br^-) and I^- , bromoperoxidases utilize Br^- and I^- , and iodoperoxidases are limited to I^- as the sole halide substrate. They generate hypohalites via the direct H_2O_2 -dependent oxidation of halides, which are

capable of halogenating aromatic moieties (121-122). Through traditional activity assays using spectrophotometric methods, it was found that chloroperoxidase activities are ubiquitously distributed in soil-water systems (14-15). However, OI formation catalyzed by these chloroperoxidases is not likely to occur in natural terrestrial environments because the substrate affinity (K_m) of these enzymes toward halides is in the mM range (126, 128), whereas environmentally relevant concentrations of I^- are in the 0.001-0.1 μ M range (10^3 - 10^6 times less than that of Cl^- [0.2-14 mM]) (34, 43, 102, 142).

Laccases are multi-copper phenolytic oxidases in which an oxidative state of +2 (Cu^{2+}) is required for substrate oxidation. They are secreted by fungi and bacteria to assist in lignin formation/degradation and can react with multiple substrates (e.g., Mn^{2+}) (109). The I^- oxidizing ability of laccase was first identified in a fungus, *Myceliophthora thermophile*, whereby I^- served as a substrate by donating one electron to the type 1 copper site (140). Moreover, the laccase mediated I^- oxidation could be enhanced in the presence of the general laccase substrate, 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (140). In recent field studies examining ^{129}I mobility, laccase activities in surface soils were correlated with OI formation (112, 141). However, bacterial laccases capable of carrying out I^- oxidation have only been identified from brine water samples, not from terrestrial sources (116).

Our previous field investigation in SRS F-area revealed relatively high amounts of IO_3^- (27.3%) and OI (OI, 23.9%) in the groundwater, indicating that I^- oxidation had occurred since I^- should be the predominant species at the ambient pH (3.2~6.8) and Eh (364~654 mV) conditions (90, 142). Although I^- could be oxidized by manganese oxides

and ferric oxides in the SRS subsurface, these processes are limited in environments with $\text{pH} < 5.0$ (137). Thus, it is probable that biogenic oxidants such as enzymes secreted by bacteria are capable of catalyzing I^- oxidation in the F-area. Indeed, 44 of 84 bacterial strains isolated from the F-area subsurface soil exhibited slow rates of extracellular I^- oxidation (71). In this study, we used one bacterial isolate of the F-area, FA-2CB*, to characterize and identify the extracellular reactive component(s) capable of carrying out I^- oxidation.

5.3 Materials and methods

5.3.1 Bacterial growth

FA-2CB* has been demonstrated to be able to oxidize I^- extracellularly and its 16S rRNA gene (GenBank accession# JQ765443) is most closely related ($> 98\%$) to that of *Bacillus cereus* Rock3-44 (71, 73). FA-2CB* was incubated in liquid M9GY medium (M9 minimal salt medium enriched with 0.4% glucose and 0.4% yeast extract, $\text{pH} 7.4 \pm 0.2$) at 30°C in the dark. When the optical density at 600 nm (OD_{600}) of the culture reached ~ 1.2 , FA-2CB* cells were harvested by centrifugation ($3200 \times g$, 20°C , 15 min), then washed and suspended in fresh 1X M9G (i.e. M9GY medium without yeast extract, $\text{pH} 7.4 \pm 0.2$) for the assays listed below.

5.3.2 Determination of the iodide oxidation rate under aerobic and anaerobic conditions

After FA-2CB* cells were suspended in 1X M9G medium, the culture was amended with I^- (10 μ M). Aerobic incubations were carried out in the dark at 30 °C on a rotary shaker (150 rpm) with loosened caps. For anaerobic incubations, the FA-2CB* culture was transferred into a 250 mL serum bottle, which was sealed using a gastight septum stopper (butyl rubber, Fisher Scientific) with a crimp seal. Oxygen was displaced from the bottle by purging with pure nitrogen gas at $\sim 8.0 \text{ L min}^{-1}$ flow rate for 5 minutes. The anaerobic incubations were also carried out in the dark at 30 °C. Background controls consisting of M9G medium amended with I^- but without bacterial cells were carried out. After 5 days incubation, the spent medium from the aerobic and anaerobic treatments were collected to determine iodide concentrations using GC-MS as described in Zhang et al. (142). The detection limits for I^- and IO_3^- were 0.34 and 1.11 nM, respectively.

5.3.3 Characterization of aerobic iodide oxidizing activity in spent medium

After FA-2CB* cells were suspended in 1X M9G medium and incubated aerobically for 24 hrs, the spent medium and cell pellets were separated by centrifugation ($3200 \times g$, 20 °C, 15 min). The spent medium was then concentrated 10 ~ 50x using centrifugal ultrafiltration units with a 3 kDa cut-off (AmiconUltra, Millipore) for further characterization of I^- -oxidizing activities.

Triiodide (I_3^-) formation was used to determine rates of I^- oxidation (10). Reaction mixtures for the determination of I_3^- formation were prepared in 96-well plates (Polystyrene, Greiner UV-Star) and consisted of 50 μ L of concentrated spent medium, 20 mM acetate buffer (pH 5.5) and KI in 150 μ L total volume. Due to the fact that I_3^- is highly reactive and volatile, high concentrations of KI (10 mM) were applied in the assay mixture in order to stabilize the presence of I_3^- in aqueous solution. Controls (i.e., reactions without KI or the spent medium) were also carried out to differentiate non-enzymatic from enzymatic reactions. Rates of I_3^- formation were monitored as the increase in absorbance at 353 nm at 30 °C as a function of time using a molar extinction coefficient (ϵ) of 25.5 $\text{mM}^{-1} \text{cm}^{-1}$ (10). One unit (U) of I^- oxidizing activity was defined as 1 μ mol I_3^- formation per hour at 30 °C.

In order to characterize the I^- oxidation capability of FA-2CB* as related to enzymatic catalysis, several common enzymatic inhibitors were tested, including: boiling (100 °C, 30 mins), sodium azide (NaN_3), ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), or sodium dodecyl sulfate (SDS).

5.3.4 Assessment of laccase activity in spent medium

In order to identify the relationship between laccase activity and iodide oxidizing activity of FA-2CB*, Cu^{2+} amendments were applied in two ways to stimulate laccase activity in spent medium. First, various concentrations of Cu^{2+} ions (as CuSO_4) were amended directly into the FA-2CB* aqueous medium during growth, in order to stimulate the production of laccases (41, 91, 117). Spent M9G medium from these cells

was collected and concentrated for I_3^- formation assays. Second, spent medium from FA-2CB* cells grown without Cu^{2+} addition was collected, concentrated and then amended with various concentrations of Cu^{2+} ions to allow the reactive I^- oxidizing component(s) to chelate with Cu^{2+} ions (17). I^- oxidation rates were determined for the Cu^{2+} amended concentrates using the I_3^- formation assay.

Molecular size fractionation was also applied to determine the relationship between laccase activity and iodide oxidizing activity of FA-2CB*. The spent medium of FA-2CB* was sequentially separated and concentrated into five fractions based on molecular size (>50, 30-50, 10-30, 3-10, and <3kDa) using centrifugal ultrafiltration devices (AmiconUltra, Millipore). Polysulfonate standards (10, 20, and 40 kDa) were used as filtration controls in order to confirm that no breakage occurred in the filtration devices. I_3^- formation rates and laccase activity (see below) were determined for each of these five size fractions. Two commercial laccases extracted from fungi, *Trametes versicolor* and *Rhus vernicifera* (Sigma-Aldrich), were used as experimental controls.

Laccase activities were measured spectrophotometrically using two laccase-specific substrates, 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS, Sigma-Aldrich) and syringaldazine (SGZ, Sigma-Aldrich). Reaction mixtures were prepared in 96-well plates and consisted of 50 μ L commercial laccases or spent medium, 20 mM acetate buffer (pH 5.5) and 1.2 mM ABTS or 60 μ M SGZ in 150 μ L total volume. To differentiate enzymatic activities from non-enzymatic reactions, controls (i.e. reactions without spent medium or ABTS/SGZ) were also carried out. Oxidized ABTS and SGZ were monitored at 30 °C as the function of time by the absorbance

increase at 420 nm ($\epsilon = 36 \text{ mM}^{-1} \text{ cm}^{-1}$) and 525 nm ($\epsilon = 65 \text{ mM}^{-1} \text{ cm}^{-1}$), respectively (55). One unit (U) of laccase activity was defined as the amount of enzyme oxidizing 1 μmol of ABTS or SGZ per hour at 30 °C.

5.3.5 The effect of common enzymatic cofactors on I⁻ oxidation by spent medium of FA-2CB*

Several common enzymatic cofactors that are involved in oxidation-reduction reactions were examined to determine their ability to enhance I⁻ oxidation in the FA-2CB* concentrated spent medium. Cofactors included flavin adenine dinucleotide (FAD), riboflavin-5'-phosphate (i.e. flavin mononucleotide, FMN), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide (NAD⁺), reduced nicotinamide adenine dinucleotide phosphate (NADPH), coenzyme A (CoA), and adenosine-5'-triphosphate (ATP) which were all purchased from Sigma-Aldrich Inc. and freshly prepared in distilled water before use. Reaction mixtures (150 μL) for the determination of I₃⁻ formation consisted of 50 μL of concentrated spent medium, 10 μL of cofactors (various concentrations), 20 mM acetate buffer (pH 5.5), and 10 mM KI. Controls (i.e. reactions without cofactors or the spent medium) were also carried out to differentiate non-enzymatic from enzymatic reactions.

5.3.6 Purification and identification of the iodide-oxidizing reactive component(s)

Spent M9G medium from FA-2CB* cultures (1~2 L) was concentrated to ~5 mL using centrifugal ultrafiltration devices with a 10 kDa cut-off (AmiconUltra, Millipore).

To fractionate the reactive Γ oxidizing component(s), the concentrated spent medium was washed with equal volume of 20 mM Tris buffer (pH 8.0) at least three times to remove the excess salt ions and directly loaded onto a 10×40 mm anion affinity column (DEAE-Sepharose CL-6B, GE Healthcare) which was equilibrated with 20 mM Tris buffer (pH 8.0) as described by the manufacturer. The column was then eluted with 25 mL of 20 mM Tris buffer (pH 8.0) using natural gravity at a flow rate $\sim 0.625 \text{ mL min}^{-1}$, and the eluent (25 mL) was collected in a 50 mL conical tube (polypropylene, VWR International). The elution and collection process was continued as a linear gradient with 25 mL of each 100, 200, 300, 400, 500, and 600 mM NaCl prepared in 20 mM Tris buffer (pH 8.0). Eluents further size fractionated based on the molecular size (>50 , 30-50, 10-30, and <10 kDa) using centrifugal ultrafiltration devices. The Γ oxidation rate of each fraction was then determined using the I_3^- formation assay. The fraction containing the reactive Γ oxidizing component(s) was then subjected to gel electrophoresis to confirm its purity (see below). The entire purification procedure was carried out at 4°C . The protein concentration of fractions for each step was quantified using the bicinchoninic acid (BCA) method (Pierce).

Purity and molecular weight determination was carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS- polyacrylamide gel consisted of a 15% resolving gel and 4% stacking gel in a 1 mm thick slab. Precision Plus Protein Dual Color standards, ranging in molecular size from 10 to 250 kDa (Bio-Rad), were used as molecular weight markers. After the sample was denatured in Laemmli loading buffer (pH 6.8; 2% SDS and 2% dithiothreitol) by boiling at 95°C for

5 min, electrophoresis was carried out in 25 mM Tris-glycine buffer (pH 8.3) following the method described by Laemmli (68). Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. The reactive fraction exhibited a single band in the gel, which was cut from the gel and digested with trypsin into several peptide fragments for amino acid sequence determination by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) at Los Alamos National Laboratory.

5.4 Results

5.4.1 Determine iodide oxidation rates under aerobic and anaerobic conditions

As shown in Fig 5.1, the capability of FA-2CB* to carry out I^- oxidation was limited to aerobic conditions, where 23.8% of amended I^- (10 μM) was oxidized and transformed into IO_3^- . Under anaerobic conditions, no significant decreases ($\leq 5\%$) in I^- concentration nor increases of IO_3^- or OI concentrations were detected, indicating that I^- oxidation was not occurring. Spent medium from heat-treated (i.e. autoclaved) FA-2CB* cells were incapable of I^- oxidation ($< 5\%$ I^- loss).

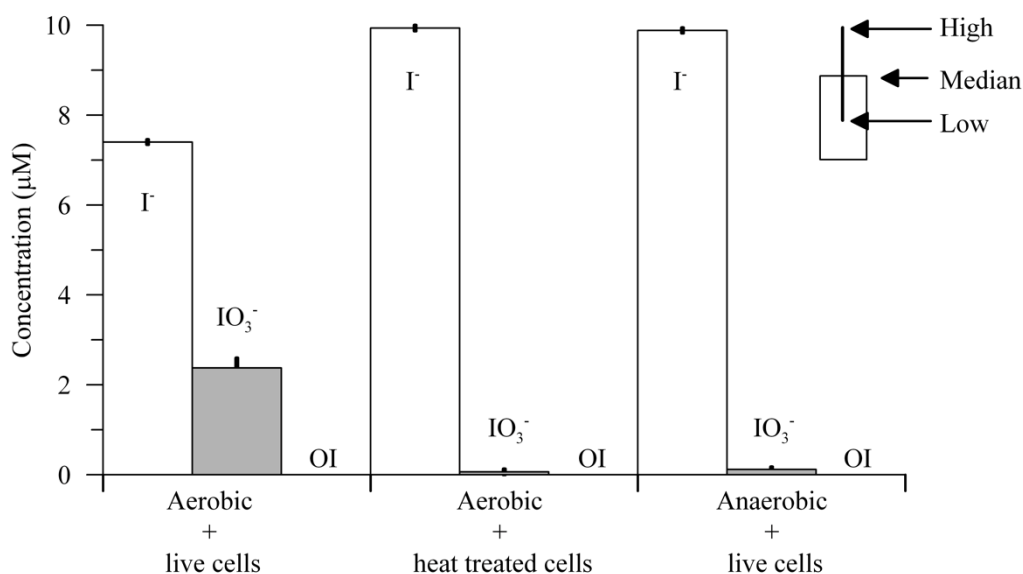


FIG. 5.1 Concentrations of iodine species in the spent medium of FA-2CB* cultures under aerobic and anaerobic treatments. Heat-treated cells of FA-2CB* cells were autoclaved. Each treatment was carried out with duplicate samples containing 10 μM I^- as the initial iodine species over a 5 day incubation. OI concentrations of each treatment were below the detection limits. Bars indicated the concentration range of duplicate samples.

5.4.2 The effect of enzymatic inhibitors on iodide oxidation by FA-2CB* spent medium

The effects of various enzymatic inhibitors on I^- oxidation in FA-2CB* spent medium are shown in Table 5.1. In the presence of 0.1 mM NaN_3 , I^- oxidation was enhanced 1.7 times, but at higher NaN_3 concentrations I^- oxidation was inhibited. I^- oxidation activity decreased with increasing EDTA concentrations from 0.1 to 10 mM. When the concentrated spent medium was treated with H_2O_2 , 1% SDS, or heat (100 °C, 30 mins), I^- oxidation was completely inhibited.

Table 5.1 Effects of enzymatic inhibitors and treatments on iodide-oxidizing activities by the extracellular extract of FA-2CB*

| Treatments | Concentration | Activity remaining (%) ^b |
|-------------------------------|---------------|-------------------------------------|
| None | | 100 |
| NaN ₃ | 0.1 mM | 167 ± 4 |
| | 1.0 mM | 67 ± 5 |
| | 10.0 mM | 30 ± 0 |
| EDTA | 0.1 mM | 67 ± 2 |
| | 1.0 mM | 17 ± 5 |
| | 10.0 mM | 17 ± 3 |
| H ₂ O ₂ | 0.05 mM | 0 ± 5 |
| | 0.5 mM | 0 ± 4 |
| | 5.0 mM | 0 ± 7 |
| SDS | 1% | 0 ± 3 |
| Heat ^a | | 0 ± 0 |

^a The heating process was carried out by boiling for 30 min.

^b The standard deviation was derived from triplicate analysis.

5.4.3 The relationship between laccase activity and iodide oxidation

Regardless of the Cu²⁺ concentration in the growth medium, which was varied from 0 to 60 μM, the I⁻ oxidizing capability of the FA-2CB* spent medium remained constant (~138 mU/mL crude concentrate) (Fig 5.2A). When the Cu²⁺ concentration in the growth medium exceeded 80 μM, the iodide oxidizing activity in the spent medium of FA-2CB* decreased (Fig 5.2A).

A similar scenario was also observed when amending Cu²⁺ directly in the concentrated spent medium of FA-2CB* that had been collected from cultures grown in Cu²⁺-free medium (Fig 5.2B). The highest I⁻ oxidizing activity was observed when the concentrated spent medium was amended with 40 μM Cu²⁺, but this value was just 1.2x higher (0.6 μU/μg crude protein) than that measured in spent medium without the

amendment of Cu^{2+} ($0.5 \mu\text{U}/\mu\text{g}$ crude protein). When the Cu^{2+} amendment concentrations exceeded $80 \mu\text{M}$, the iodide oxidizing activity of the spent medium was significantly inhibited (Fig 5.2B).

The concentrated spent medium of FA-2CB* was fractionated based on molecular size, >50 , 30 - 50 , 10 - 30 , 3 - 10 , and <3 kDa, and then examined for I^- oxidizing activity and laccase activity, which was assessed using two common laccase substrates, ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) and SGZ (syringadazine). As shown in Fig 5.3, the component(s) responsible for I^- oxidation was 10 - 30 kDa, whereas the fractions exhibiting laccase activity were 30 - 50 kDa ($\sim 84\%$ of total observed laccase activities) and >50 kDa ($\sim 16\%$ of total laccase activities).

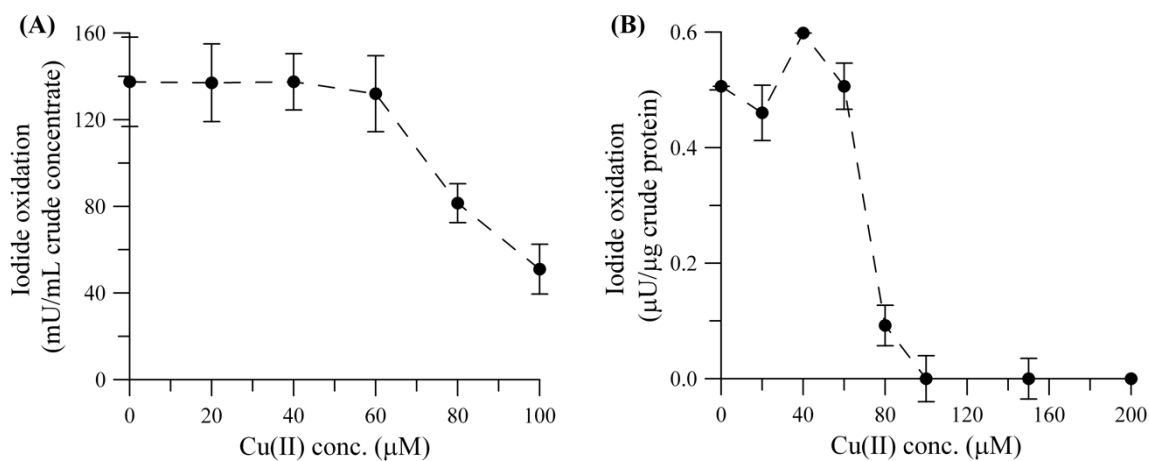


FIG. 5.2 The influence of Cu^{2+} ions on iodide oxidation activity of concentrated spent medium from FA-2CB* in which Cu^{2+} ions were amended (A) to the culture medium of FA-2CB* during growth or (B) into the concentrated spent medium prior to the I_3^- formation assays. Symbols represent the average value of triplicate analysis. One unit (U) of iodide oxidation is defined as $1 \mu\text{mol I}_3^-$ generated in 1 hour at 30°C .

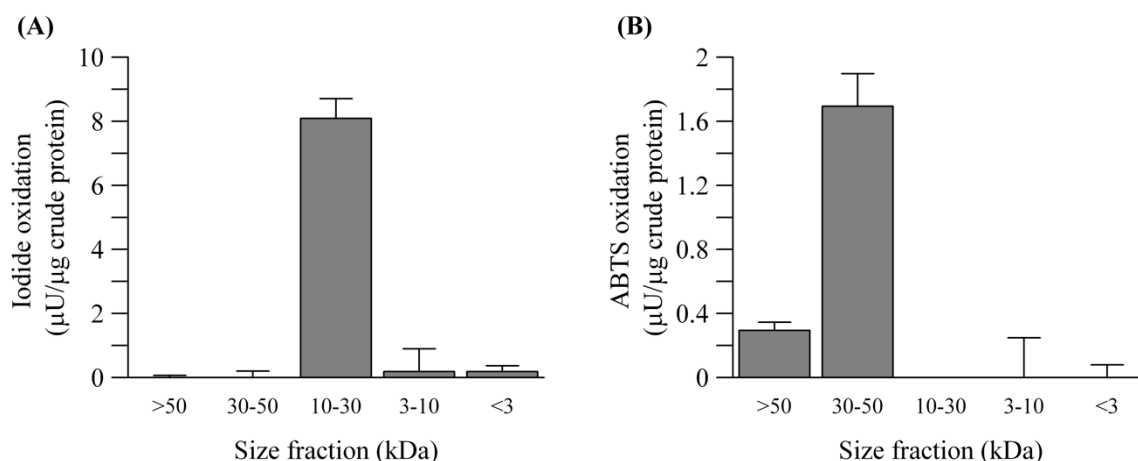


FIG. 5.3 (A) Formation of I_3^- as the indicator of I^- oxidation and (B) oxidation of ABTS as an indicator of laccase activity, in each molecular size fraction of FA-2CB* concentrated spent medium. Error bars represent one standard deviation of triplicate analysis. One U of iodide oxidation is defined as $1 \mu\text{mol } I_3^-$ generated in 1 hour at 30°C . One U of laccase activity is defined as $1 \mu\text{mol}$ of ABTS oxidized per hour at 30°C .

5.4.4 Influence of common enzymatic cofactors on iodide oxidation by FA-2CB* spent medium

A total of 7 cofactors that are commonly associated with enzymatic oxidation-reduction reactions were examined for their influence on I^- oxidation by FA-2CB* spent medium (Table 5.2). Results showed that in the presence of FMN (flavin mononucleotide), NADH (reduced nicotinamide adenine dinucleotide), and NADPH (reduced nicotinamide adenine dinucleotide phosphate), the capability for I^- oxidation by concentrated FA-2CB* spent medium was enhanced, whereas no significant enhancement was observed in the presence of FAD (flavin adenine dinucleotide), NAD^+ (nicotinamide adenine dinucleotide), CoA (coenzyme A), or ATP (adenosine-5'-triphosphate). The greatest enhancement in I^- oxidation (3.3 times higher than without cofactors) was observed when $14 \mu\text{M}$ FMN was amended to the $150 \mu\text{l}$ assay mixture

containing 50 μl of concentrated FA-2CB* spent medium. When the FMN concentration was increased to 139 μM , I^- oxidation activity was enhanced just 1.3 fold and at this high concentration (139 μM), FMN was capable of oxidizing I^- by itself. In general, the I^- oxidation capability of concentrated FA-2CB* spent medium was increased when NADH and NADPH concentrations were increased. However, NADPH provided a greater enhancement in I^- oxidation (2.7 fold enhancement at 58 μM) than did NADH (1.5 fold enhancement at 100 μM).

Table 5.2 Effects of various cofactors on iodide-oxidizing activity of spent medium from FA-2CB*

| Cofactors | Biochemical function | Oxidation status | Concentration (μM) | Abiotic I_3^- formation (μM) ^a | Total I_3^- formation (μM) ^a |
|-------------------|----------------------|------------------|---------------------------------|---|---|
| None ^b | | | | 0.00 \pm 0.04 | 0.16 \pm 0.02 |
| FAD | Redox reaction | Oxidized form | 80 | 0.00 \pm 0.04 | 0.15 \pm 0.09 |
| FMN | Redox reaction | Oxidized form | 14 | 0.00 \pm 0.09 | 0.61 \pm 0.10 |
| | | | 139 | 0.27 \pm 0.14 | 0.50 \pm 0.10 |
| NAD ⁺ | Redox reaction | Oxidized form | 90 | 0.00 \pm 0.03 | 0.16 \pm 0.04 |
| NADH | Redox reaction | Reduced form | 10 | 0.03 \pm 0.02 | 0.24 \pm 0.02 |
| | | | 100 | 0.04 \pm 0.04 | 0.27 \pm 0.03 |
| NADPH | Redox reaction | Reduced form | 6 | 0.00 \pm 0.04 | 0.24 \pm 0.04 |
| | | | 58 | 0.00 \pm 0.04 | 0.49 \pm 0.06 |
| CoA | Oxidation | | 87 | 0.00 \pm 0.04 | 0.14 \pm 0.08 |
| ATP | Energy transfer | | 10 | 0.00 \pm 0.04 | 0.16 \pm 0.06 |

^a Abiotic I_3^- formation indicates the amount of I_3^- formed in 6 hours in reactions without spent medium, whereas total I_3^- formation indicates the amount of I_3^- formed in reactions with FA-2CB* spent medium. The standard deviation is derived from triplicate analysis.

^b "None" indicates that cofactors were not added to the reaction mixture.

5.4.5 Purification of the iodide-oxidizing reactive component(s)

After the purification process using DEAE-Sepharose anion exchange chromatography and molecular size fractionation (10-30 kDa), the specific I^- oxidizing

activity of the reactive fraction was 99.09 U/g protein, whereas that from the concentrated spent medium following ultrafiltration (with 10 kDa cut-off) was only 0.55 U/g protein (Table 5.3). Moreover, I_3^- production by 0.165 μ g protein of the purified reactive fraction increased in a linear fashion over 5-6 hours ($R^2=0.99$, $p<0.05$) (Fig 5.4A). After subjecting ~ 15 μ g protein of the purified reactive fraction to SDS-PAGE, two bands were observed with the estimated molecular size of 16.4 (band A) and 15.6 kDa (band B) (Fig 5.4B). The band with molecular size of 16.4 kDa was cut from the gel and digested with trypsin for amino acid sequence determination by MALDI-TOF MS at Los Alamos National Laboratory.

Table 5.3 Summary of purification of Γ oxidizing component(s) from FA-2CB* spent medium

| Procedure | Protein (μ g) | I_3^- formation (μ M) ^b | Specific Γ oxidizing activity (U/g protein) ^c |
|---|-----------------------|--|--|
| Ultrafiltration-concentrated spent medium | 7.1 | 0.16 \pm 0.02 | 0.55 \pm 0.08 |
| Reactive fraction ^a | 0.165 | 0.65 \pm 0.08 | 99.09 \pm 11.82 |

^a The reactive fraction was purified from the DEAE-Sepharose anion exchange column and 10-30 kDa fractionation.

^b The I_3^- concentration was determined after incubating the assay mixture for 6 h at 30 °C. Cofactors were not used for this assay. Standard deviations were derived from triplicate analysis.

^c One unit (U) of Γ oxidizing activity was defined as 1 μ mol I_3^- formed per hour at 30 °C.

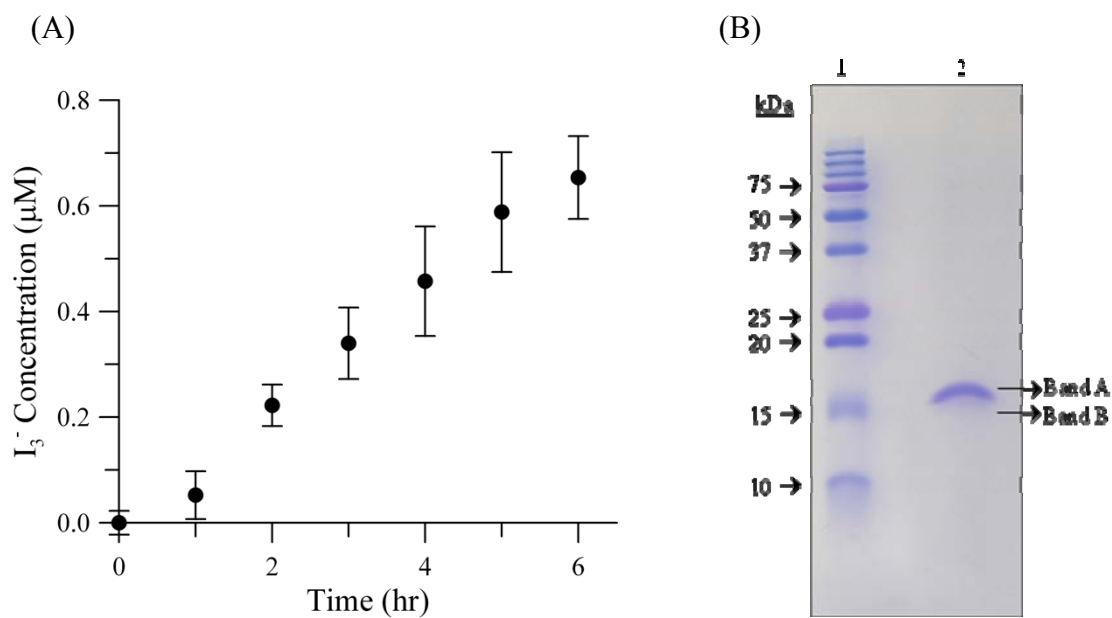


FIG. 5.4 (A) I^- oxidation (measured as I_3^- production) over time by the purified, reactive fraction (0.165 μg protein; no cofactors) and (B) visible protein bands in a SDS-PAGE gel run with the purified, reactive fraction containing $\sim 15 \mu\text{g}$ protein (lane 2) and molecular standards (lane 1). Error bars indicate the standard deviation ($n=3$).

5.5 Discussion

In this study, we characterized the I^- oxidizing component(s) from the spent medium of a strain, FA-2CB*, isolated from ^{129}I -contaminated soils of the SRS (71). The I^- oxidizing activity of the spent medium exhibited an oxygen requirement (Fig 5.1), heat-intolerance (Fig 5.1 & Table 5.1) and was not dependent on H_2O_2 and Cu^{2+} ions (Table 5.1 & Fig 5.2). In the presence of NADH, NADPH and FMN the I^- oxidizing activity of FA-2CB* spent medium was enhanced 1.3, 2.7 and 3.3 fold, respectively (Table 5.2). These results lead us to conclude that an oxidase-type enzyme, likely an

superoxide- or H₂O₂-producing oxidase, is responsible for the I⁻ oxidizing activity observed in spent medium from FA-2CB*.

5.5.1 An oxidase-like enzyme mediated the I⁻ oxidation

The fact that I⁻ oxidation by the concentrated FA-2CB* spent medium was inactivated upon boiling for 30 min (Table 5.1), indicated that the reactive component(s) was likely an enzyme. Two types of oxidoreductases, oxidases and peroxidases, are often implicated for halogenation processes in soils (50, 116, 121, 123, 130, 140). The activities of oxidase and peroxidases can be distinguished by their electron acceptors, which are oxygen and hydrogen peroxide (H₂O₂), respectively. As shown in Fig 5.1, I⁻ oxidation activity by FA-2CB* spent medium was significant only under aerobic conditions, in which case 23% of amended I⁻ (10 μM) was transformed into IO₃⁻. Moreover, the I⁻ oxidizing activity of the concentrated FA-2CB* spent medium was completely inhibited in the presence of 0.05 to 5 mM H₂O₂ (Table 5.1). These results indicate that the reactive enzyme(s) was not a peroxidase, but rather an oxidase-like enzyme.

5.5.2 I⁻ oxidation was not mediated by laccase activity

In studies conducted to date, only one type of oxidase, laccases secreted from fungi *Myceliophthora thermophila* and brine water iodide-oxidizing *α-Proteobacteria*, have been directly shown to carry out I⁻ oxidation (116, 140). Laccases are widely produced by plants, fungi and bacteria and are classified as multicopper oxidases.

Laccase activity can be stimulated by the addition of Cu^{2+} ions, either as a supplement to medium during the growth of laccase-bearing fungi or bacteria or when directly applied to enzyme mixtures (17, 41, 91, 117); however, the optimal concentrations of Cu^{2+} ions vary (0.02-5 mM), depending on fungal or bacterial species or growth conditions (17, 41, 91, 117). In this study, we tested two means of Cu^{2+} amendment, (i) additions of 0 – 100 μM Cu^{2+} during the growth of FA-2CB* and (ii) additions of 0 – 200 μM Cu^{2+} to the concentrated FA-2CB* spent medium, to examine the possibility that the Γ oxidizing activity exhibited by FA-2CB* spent medium is mediated by a laccase.. Significant enhancement of Γ oxidation not observed upon either Cu^{2+} amendment (Fig 5.2).

To further explore the relationship between Γ oxidizing capability and laccase activity, concentrated FA-2CB* spent medium was fractionated into 5 groups by size (>50, 30-50, 10-30, 3-10, <3 kDa) and each was tested for Γ oxidizing and laccase activity. The results demonstrate that Γ oxidation activity was limited to the 10-30 kDa fraction, whereas the laccase activity, measured as the oxidation of ABTS, was found in fractions >30 kDa. Thus, we conclude that laccase activity by FA-2CB* under our experimental conditions was likely not responsible for the observed Γ oxidation.

5.5.3 Was Γ oxidation mediated by a NADPH oxidase?

Several lines of evidence lead us to hypothesize that the reactive Γ oxidizing enzyme could be an oxidase, possibly a NADPH oxidase. First, Γ oxidation by the concentrated FA-2CB* spent medium was significantly enhanced upon addition of NADH, NADPH, and FMN (Table 5.3). Among these cofactors, 1 μg FMN and 10 μg

NADPH could enhance I^- oxidation 3.3 and 2.7 fold, respectively (Table 5.2). The enhancement of I^- oxidation by an oxidized flavin molecule (Table 5.2), such as FMN, which are well recognized electron transfer shuttles in many oxidases (81), strongly supports our hypothesis that the I^- oxidizing activity in FA-2CB* spent medium was mediated by an oxidase-like enzyme. Second, in the presence of 58 μM NADPH, a stronger enhancement of I^- oxidizing activity was observed than in the presence of 100 μM NADH (Table 5.2). Also, we observed a concentration-dependent enhancement (~ 2 fold) of I_3^- oxidation activity upon inclusion of 6 to 58 μM NADPH in the reaction mixture (Table 5.2). These results suggest that the oxidase(s) from FA-2CB* involved in I^- oxidation preferably utilizes NADPH over NADH to activate molecular oxygen.

Oxidases that preferentially utilize NADPH and flavin molecules as cofactors are NADPH oxidases (EC 1.6.3.1) (21, 76). A subset of NADPH oxidases, including the NOX family, is membrane-bound complexes that can generate reactive oxygen species. The NOX enzymes have been recognized among facultative bacteria, such as *Bacillus licheniformis*, *Bacillus megaterium*, *Lactobacillus sanfranciscensis* and *Lactococcus lactis* (76, 79, 87, 101). NOX oxidases can transfer electrons from NADPH or NADH to oxygen molecules (O_2) which are subsequently transformed to superoxide anions (Fig 5.5). Superoxide anion is a highly reactive molecule and can disproportionate rapidly into H_2O_2 and O_2 in the presence of water molecules. In a previous study, H_2O_2 was shown to oxidize I^- , especially under acidic conditions (73). Superoxide anions in the aqueous form have similar properties as H_2O_2 , that is, they are highly reactive toward electron sequestration. Furthermore, bacterial production of reactive oxygen species has

been demonstrated to oxidize Mn^{2+} and Fe^{2+} into insoluble forms of Mn(IV) and Fe(III) (69, 100) and Mn- and Fe-oxides have been shown to oxidize iodide (36, 42, 137)..

Thus, regardless of the produced superoxide anion, i.e., if they rapidly transform to H_2O_2 or if they remain in the original form, they should be capable of oxidizing I^- to reactive iodine species (i.e. $I_2/I_3^-/HOI$) (Fig 5.5), which are then capable of iodinating soil aromatic moieties.

However, our hypothesis that the reactive I^- oxidizing enzyme is a superoxide-producing NADPH oxidase is based on preliminary results that require confirmation. The actual identity of the reactive enzyme still needs to be established. In order to determine whether I^- oxidation by spent medium of FA-2CB* is associated with the production of superoxide anions, several assays are being conducted: 1) substrate (NADPH and NADPH+FMN) kinetics for I^- oxidation, 2) the production of superoxide anions and H_2O_2 , and 3) peptide sequence of the reactive enzymes.

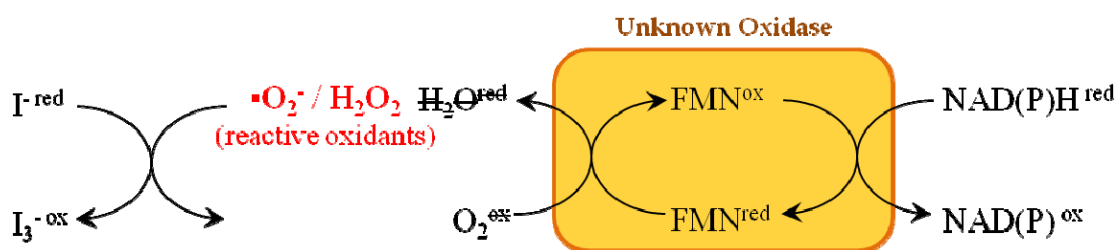


FIG. 5.5 The hypothesized pathway of I^- oxidation mediated by FA-2CB* secreted oxidase.

CHAPTER VI

SUMMARY

^{129}I has recently been recognized as one of the key risk radionuclides in the groundwater at two DOE nuclear disposal facilities, the Savannah River and Hanford Sites, due to its longevity, excessive inventory, perceived high mobility in water-soil systems, and biophilic nature. Mobility of ^{129}I in water-soil systems depends on its speciation, which is greatly influenced by the presence of natural organic matter (NOM). Due to environmental pH and Eh conditions, I^- , which has the lowest oxidation state (-1) among all iodine species and higher mobility than IO_3^- , was expected to be the predominant species in groundwater (13, 54, 65, 103, 107). However, field measurements demonstrate that I^- , IO_3^- and OI can comprise a significant fraction of the total iodine in groundwater (52-53, 90, 107, 142), indicating that biogeochemical processes, beyond pH and Eh, mediate iodine speciation in the subsurface. For example, OI formation requires that reactive intermediate iodine species ($\text{I}_2/\text{HOI}/\text{I}_3^-$), arising through the oxidation of I^- or reduction of IO_3^- , attack organic matter through electrophilic substitution on aromatic moieties (25, 82, 97, 104, 139, 141). Thus, a soil with elevated NOM concentration has been demonstrated to have a higher tendency to serve as an iodine sink through the iodination process and act as a barrier with decreased ^{129}I mobility (53-54, 137).

It has been hypothesized that microbes influence I^- oxidation and OI formation in water-soil systems due to iodine's biophilic nature, strong association with organic

matter and the redox sensitivity, along with insights into the capabilities of marine microbes that can influence the biogeochemical cycle of iodine (3, 32). Given the high abundance of microbes occurring in water-soil systems, especially in organic-rich soils (e.g. 10^8 - 10^9 bacteria per gram soil) (118, 129), it is quite possible that microorganisms play an important role in the speciation and mobility of ^{129}I in the subsurface. This assumption is supported by recent field observations and laboratory experiments (3-4, 6, 83-84, 107, 112). These laboratory studies have demonstrated that soils/sediments treated with heat, chloroform, γ -irradiation, or prokaryotic antibiotics, exhibit considerable reduction in iodine binding capacity onto soils (6, 83-85). Inoculation of sterilized soils with fresh soil or viable microorganisms can restore the iodine-organic matter binding potential (84). However, details concerning the mechanisms, identities, and functions of soil bacteria in regards to iodide oxidation and OI formation are lacking. In this study we examined the capacity of bacteria that were isolated from surface and subsurface soils of an ^{129}I -contaminated region of SRS' F-area to transform iodine and influence its mobility in the subsurface..

6.1 Accumulation of I^- by bacteria

Because of its biophilic nature, iodine accumulation has long been observed in various organisms, such as mammals, vertebrates, marine algae and marine bacteria. Among the culturable aerobic bacterial strains isolated from F-area soils, I^- accumulation was found in 3 out of 136 strains tested (FA-30, FA-2CB*, and FA-191), in the presence of H_2O_2 (5 mM). These three strains were closely related to *Streptomyces/Kitasatospora*

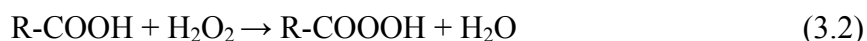
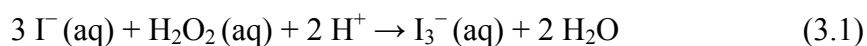
spp., *Bacillus mycooides*, and *Ralstonia/Cupriavidus* spp, suggesting that the Γ^- accumulation phenotype was not limited to any particular phylogenetic group. Compared to IAB isolated from the marine environment, the Γ^- accumulating capacity of the F-area IAB was quite small. With environmentally relevant Γ^- concentrations (0.1 μM), the F-area IAB only accumulated 0.2 to 2.0% of the total Γ^- , whereas two previously described marine IAB, a *Flexibacter aggregans* strain and an *Arenibacter troitsensis* strain, could accumulate 2 to 50%.

Specific mechanisms for marine IAB accumulation of Γ^- was proposed by Amachi et al. (7) whereby extracellular H_2O_2 , generated by glucose oxidase, oxidizes Γ^- to I_2 or HOI via an unidentified haloperoxidase. HOI is then transported across the cell membrane via facilitated diffusion. Once inside the cell, HOI either is reduced to Γ^- or forms OI. F-area IAB, however, exhibited a different mechanism. For example, Γ^- accumulation by FA-30 was stimulated by the addition of H_2O_2 , was not inhibited by high concentrations of chloride ions (27 mM), did not exhibit substrate saturation kinetics with regard to Γ^- concentration (0.1 to 10 μM Γ^-), and increased at pH values of <6 . These data indicated that the mechanism for Γ^- accumulation by F-area IAB is unlikely mediated by specific enzymatic catalysis or cell surface electrostatic adsorption. Rather, it is likely that Γ^- was oxidized by H_2O_2 to reactive iodine species, which could subsequently attack the organic molecules containing aromatic functional groups on bacterial surfaces by electrophilic substitution to form OI. In chapter 2 we demonstrate that readily culturable, aerobic bacteria of the F-area aquifer do not accumulate significant amounts of Γ^- , and that these IAB are most likely not responsible for the high

fraction of OI observed in F-area groundwater (142). However, this mechanism may contribute to the long-term fate and transport of ^{129}I and to the biogeochemical cycling of iodine over geologic time scales.

6.2 Oxidation of I^- by H_2O_2 -dependent reaction with bacterially secreted organic acids

Oxidation of I^- via a single-step electron transfer is thermodynamically unfavorable unless strong oxidants are available (77). In water-soil systems, abiotic oxidants (e.g. MnO_2 and Fe_2O_3) are capable of oxidizing I^- but their roles are limited in acidic environments, where pH values ≤ 5.0 (36, 42, 137). In order to investigate the extracellular I^- oxidizing capacities from F-area aerobic bacteria, the spent medium of 84 F-area bacterial cultures was examined. The enhanced I^- oxidation (2-10 fold) that occurred with 27 of the bacterial strains in the presence of 5 mM H_2O_2 was found to be due to the organic acids secreted by the bacteria. Two mechanisms were found to be responsible for the enhanced I^- oxidation. First, organic acids acidified the spent medium by dissociation of hydrogen ions from their carboxyl groups. The lowered pH of the spent medium subsequently enhanced the rate of H_2O_2 hydrolysis which sequestered electrons from I^- (Equation 3.1). Second, organic acids react with H_2O_2 to form peroxy carboxylic acids, which are strong oxidizing agents (Equation 3.2). The instability of peroxy carboxylic acids in aqueous solution leads to their spontaneous decomposition, whereby 2 electrons are consumed from I^- by each peroxy carboxyl group (Equation 3.3).



With decreasing pH from 9 to 4, I_3^- concentrations, which were used as indicators for H_2O_2 -dependent I^- oxidation, increased exponentially from 8.4 to 825.9 μM . Organic acids with ≥ 2 carboxy groups, enhanced H_2O_2 -dependent I^- oxidation 1.5 – 15 fold as a function of increasing pH above pH 6.0, but had no effect at $\text{pH} \leq 5.0$. The results indicate that, as pH decreases (≤ 5.0), increasing H_2O_2 hydrolysis is the driving force behind I^- oxidation. However, at pH of ≥ 6.0 , spontaneous decomposition of peroxy carboxylic acids, generated from H_2O_2 and organic acids, contributes significantly to iodide oxidation. The results reveal an indirect microbial mechanism, organic acid secretion coupled to H_2O_2 production, that could enhance I^- oxidation and OI formation in soils and sediments.

6.3 Oxidation of I^- by bacterial extracellular oxidase-like enzymes

I^- -oxidizing bacteria (IOB) have been isolated from natural gas brine waters or sediments where the environments are naturally high in iodine (10) and from a marine fish aquarium where I_2 formation was implicated in a fish kill (45). IOB were also isolated from seawater, however only after enrichment for several months in the presence of 1 mM I^- (10). Using similar approaches, efforts to isolate IOB from terrestrial sources have been unsuccessful (10). To determine if bacteria from SRS F-area sediments could influence ^{129}I speciation by extracellular activity, I^- oxidizing

activity was evaluated from the culturable bacterial strains and from soils. All bacterial strains isolated directly from sediment material (n=325) and from sediment enrichment cultures containing 1 mM I^- (n=29) tested negative for I^- oxidation on R2A agar plates containing I^- and starch. However, tubes from 2/24 enrichment cultures exhibited a distinct yellow coloration, indicative of I^- 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 I^- to IO_3^- and OI, whereas I^- oxidation did not occur in treatments with autoclaved soils. Screening of 84 strains for I^- 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 oxidizing I^- at slow rates. The IOB phenotype was observed across a number of distinct bacterial phyla, indicating that I^- oxidation can be supported by a variety of terrestrial bacteria.

A growing body of literature has implicated microbial enzymes, such as oxidases, laccases, perhydrolases and peroxidases, in the formation of halogenated organic matter compounds in soils (6, 20, 48, 67, 84-85, 89, 95, 98, 112, 140). In recent field studies that examining ^{129}I mobility in soils, laccase activities were correlated with OI formation (112, 141). However, bacterial laccases capable of carrying out I^- oxidation have been identified from the brine water IOB (116), but not from terrestrial bacteria. In chapter V we characterize the extracellular I^- oxidizing component(s) of one F-area IOB closely related to *Bacillus cereus*, strain FA-2CB*. Oxidase-like enzyme(s) were implicated as the reactive I^- oxidizing component(s) because I^- oxidation was inhibited by treatments

with heat, H_2O_2 and anaerobic incubation. It was also shown that the reactive enzyme was unlikely a laccase. In the presence of cofactors NADH (reduced nicotinamide adenine dinucleotide, 100 μM), NADPH (reduced nicotinamide adenine dinucleotide phosphate, 58 μM) or FMN (riboflavin-5'-phosphate, 14 μM), the Γ oxidizing activity in FA-2CB* spent medium was enhanced 1.3, 2.7 and 3.3 times, respectively. In aggregate, the results led us to hypothesize that an oxidase-type enzyme was responsible for the Γ oxidizing activity observed in the FA-2CB* spent medium. Additional experiments are underway to identify the reactive enzyme, including: 1) substrate (NADPH and NADPH+FMN) kinetics for Γ oxidation, 2) the production of superoxide anions and H_2O_2 , and 3) protein identification.

The research presented in this thesis demonstrates that SRS aerobic bacteria are capable of transforming Γ by (1) cellular accumulation, (2) organic acids and/or H_2O_2 secretion (i.e. peroxy carboxylic acids formation), and (3) extracellular enzymatic activity. In summary, a better understanding of the role that bacteria play in Γ oxidation and OI formation is expected to help to reduce ^{129}I mobility in water-soil systems.

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