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Superoxide Production by a Manganese-Oxidizing Bacterium Facilitates Iodide Oxidation

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The release of radioactive iodine (i.e., iodine-129 and iodine-131) from nuclear reprocessing facilities is a potential threat to human health. The fate and transport of iodine are determined primarily by its redox status, but processes that affect iodine oxidation states in the environment are poorly characterized. Given the difficulty in removing electrons from iodide (I^-), naturally occurring iodide oxidation processes require strong oxidants, such as Mn oxides or microbial enzymes. In this study, we examine iodide oxidation by a marine bacterium, *Roseobacter* sp. AzwK-3b, which promotes Mn(II) oxidation by catalyzing the production of extracellular superoxide (O_2^-). In the absence of Mn^{2+}, *Roseobacter* sp. AzwK-3b cultures oxidized ~90% of the provided iodide (10 μM) within 6 days, whereas in the presence of Mn(II), iodide oxidation occurred only after Mn(IV) formation ceased. Iodide oxidation was not observed during incubations in spent medium or with whole cells under anaerobic conditions or following heat treatment (boiling). Furthermore, iodide oxidation was significantly inhibited in the presence of superoxide dismutase and diphenylene iodonium (a general inhibitor of NADH oxidoreductases). In contrast, the addition of exogenous NADH enhanced iodide oxidation. Taken together, the results indicate that iodide oxidation was mediated primarily by extracellular superoxide generated by *Roseobacter* sp. AzwK-3b and not by the Mn oxides formed by this organism. Considering that extracellular superoxide formation is a widespread phenomenon among marine and terrestrial bacteria, this could represent an important pathway for iodide oxidation in some environments.

With the development of nuclear power, large inventories of radioiodine, including iodine-129 and iodine-131, have been deposited in the environment. For example, it has been estimated that 212 PBq of iodine-131 and 8.06 GBq of iodine-129 were released into the environment from the Dai-ichi nuclear power plant after the Fukushima accident (1). Iodine (I) is an essential element for all mammals (including humans) because of its role as a constituent of thyroid hormones; however, radioisotopes of this element can be dangerous carcinogens. Once introduced into the body, iodine-131 accumulates in the thyroid gland and can lead to cancer due to high beta decay energy (2, 3). However, because the half-life of iodine-131 is just 8 days, the period of dangerous exposure after a release event is relatively short (days to months). In contrast, iodine-129 is less hazardous (low-energy beta and gamma emissions) but has a half-life of ~16 million years. In order to evaluate the potential human exposure risks and environmental impacts of these radioisotopes, a comprehensive understanding of the environmental behavior of iodine is needed.

Changes in redox potential control the environmental behavior of iodine, including its mobility and biogeochemical cycling (4–8). Iodide (I^-) (oxidation state −1) is considered the main iodine species that is released from nuclear power reprocessing facilities because of the redox properties of the reprocessing wastewater (8). When I^- is released into highly oxic environments (oxidation reduction potential of >0.5 to 0.8 V, depending on the pH), it will spontaneously transform into elemental iodine (I_2) (oxidation state 0). I_2 can then be emitted to the atmosphere, spontaneously transformed into iodate (IO_3^-) (oxidation state +5), methylated, or incorporated into organic matter (7, 9). However, in many environments (e.g., most subsurface waters and anaerobic sediments, etc.), the oxidation of I^- to I_2 is not thermodynamically favored, because this one electron transfer requires the presence of a strong oxidant or enzymatic catalyst (10).

Studies related to marine biogeochemical cycling of iodine have focused primarily on IO_3^- reduction to I^- (11–14) or I^- incorporation into brown algae (15, 16). The brown alga *Laminaria digitata* utilizes an iodoperoxidase to transform I^- to I_2 on the cell surface for subsequent uptake (17). Inside the algal cells, I_2 is reduced back to I^- to serve as an antioxidant to protect against reactive oxygen species (ROS) formed during photosynthesis. The accumulated I^- tends to be released into the atmosphere as iodine oxide aerosol through complex chemical reactions within algal cells and the marine boundary layers (18).

Besides brown algae, microorganisms are also capable of catalyzing I^- oxidation in marine systems. It has long been suspected that microorganisms play a role in I^- oxidation (4, 19–21), yet studies detailing potential mechanisms are scarce. In the late 1960s, a marine bacterium, *Pseudomonas iodooxidans*, was found to be associated with a fish kill in a marine aquarium (22). It was surmised that the bacteria had oxidized I^- to highly toxic I_2. However, the culture stock for this organism was lost; thus, further information concerning the mechanism of iodide oxidation and

Received 5 February 2014 **Accepted** 9 February 2014
Published ahead of print 21 February 2014
Editor: R. M. Kelly
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00400-14.

doi:10.1128/AEM.00400-14

its importance in biogeochemical cycling of iodine remains unclear. Recently, marine iodide-oxidizing bacteria have been isolated by enriching seawater with high I^- concentrations (1 mM) and from brine water naturally rich in I^- (63 μM to 1.2 mM) (23). These iodide-oxidizing bacteria are phylogenetically divided into two groups within the *Alphaproteobacteria* (23). One of the groups is related to the *Roseobacter* lineage and has been shown to utilize a nonspecific, laccase-like multicopper oxidase to oxidize I^- to I_2 (23, 24). It was concluded that these microorganisms may produce and release I_2 as an antibiotic, providing them an ecological advantage in iodide-rich environments (23). However, the laccase-like enzyme responsible for I^- oxidation has a K_m value of 2.64 mM, suggesting that these microorganisms play a minor role in I^- oxidation in natural seawater, where I^- concentrations are typically <0.45 μM.

In addition to biotic processes, laboratory studies have shown that I^- can be oxidized by mineral and synthetic oxides, such as those of manganese (Mn), iron (Fe), or titanium (Ti), under acidic conditions (6, 25, 26). These minerals, which are plentiful in terrestrial ecosystems and can be abundant in marine surface waters following dust deposition, can be formed via microbial processes. It is thought that intermediates of the biogenic mineral oxide formation process possess higher oxidizing potential than the final mineral structure, primarily because aging increases crystallinity and hence decreases reactivity (27). The interaction between biogenic Mn oxides and I^- has not yet been characterized.

Recently, it was demonstrated that a marine bacterium, *Roseobacter* sp. AzwK-3b, indirectly oxidizes Mn^{2+} through the production of superoxide (O_2^-) carried out by an extracellular, NADH-stimulated protein(s) (28–30). The *Roseobacter* clade is a widely distributed genus of marine bacteria that typically comprises up to 15 to ~20% of the bacterial community in coastal and marine environments (31, 32); thus, *Roseobacter* sp. Azwk-3b is a good candidate for evaluation of the role of biogenic Mn oxides in I^- oxidation. In addition, superoxide is a potent reactive oxygen species that could potentially oxidize I^-. Its decomposition product, hydrogen peroxide, is also known to facilitate iodide oxidation (33). The aim of this study was to investigate the iodide-oxidizing capability of *Roseobacter* sp. Azwk-3b and the underlying mechanisms.

MATERIALS AND METHODS

Bacterial growth. *Roseobacter* sp. AzwK-3b was grown on K-ASW medium (K medium prepared in artificial seawater), consisting of 2 g peptone/liter, 0.5 g yeast extract/liter, 20 mM HEPES (pH 7.2), 25% double-distilled water, and 75% artificial seawater at room temperature under ambient room lighting on a rotary shaker (200 rpm) (29). The artificial seawater was modified from the one described previously by Templeton et al. (32) and consisted of 0.5 M NaCl, 30 mM $MgCl_2$, 14 mM KCl, 1 mM $CaCl_2$, 2 mM NH_4Cl, 30 μM K_2HPO_4, and 2 mM $NaHCO_3$.

Iodide and Mn oxidation by *Roseobacter* sp. AzwK-3b cultures. Cultures (150 ml) of *Roseobacter* sp. AzwK-3b were grown in K-ASW medium amended with I^- (10 μM potassium iodide [KI]) or I^- (10 μM KI) and Mn^{2+} (as $MnCl_2$) (100 μM). At various time points over the 16-day incubation, samples (5 ml) were collected to determine I^- and Mn(III,IV) oxide concentrations and the optical density at 600 nm (OD_{600}) of the culture. Mn(III,IV) concentrations were determined colorimetrically at 620 nm by using a leucoberbelin blue (LBB) assay (29). A potassium permanganate ($KMnO_4$) calibration curve was used for Mn(III,IV) quantification with a normalization factor of 2.5 by converting electron equivalents from +7 to +4 (29). Iodide concentrations were determined as described in "Quantification of iodide concentrations" below.

Superoxide formation by *Roseobacter* sp. AzwK-3b cultures. *Roseobacter* sp. AzwK-3b cells were grown in K-ASW medium as described above (in the absence of I^- and Mn^{2+} to preclude the interaction of these ions with superoxide). Samples (2 ml) of the culture were collected at various times during the experiment and centrifuged at 8,000 × *g* for 10 min at 25°C. Superoxide was quantified in the supernatant by using a chemiluminescent probe, MCLA [2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol(1,2-a)pyrazin-3(7H)-one] (Sigma-Aldrich), in a 96-well plate assay as developed previously by Godrant et al. (34) and modified by Learman et al. (30). Supernatant (280 μl/well) was added to five sets of wells (triplicates of each set) containing xanthine (50 μM) and diethylenetriaminepentaacetic acid (DTPA) (100 μM). The first set of wells represented a blank measurement by adding superoxide dismutase (SOD) (50 kU/liter; Sigma) to remove superoxide. The second set, containing only the sample with xanthine and DTPA, represented superoxide production in the spent medium. The third through fifth sets contained increasing amounts (17, 50, and 83 mU/liter) of xanthine oxidase (XO) (Sigma) as a source of a superoxide standard for calibration purposes. Prior to establishing the calibration factor, the signal of the second set (spent medium only without XO and SOD) was subtracted from the signal of the calibration standard (i.e., spent medium with XO). The signals of all wells were corrected by subtracting the signal of the blank (i.e., spent medium with SOD). A steady luminescence value was observed for all reactions 3 min after the addition MCLA. Data points for all reactions were collected 5 min after the addition of MCLA.

To convert the luminescence signal obtained in the plate assay to a superoxide production rate, the activity of XO in K-ASW medium was determined by using a colorimetric method with nitroblue tetrazolium (NBT) (35). A mixture of XO (5 U/liter) and xanthine (5 μM) was combined with 1 mM NBT in a 1:1 ratio and allowed to incubate at room temperature. The irreversible reaction product monoformazan (MF^+) was measured over the course of 5 min at an absorbance of 530 nm. There was a linear correlation between absorbance and time for the reaction; thus, the slope was used to determine the MF^+ formation rate. The MF^+ formation rate was converted to a superoxide production rate based on the molar extinction coefficient of MF^+ at 530 nm, 12,800 M^{-1} cm^{-1}, and the stoichiometry of 1 part MF^+ production from NBT by 2 parts superoxide (35). This rate was then used to calculate the increase in the rate of superoxide production resulting from the XO additions in the third through fifth reaction sets of the 96-well MCLA assay described above. In this manner, a calibration factor for converting MCLA luminescence to a superoxide production rate in the absence of XO was achieved (30).

Correlation of iodide oxidation and Mn^{2+} concentrations. To evaluate the influence of the Mn^{2+} concentration on iodide oxidation by superoxide, potassium dioxide (KO_2) (Sigma) was used as a source of superoxide. Because superoxide can rapidly break down to hydrogen peroxide once it reacts with H_2O (the decomposition rate constant ranges from 10^3 to 10^9 M^{-1} s^{-1}) (36), the iodide solution (10 μM; 50 ml) in K-ASW medium was poured directly into a test tube containing KO_2 powder (3.5 mg) to avoid the loss of superoxide. After a 30-min incubation period on a shaker (200 rpm), the iodide concentration in the solution was determined.

To quantify the concentration of superoxide produced by the KO_2 powder in the reaction mixture, 50 ml K-ASW medium was poured directly into a tube containing 3.5 mg of KO_2 powder. This KO_2 solution was immediately (within a minute) mixed in a 1:1 ratio with 1 mM NBT. The reaction product, MF^+, was quantified as described above.

Iodide oxidation by biogenic Mn oxides. To prepare biogenic Mn(III,IV) oxides [defined here as Mn(III,IV) oxides formed through the enzymatic production of superoxide], cultures of *Roseobacter* sp. AzwK-3b were grown in K-ASW medium until the OD_{600} reached ~0.1. Cells were then removed from the culture medium by centrifugation (8,000 × *g* for 20 min), and the supernatant was filtered through a 0.2-μm sterile cellulose acetate disc filter (VWR International). The cell-free filtrate was then amended with 100 μM Mn^{2+} and incubated for 7 days

under ambient room lighting. The black precipitate (Mn oxides formed by biogenic superoxide [30]) formed over this period was collected by centrifugation (10,000 × *g* for 30 min) and rinsed twice with K-ASW medium. Mn oxides (final concentration, 30 μM) were added to a Falcon tube containing K-ASW medium and iodide (10 μM), which was then incubated for 15 days at room temperature under ambient room lighting on a rotary shaker (200 rpm). Iodide concentrations were determined as described below.

Iodide oxidation by spent medium and 5-fold-concentrated cell suspensions of *Roseobacter* sp. AzwK-3b. When the OD_{600} of the *Roseobacter* sp. AzwK-3b culture (125 ml) reached ~0.10, the cells were collected by centrifugation (8,000 × *g* for 10 min) and washed twice with fresh, sterile K-ASW medium. The supernatant from the initial centrifugation step was filtered through a 0.2-mm sterile disc filter (cellulose acetate; VWR) to obtain the spent medium for subsequent assays. Washed cells were suspended in 25 ml of fresh, sterile K-ASW medium, resulting in a 5-fold-concentrated cell suspension (OD_{600}, ~0.5). Iodide oxidation assays were performed with either spent medium (25 ml) or the 5-fold-concentrated cell suspension (25 ml) by adding iodide (final concentration, 10 μM) and incubating the mixture at room temperature under ambient room lighting on a rotary shaker at 200 rpm (standard conditions). At day 3 and day 5, samples (1 ml) were collected for iodide concentration determinations. Similar incubations were carried out under anaerobic conditions by purging 200-ml serum bottles, containing spent medium (25 ml) or a 5-fold-concentrated cell suspension (25 ml), with nitrogen gas (oxygen free) at a flow rate of 100 ml/min for approximately 5 min. To assess the biotic nature of iodide oxidation by *Roseobacter* sp. AzwK-3b, spent medium (25 ml) and a 5-fold-concentrated cell suspension (25 ml) were heat treated by boiling for 45 min, and each mixture was then incubated in the presence of iodide (final concentration, 10 μM) and tested for iodide oxidation under standard conditions.

To evaluate the potential role of NADH oxidoreductases and other NADH-oxidizing enzymes in iodide oxidation, NADH (200 μM; Sigma-Aldrich) and an irreversible inhibitor of NADH oxidoreductase, diphenyleneiodonium chloride (DPI) (50 μM; Sigma-Aldrich) (37), were added to spent medium and to a 5-fold-concentrated cell suspension and incubated under standard conditions for 3 days. The impact of SOD (Sigma-Aldrich) and catalase (Sigma-Aldrich) on the iodide-oxidizing capability was also evaluated by the daily addition of SOD (0.1 U/ml/day) and catalase (20, 200, and 2,000 U/ml/day) to spent medium and to a 5-fold-concentrated cell suspension and incubation under standard conditions for 3 days.

Quantification of iodide concentrations. Preliminary results indicated that cultures of *Roseobacter* sp. AzwK-3b did not generate detectable levels of gaseous iodine, such as methyl iodine or I_2. Therefore, the sum of iodate and organic iodine should account for the transformed iodide in the incubation mixtures. Thus, losses in iodide concentrations were used to represent oxidized iodide in this study. Quantification of iodide concentrations were performed as described previously by Zhang et al. (38). After the removal of cells by centrifugation (8,000 × *g* for 20 min), iodide was oxidized to iodine by using 2-iodosobenzoate (Sigma) and subsequently iodinated with *N*,*N*-dimethylaniline (Sigma) to yield 4-iodo-dimethylaniline. This iodinated derivative was extracted with cyclohexane (Sigma) and quantified by gas chromatography-mass spectrometry (GC-MS) (Thermo Scientific). The detection limit for iodide was 0.34 nM (38). An analytical error of ±10% for the iodide quantification technique was determined in this study (i.e., 1 μM iodide); thus, iodide transformations of <10% cannot be distinguished from background and should be treated with caution.

RESULTS

Iodide and Mn oxidation by *Roseobacter* sp. AzwK-3b cultures with production of superoxide. In the presence of Mn^{2+} (100 μM), *Roseobacter* sp. AzwK-3b cultures produced Mn(III,IV), which reached a concentration of ~75 to 80 μM after 4 days and remained near this level for the duration of the 16-day incubation (Fig. 1A). In cultures incubated in the absence of Mn^{2+} and I^-, the production rate of superoxide increased from day 0 to day 4, peaking at 0.45 μM/min (Fig. 1B). Following day 4, the rate of superoxide production exhibited a pattern of exponential decrease (Fig. 1B).

In the absence of Mn^{2+}, peak rates of iodide disappearance corresponded to peak periods of superoxide production (days 3 to 7). Similar to the pattern of superoxide production, iodide concentrations exhibited a pattern of exponential decrease over the later portion of the 16-day incubation (Fig. 1B and C). In contrast, iodide disappearance did not correlate with peak periods of superoxide production in cultures containing both Mn^{2+} and I^- (Fig. 1B and D). In this case, iodide concentrations did not exhibit a significant decrease over the first 4 days of the incubation. It was not until Mn(III,IV) oxide production had ceased (days 6 to 8) that iodide transformation occurred, albeit at a lower rate than the peak rate observed in incubation mixtures without Mn^{2+}.

Correlation of iodide oxidation and Mn^{2+} concentrations. To elucidate the influence of Mn^{2+} concentrations on iodide oxidation, an abiotic reaction in K-ASW medium (pH 7.2) was carried out by using 10 μM iodide, various concentrations of Mn^{2+}, and a high concentration (26 mM) of superoxide supplied as KO_2. The results showed that increasing Mn^{2+} concentrations from 0 to 1 mM resulted in lower levels of iodide oxidation in K-ASW medium (see Fig. S1 in the supplemental material).

Iodide oxidation by Mn oxides formed in *Roseobacter* sp. AzwK-3b cell-free filtrate. Iodide oxidation in the presence of Mn oxides (30 μM) derived from *Roseobacter* sp. AzwK-3b cell-free filtrate (Mn oxides formed by biogenic superoxide) was evaluated in fresh K-ASW medium (pH ~7). A significant decrease (7.2% ± 1.5%; <10%) in iodide concentrations was not observed after a 15-day incubation (data not shown). As mentioned in Materials and Methods, it is important to note that we established an analytical error of ±10% for the iodide quantification technique employed in this study (i.e., 1 μM iodide); thus, iodide transformations of <10% cannot be distinguished from background and should be treated with caution.

Iodide oxidation by spent medium and 5-fold-concentrated cell suspensions from *Roseobacter* sp. AzwK-3b cultures. In order to determine whether the iodide oxidation observed in *Roseobacter* sp. AzwK-3b cultures was mediated by enzymatic or other cellular activity, the effects of heating and anaerobic conditions were examined. In the spent medium from *Roseobacter* sp. AzwK-3b, 25.3% ± 5.0% of the amended iodide (10 μM) was oxidized after a 5-day incubation under aerobic conditions, whereas just 6.5% ± 2.4% of the iodide was oxidized under anaerobic conditions (Fig. 2A). In boiled spent medium, only 6.2% ± 2.3% of the iodide was oxidized under aerobic conditions (Fig. 2A). Similar results were observed with a 5-fold-concentrated cell suspension of *Roseobacter* sp. AzwK-3b (Fig. 2B). Under aerobic conditions, 67.7% ± 1.5% of the iodide was oxidized after a 5-day incubation, whereas the percentage of iodide oxidized was within background levels (±10%) in incubation mixtures containing heat-treated cells or under anaerobic conditions (6.5% ± 4.8% and 4.9% ± 3.6%, respectively).

The addition of the superoxide scavenger SOD (0.1 U/ml/day) resulted in 96% and 82% decreases in the iodide-oxidizing activity of spent medium and a 5-fold-concentrated cell suspension, respectively (Table 1). The addition of NADH (200 μM) resulted in

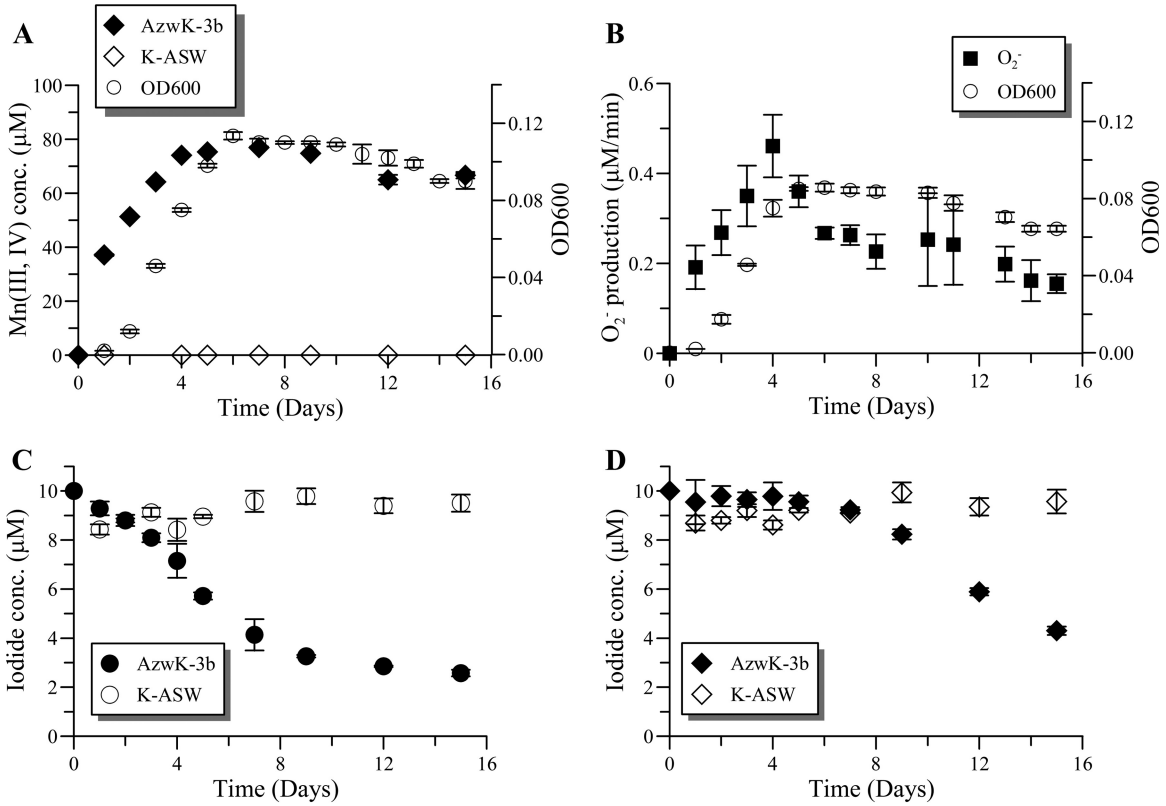

FIG 1 (A) Formation of Mn(III,IV) oxides, presented as Mn(IV)-equivalent concentrations, in the *Roseobacter* sp. AzwK-3b culture in the presence of 100 μM Mn^{2+}. (B) Production rate of superoxide anions through time in the *Roseobacter* sp. AzwK-3b culture in the absence of iodide and Mn^{2+}. (C and D) Iodide-oxidizing capability of a *Roseobacter* sp. AzwK-3b culture in the absence (C) and presence (D) of 100 μM Mn^{2+} through a 15-day time course. Error bars represent standard deviations of triplicate experimental samples. K-ASW, medium-only control.

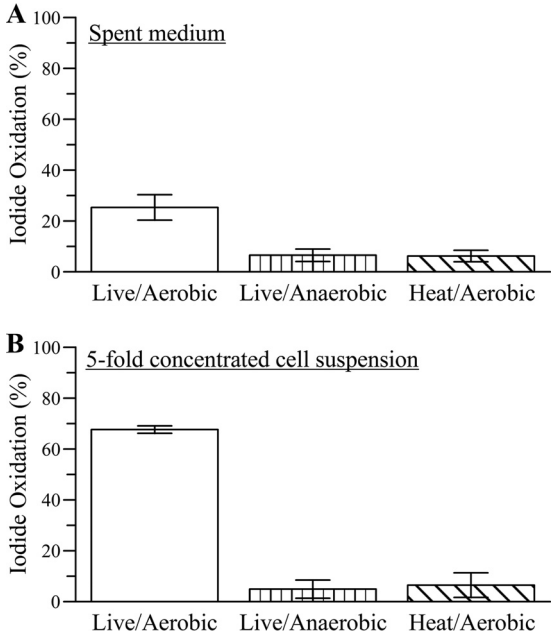

FIG 2 Iodide-oxidizing capability of spent medium (A) and a 5-fold-concentrated cell suspension of *Roseobacter* sp. AzwK-3b (B) under aerobic (solid white bars) and anaerobic (bars with vertical lines) conditions and with heat-treated (boiled) spent medium or concentrated cells under aerobic conditions (bars with diagonal lines). Error bars represent standard deviations from triplicate experimental samples.

30% and 13% increases in iodide oxidation by the spent medium and a 5-fold-concentrated cell suspension of *Roseobacter* sp. AzwK-3b, respectively (Table 1). The addition of DPI (50 μM) resulted in nearly complete inhibition (96%) of iodide oxidation activity by spent medium and moderate inhibition (63%) of iodide oxidation activity by a 5-fold-concentrated cell suspension (Table 1).

To determine if the primary breakdown product of the superoxide anion, H_2O_2, played a major role in iodide oxidation by *Roseobacter* sp. AzwK-3b, various amounts of catalase (20 to 2,000

TABLE 1 Iodide-oxidizing activity in spent medium and in a 5-fold-concentrated cell suspension of *Roseobacter* sp. AzwK-3b with various enzymatic treatments

Enzymatic treatment	Mean iodide-oxidizing activity (%) ± SD[a]	
	Spent medium	5-fold-concentrated cell suspension
None	25.3 ± 5.0	67.7 ± 1.5
SOD, 0.1 U/ml/day	1.1 ± 1.5	12.4 ± 1.5
NADH, 200 μM	33.0 ± 0.1	76.4 ± 1.3
DPI, 50 μM	0.9 ± 0.9	25.2 ± 3.5
Catalase, 20 U/ml/day	27.7 ± 3.7	60.2 ± 4.5
Catalase, 200 U/ml/day	21.6 ± 5.8	69.4 ± 3.2
Catalase, 2,000 U/ml/day	20.8 ± 6.5	58.7 ± 5.4

[a] Shown are means and standard deviations from triplicate experimental samples.

U/ml) were added daily to spent medium or a 5-fold-concentrated cell suspension of *Roseobacter* sp. AzwK-3b in the presence of 10 μM iodide over a 3-day incubation period. The addition of catalase did not result in a significant difference in the amount of iodide oxidized by spent medium or by a 5-fold-concentrated cell suspension, unless extremely high levels of the enzyme (2,000 U/ml/day) were added daily, in which case the catalase addition resulted in about a 15% decrease in iodide oxidation activity (Table 1). Additionally, iodide oxidation was assessed in K-ASW medium amended with high concentrations (26 mM) of either KO_2 (as a source of superoxide anions) or H_2O_2. Over a 30-min incubation period, 58.3% ± 0.2% of the iodide (10 μM) was oxidized in the presence of KO_2, whereas only 6.9% ± 4.5% (within background levels of the iodide detection assay, ±10%) was oxidized in the presence of H_2O_2 (see Fig. S1 in the supplemental material).

DISCUSSION

Mechanism of iodide oxidation in *Roseobacter* sp. Azw-3b cultures. The initial hypothesis of this study was that iodide could be directly oxidized by Mn(III,IV) oxides formed by *Roseobacter* sp. AzwK-3b. In this study, the production of Mn(III,IV) oxides from a *Roseobacter* sp. AzwK-3b culture had a pattern comparable to that of superoxide production (Fig. 1A and B). These results are consistent with those of Learman et al. (30), from which it was concluded that Mn(III,IV) oxide formation by *Roseobacter* sp. AzwK-3b is mediated by the generation of superoxide carried out by an extracellular protein(s). However, in our experiments, iodide oxidation did not correlate with either peak periods of Mn(III,IV) oxide production or superoxide production in cultures containing both Mn^{2+} and I^- (Fig. 1B and D). It was not until most of the Mn^{2+} had been depleted and Mn(III,IV) oxide production had ceased that iodide oxidation was detected. In contrast, rates of iodide disappearance were highest during peak periods of superoxide production in the absence of Mn^{2+} (day 3 to day 7) (Fig. 1B and C). Taken together, the results indicated that Mn^{2+} ions or Mn oxides inhibited iodide oxidation by *Roseobacter* sp. AzwK-3b. The most obvious explanation for the observed inhibition is that the Mn^{2+}/MnO_2 couple has a lower redox potential (−0.3 V) than the I^-/I_2 couple (0.62 V) at neutral pH; that is, Mn^{2+} ions were the preferred electron donor for superoxide, outcompeting iodide under the experimental conditions (oxic; pH ~7).

Indeed, increasing concentrations of Mn^{2+} resulted in lower levels of iodide oxidation in K-ASW medium (pH 7.2) containing 26 mM superoxide (see Fig. S1 in the supplemental material). It should be noted that superoxide concentrations were orders of magnitude higher in this abiotic reaction than in *Roseobacter* sp. AzwK-3b cultures, which could result in a greater availability of superoxide per molecule of iodide at a given Mn^{2+}/iodide ratio. This could explain why nearly complete inhibition of iodide oxidation was initially observed in cell cultures with an initial Mn^{2+}/iodide ratio of 10, whereas in the abiotic reaction with a similar Mn^{2+}/iodide ratio, iodide oxidation was inhibited by just 30% (Fig. 1D; see also Fig. S1 in the supplemental material).

After the period of active Mn(III,IV) oxide formation (days 7 to 15), ~40% of the iodide was oxidized (Fig. 1D). These results may indicate that iodide oxidation is stimulated by biogenic Mn(III,IV) oxides following their production. However, when incubated in fresh K-ASW medium (pH ~7) containing biogenic Mn oxides extracted from spent medium, iodide concentrations decreased only 7.2% ± 1.5%, which is within the analytical error of the assay (10%). Currently, our favored interpretation of these results is that iodide oxidation is facilitated by low levels of superoxide produced after day 7 in the *Roseobacter* sp. AzwK-3b culture, once Mn^{2+} concentrations have decreased to the point where inhibition is minimal.

Because iodide oxidation by *Roseobacter* sp. AzwK-3b did not correlate with the production of Mn(III,IV) oxides, we hypothesized that superoxide, or a breakdown product of this radical (i.e., H_2O_2), was a more potent agent for iodide oxidation under these experimental conditions. Previous studies found that the production of extracellular superoxide by *Roseobacter* sp. AzwK-3b was associated with extracellular oxidoreductase activity that is stimulated by NADH (28, 30). The results of the heat treatments and anaerobic incubations performed here demonstrated that iodide oxidation by *Roseobacter* sp. AzwK-3b was mediated by a heat-labile, oxygen-requiring process. Furthermore, a series of experiments performed with NADH, the superoxide scavenger SOD, and the general NADH oxidoreductase inhibitor DPI indicated that iodide oxidation by *Roseobacter* sp. AzwK-3b was mediated by superoxide (or a breakdown product such as H_2O_2) and that the generation of the extracellular oxidants was, at least partially, associated with the activity of a protein(s) (probable oxidoreductase) that is stimulated in the presence of NADH. Interestingly, the addition of NADH (200 μM) resulted in 30% and 13% increases in iodide oxidation by spent medium and by a 5-fold-concentrated cell suspension of *Roseobacter* sp. AzwK-3b, respectively (Table 1). The disparity of these responses could be explained by the fact that whole cells should be capable of regenerating reducing equivalents such as NADH to a greater extent than spent medium; thus, the stimulatory effect of NADH would be expected to be greater in spent medium.

Two lines of evidence suggested that superoxide is a more potent oxidant with respect to iodide than H_2O_2 under the experimental conditions used here (i.e., K-ASW medium, pH 7.2). First, the addition of catalase did not result in a significant difference in the amount of iodide oxidized by spent medium or by 5-fold-concentrated cells, unless extremely high levels of the enzyme (2,000 U/ml/day) were added daily (Table 1). In contrast, daily additions of just 0.1 U/ml of the superoxide scavenger SOD resulted in 80 to 90% inhibition of iodide oxidation activity. Second, superoxide supplied in the form of KO_2 (26 mM) supported higher levels of iodide oxidation (58%) than did H_2O_2 (26 mM; 7% iodide oxidation) (see Fig. S2 in the supplemental material).

Implication for iodide oxidation in natural environments. A recent study showed that extracellular superoxide production is a widespread phenomenon among many bacterial lineages in both marine and terrestrial environments (28). Thus, it is possible that superoxide of microbial origin can facilitate oxidation processes, such as Mn^{2+} or iodide oxidation, in a variety of environments. The presence of competing electron sources, such as metal ions or organic matter, could ultimately determine the extent to which superoxide-mediated iodide oxidation occurs in a given environment. In this study, oxidation of iodide (10 μM) in the *Roseobacter* sp. AzwK-3b culture was inhibited if excess Mn^{2+} (100 μM) was added (Fig. 1C). This could be explained by the fact that Mn^{2+} has a higher reaction rate with superoxide than does iodide at a near-neutral pH (36). In addition to Mn^{2+}, other metals, such as iron and copper ions, also exhibit higher reaction rates with superoxide

than with iodide (36, 39). In terrestrial environments (i.e., soils or groundwater), which typically contain higher concentrations of Mn^{2+} or Fe ions (μM to mM range) than iodide (low μM), iodide oxidation mediated by superoxide may not be a significant pathway in bulk pore water. However, microorganisms often exist as biofilms in soils and sediments, where localized regions (micrometer to millimeter scale) of high levels of superoxide production could catalyze low levels of iodide oxidation in certain subsurface environments.

In contrast to soil systems, microbially produced superoxide could play an important role in iodine biogeochemical cycling in marine systems, where the concentrations of trace metals (nM) are typically lower than iodide concentrations (μM). As such, we tested the ability of *Roseobacter* sp. AzwK-3b cultures to oxidize iodide (10 μM) in seawater collected from Galveston Bay, TX, but we did not detect iodide oxidation at levels above the sensitivity of our assay (±10%) (data not shown). Galveston Bay seawater is rich in organic matter, which could have acted as a preferred electron donor for the superoxide produced by *Roseobacter* sp. AzwK-3b. Alternatively, *Roseobacter* sp. AzwK-3b cells may not have produced sufficient levels of superoxide to support iodide oxidation when grown in Galveston Bay seawater. Regardless of the reason behind the inability of *Roseobacter* sp. AzwK-3b to promote iodide oxidation in Galveston Bay seawater, it would be worthwhile to evaluate the capacity of microbially produced superoxide as an iodide oxidant in seawater exhibiting a range of chemistries, especially those from more oligotrophic regions.

ACKNOWLEDGMENTS

This work was funded by the U.S. Department of Energy (DOE) Subsurface Biogeochemistry Research Program within the Office of Science (DE-FC02-07 ER65222). Danielle Creeley was partially funded by Welch grant BD0046 and the NSF-REU program.

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