Nitrate-Utilizing Microorganisms Resistant to Multiple Metals from the Heavily Contaminated Oak Ridge Reservation

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ABSTRACT Contamination of environments with nitrate generated by industrial processes and the use of nitrogen-containing fertilizers is a growing problem worldwide. While nitrate can be removed from contaminated areas by microbial denitrification, nitrate frequently occurs with other contaminants, such as heavy metals, that have the potential to impede the process. Here, nitrate-reducing microorganisms were enriched and isolated from both groundwater and sediments at the Oak Ridge Reservation (ORR) using concentrations of nitrate and metals (Al, Mn, Fe, Co, Ni, Cu, Cd, and U) similar to those observed in a contaminated environment at ORR. Seven new metal-resistant, nitrate-reducing strains were characterized, and their distribution across both noncontaminated and contaminated areas at ORR was examined. While the seven strains have various pH ranges for growth, carbon source preferences, and degrees of resistance to individual and combinations of metals, all were able to reduce nitrate at similar rates both in the presence and absence of the mixture of metals found in the contaminated ORR environment. Four strains were identified in groundwater samples at different ORR locations by exact 16S RNA sequence variant analysis, and all four were found in both noncontaminated and contaminated areas. By using environmentally relevant metal concentrations, we successfully isolated multiple organisms from both ORR noncontaminated and contaminated environments that are capable of reducing nitrate in the presence of extreme mixed-metal contamination.

IMPORTANCE Nitrate contamination is a global issue that affects groundwater quality. In some cases, cocontamination of groundwater with nitrate and mixtures of heavy metals could decrease microbially mediated nitrate removal, thereby increasing the duration of nitrate contamination. Here, we used metal and nitrate concentrations that are present in a contaminated site at the Oak Ridge Reservation to isolate seven metal-resistant strains. All were able to reduce nitrate in the presence of high concentrations of a mixture of heavy metals. Four of seven strains were located in pristine as well as contaminated sites at the Oak Ridge Reservation. Further study of these nitrate-reducing strains will uncover mechanisms of resistance to multiple metals that will increase our understanding of the effect of nitrate and metal contamination on groundwater microbial communities.

Anthropogenic disruption of the nitrogen cycle is a growing worldwide problem that has resulted in a wide range of environmental and human health-related issues. In particular, increased use of nitrogen-based fertilizers (1–3) and fossil fuels has led to a situation in which human activity is generating reactive nitrogen species at a rate that exceeds their removal by natural processes (4). This has led to a host of negative environmental consequences, such as coastal eutrophication (5), acid rain (2), and decreased groundwater quality due to nitrate contamination (6–8). Consumption

of nitrate-contaminated groundwater can cause methamoglobinemia in infants and may also be a risk factor for specific types of cancer (9).

The Oak Ridge Reservation (ORR), Tennessee, contains a site that is highly contaminated with both nitrate and a variety of heavy metals. This stems from the discharge, from 1951 to 1983, of acidic uranium- and nitrate-containing waste from nuclear processing activity at the ORR Y-12 plant into four 9.5 million-liter-capacity ponds. In 1978, the liquid in the ponds had pH values ranging from 0.8 to 5.3, with nitrate concentrations as high as 74 g/liter (1.2 M), together with high concentrations of various metals, including aluminum (4.9 g/liter), manganese (0.024 g/liter), and nickel (0.13 g/liter), as well as uranium (0.32 g/liter) (10). In 1983, the liquid in the ponds was neutralized and the resulting sludge was allowed to settle. The liquid was removed and the ponds were capped and turned into a parking lot (10, 11). There are currently five large contamination plumes of groundwater extending from the so-called S-3 ponds (11). Contaminants in the surrounding groundwater include not only nitrate (up to 11.6 g/liter, 190 mM) and U (140 mg/liter) but also multiple metals, such as aluminum (560 mg/liter), manganese (170 mg/liter), and nickel (9.4 mg/liter) (12). Consequently, the ORR S-3 ponds are one of the most heavily contaminated sites in the United States in terms of nitrate and metals.

Nitrate is typically removed naturally from soil and groundwater environments by denitrification, an anaerobic or microaerophilic respiratory process in which microorganisms couple the oxidation of reduced electron sources to the reduction of nitrate instead of oxygen. The nitrite that is formed is then reduced to nitric oxide, nitrous oxide, and finally nitrogen by nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively, although many microorganisms carry out only some steps of the pathway (13). Nitrate reductase catalyzes the first step of the denitrification pathway, and there are two types (13). Respiratory nitrate reductase is a trimeric transmembrane protein encoded by *narGHI* that requires molybdenum (Mo) in the form of a molybdopterin guanine dinucleotide (MGD) cofactor at its active site within NarG. NarH contains iron-sulfur clusters, while NarI contains cytochrome *b* (14, 15). In contrast, some organisms contain a dimeric periplasmic nitrate reductase encoded by *napAB*. NapA also requires Mo coordinated with a MGD cofactor, and NapB contains cytochrome *c* (16, 17).

Several environmental factors are known to be important for microbial denitrification in soil, including a source of reductant as well as low concentrations of oxygen (13, 18). Soil pH can also affect denitrification if pH values are below 6.0, resulting in decreased rates and the formation of nitrous oxide rather than nitrogen as the primary end product (19, 20). Mo availability also influences nitrate reduction in soils. Indeed, at the ORR nitrate-contaminated site, Mo is present at only picomolar concentrations in some of the most highly contaminated groundwater samples in spite of the high concentrations of a variety of other metals (12, 21). It was proposed that high concentrations of soluble Fe and Al in the contaminated environment form precipitates as the acidic contaminated groundwater mixes with the surrounding soil. These precipitates incorporate and adsorb molybdate, the soluble form of Mo, making it unavailable to microorganisms for nitrate reduction (21).

High concentrations of metals in contaminated environments are another factor that can impede nitrate reduction. Wastewaters from mining operations typically contain high concentrations of not only metals, such as Fe and Ni, but also nitrate due to the use of ammonium nitrate-based explosives and cyanide as a leaching agent (22). When simulated mine water was investigated for the effects of metals on nitrate removal rates, it was found that 50 and 100 mg/liter Ni decreased them by 18 and 65%, respectively (22). The pickling process used in stainless steel production results in metallurgic effluents containing high concentrations of nitrate and various metals, including Fe, Cr, and Ni, and these have been tested for their impact on denitrification by wastewater sludge (23). Fe (25 mg/liter) and Cr (100 mg/liter) were found to de-crease overall denitrification, while Ni (5 mg/liter) caused the accumulation of denitri-fication intermediates (NO_2^- and N_2O) (23). In addition, in a study of saltmarsh

TABLE 1 Metal and nitrate concentrations in ORR wells and derivation of COMM

Well	pH	Concn of[a]: Nitrate	Al	Mn	U	Ni	Co	Fe	Cu	Cd	Cr
FW300 (NC)	6.6	0.059	1.12	1.96	0.049	0.024	0.005	0.09	0.007	0.001	0.001
GW066 (NC)	6.2	0.014	0.812	0.231	0	0.027	0.002	0.897	0.051	0.001	0.008
FW301 (NC)	6.1	0.306	0	0.047	0.004	0.032	0.001	0.07	0.005	0	0.002
FW104 (C)	5.2	146	215	3,020	78	19	1.68	9.82	0.567	0.944	0.016
FW109 (C)	3.7	9.2	1,680	667	79.6	20.2	7.28	0.784	0.241	0.632	0.068
FW411 (C)	3.7	24.5	2,750	704	141	37.9	9.63	3.03	0.302	1.51	0.031
FW106 (C)	3.6	43.4	4,020	446	160	79.5	5.52	0.787	7.61	1.07	5.49
FW021 (C)	3.4	72.7	4,240	3,150	39.8	125	30.5	0.6	2.2	1.67	0.029
FW126 (C)	3	188	20,700	1,700	576	157	15.1	1.87	15	10.2	11.3
COMM			1,000	100	100	100	30	10	10	5	0

[a]The concentrations of nitrate and of the various metals are in mM and μM, respectively. The wells listed were used as inocula for enriching the ORR strains (Table 2). Wells designated NC are considered noncontaminated (<126 nM uranium), while wells designated C are contaminated (>126 nM uranium).

sediments, it was reported that denitrification rates were decreased by many metals, including Pb, Ni, Cr, Zn, Cu, Fe, and Cd, at 1 g/liter. It was concluded that metal pollution could alter the dynamics of the nitrogen cycle in marine sediments (24).

In previous studies at ORR, the nitrate-reducing microbial community in noncontaminated and contaminated environments was investigated using 16S rRNA, *nirK*, and *nirS* gene sequences (25), but key microorganisms were not isolated. In the present study, our goal was to characterize indigenous microorganisms that are able to reduce nitrate under conditions of extreme metal contamination. We would then map the distribution of those strains in 93 different noncontaminated and contaminated ORR groundwater wells using a library of 16S rRNA gene sequences. Concentrations of nitrate and of a mixture of metals based on those found in the contaminated ORR environments were used to enrich for and isolate a total of seven nitrate-reducing bacterial strains. All seven strains retained nitrate reductase activity when grown in the presence of a contaminated ORR environment metal mix (COMM), and four of the seven strains were identified in both noncontaminated and contaminated groundwater wells at ORR by exact sequence variant (ESV) analysis. We propose that these strains can serve as models for understanding how nitrate contamination can be mitigated even in the presence of high concentrations of a mixture of metals in extremely contaminated environments.

RESULTS

Isolation of metal-resistant, nitrate-reducing ORR strains. To determine if there were microorganisms at the ORR-contaminated site that could obtain energy for growth by nitrate reduction in the presence of high concentrations of metals, a multiple-metal mixture (COMM) containing eight metals (Al, Mn, Fe, Co, Ni, Cu, Cd, and U) was formulated that mimics the metals that are present in the groundwater in highly contaminated wells near the S-3 ponds at the ORR site (12). The composition of COMM is shown in Table 1. This is representative of the metal concentrations measured in a previous analysis of 80 different groundwater wells at the contaminated ORR site (12). The exceptions are concentrations of Mn and Al, which in COMM are significantly lower than those in the most highly contaminated wells in order to minimize precipitation issues with the growth medium used here. To isolate new strains, a series of over 2,000 high-throughput enrichments were set up in which groundwater and sediment from noncontaminated and contaminated areas at ORR were used as inocula. Noncontaminated water is defined as containing less than 126 nM uranium, which is the EPA limit allowed in drinking water, and this is found in ORR wells outside the contamination plumes from the S-3 ponds. The COMM was added to about 90% of the enrichments, and the remainder contained either a 10-fold lower concentration (0.1× COMM) or an elevated concentration of U (100 to 400 μM; the COMM contains 100 μM U) in the absence of other metals. Nitrate was used at concentrations of either 20 mM or 50 mM,

TABLE 2 Isolation conditions for the seven metal-tolerant nitrate-reducing ORR strains[a]

Strain	Closest species	Inoculum	pH		C source	Nitrate concn (mM)	Metal	Aeration	pH (optimum)
			Inoculum	Isolation					
MT058	*Pantoea ananatis*	FWB-306 (NC)	6.8	5.5	Glucose	20	COMM	AN	7.5
MT049	*Serratia proteamaculans*	GW066 (NC)	6.2	5.5	Lactate/fumarate	20	COMM	AN	6.5
MT123	*Castellaniella defragrans*	FW104 (C)	5.2	6.5	Ethanol	50	0.1× COMM	MAE	5.5
MT094	*Bacillus cereus*	FW104 (C)	5.2	5.5	Compost	20	0.1× COMM	AE	7
MT066	*Bacillus* sp. strain FJAT-18043	FW106 (C)	3.6	5.5	Compost	20	COMM	AN	6
MT086	*Paenibacillus lactis*	FW126 (C)	3	5.5	Compost	20	COMM	AN	7
MT124	*Paenibacillus* sp. strain Root52	FW126 (C)	3	7	Formate	0	200 μM U	MAE	7.5

[a]Inocula designated NC are from noncontaminated wells, while those designated C are from contaminated wells. Groundwater was used for all inocula except FWB-306, where sediment was used. For aeration, AE represents aerobic, AN represents anaerobic, and MAE represents microaerophilic. The pH optimum is taken from Fig. 1.

which are also based on those in the contaminated wells (Table 1). Other parameters that were varied for enrichments include carbon source (ethanol, lactate, fumarate, pyruvate, glucose, cellulose, or compost), pH (3.0 to 7.0), oxygenation (aerobic, microaerophilic [3% O_2], or anaerobic), and the presence of sulfate (20 mM) rather than nitrate or with no terminal electron acceptor added. Enrichments were incubated at 22°C, and growth was monitored over the course of 3 months. Isolates were obtained from enrichments exhibiting growth after plating on solid medium.

By 16S rRNA gene sequencing, twenty-one new strains were obtained and all but six sequences were observed for multiple independent isolates. These are listed in Table S1 in the supplemental material with their closest relative species by 16S rRNA gene sequence. They represent a diversity of bacteria, including one *Actinobacterium*, eight *Firmicutes* of the *Bacilli* class, and ten *Proteobacteria* from the classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. The most common isolates were various *Rhodanobacter* strains, one of which was isolated 17 separate times, and various *Bacillus* strains, of which there were four different strains, each isolated between 3 and 7 separate times.

Seven of these 21 ORR strains were selected for further in-depth study based on their ability to grow anaerobically by nitrate reduction (Table 2). These were designated MT049, MT058, MT066, MT086, MT094, MT123, and MT124. Their enrichment conditions along with their closest relatives by 16S rRNA gene sequence analysis are shown in Table 2. Of the seven strains, five were isolated from enrichments inoculated with highly contaminated groundwater from the area near the ORR S-3 ponds, while one (MT049) was from a noncontaminated ORR groundwater sample and the other (MT058) was from an uncontaminated ORR soil sample. They consist of two *Bacillus* (MT066 and MT094) and two *Paenibacillus* (MT086 and MT124) Gram-positive strains and one each of *Pantoea* (MT058), *Castellaniella* (MT123), and *Serratia* (MT049) Gram-negative strains. Six of the seven strains were isolated in the presence of at least 20 mM nitrate, while four were isolated in the presence of COMM, two in the presence of 0.1× COMM, and one in the presence of 200 μM U with no other metals added (Table 2). The carbon sources that the strains were isolated on included formate, ethanol, glucose, a lactate-fumarate mixture, and compost tea. The latter was prepared from compost obtained from a commercial composting facility and was used to enrich both *Bacillus* strains and one of the *Paenibacillus* strains.

Carbon source and pH dependence of ORR strains. The pH profiles for the seven ORR strains grown anaerobically in the presence of 20 mM nitrate and the indicated carbon source (Table S2) are shown in Fig. 1 (standard deviations are given in Table S3). In most cases, the pH values of the ORR groundwater samples that the strains were isolated from were lower than the pH of the enrichment cultures used for isolation (Table 2) or the pH optima observed for the isolates (Fig. 1 and Table 2). For example, both *Paenibacillus* species (MT086 and MT124) were isolated from groundwater (well FW126) that had the lowest pH (3.0) of all of the source wells and the highest concentrations of most of the contaminating metals (Table 1). However, MT086 was

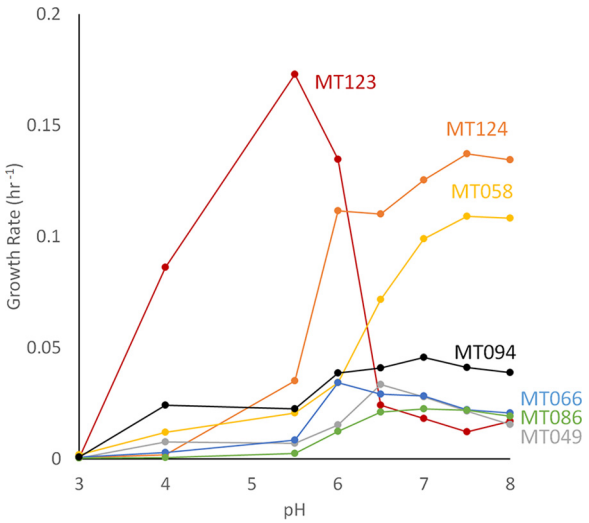

FIG 1 pH dependence of growth of the ORR strains. The seven strains were grown in triplicate anaerobically with 20 mM nitrate at different pH values using the carbon sources shown in Table S2. Maximum growth rates were calculated and the average values are shown in the figure. Standard deviations between the biological triplicates are given in Table S3. Strains are MT049 (*Serratia*), MT058 (*Pantoea*), MT066 (*Bacillus*), MT086 (*Paenibacillus*), MT094 (*Bacillus*), MT123 (*Castellaniella*), and MT124 (*Paenibacillus*).

enriched at pH 5.5 and grows optimally from pH 6.0 to 8.0 (Fig. 1), while MT124 was enriched at pH 7.0 and has a growth pH range from pH 5.5 to 8.0 with an optimum at pH 7.5 (Fig. 1 and Table 2). None of the isolates grew at pH 3.0; however, all three Gram-negative isolates (MT049, MT058, and MT123) as well as one of the *Bacillus* Gram-positive isolates (MT094) had measurable growth at pH 4.0 (Fig. 1), which in each case is lower than the pH of the groundwater from which they were isolated (Table 2). The *Castellaniella* species strain MT123 was the most acidophilic, with an optimum at pH 5.5, which is lower than the pH of the enrichment (pH 6.5). All of the other strains had significantly higher pH optima than the media used for isolation. For example, the *Bacillus* sp. strain MT066 had a broad pH optimum surrounding 7.0 and was enriched at pH 5.5.

The pH optimum for each of the seven ORR strains was used for carbon source and metal resistance experiments, and these parameters are summarized in Table S2. Growth was measured using six different carbon sources (formate, ethanol, fumarate, lactate, pyruvate, and glucose) under anaerobic, nitrate-reducing conditions with 20 mM nitrate. This is within the range of nitrate concentrations in the contaminated wells near the S-3 ponds, which vary over the range of 9.2 to 188 mM (Table 1). The results are shown in Fig. 2 (the standard deviations are given in Table S4). Three of the strains (MT066, MT086, and MT123) were unable to grow on any carbon source unless nitrate was present, while the other four strains exhibited increased growth in the presence of nitrate on at least one of the carbon sources. Among the strains that could grow in the absence of nitrate, growth was observed only on pyruvate and glucose. For example, the *Paenibacillus* sp. strain MT124 only grew on glucose and the *Pantoea* sp. strain MT058 only grew on pyruvate and glucose in the absence of nitrate. Most of the strains, including the two *Bacillus* strains (MT066 and MT094) as well as the *Castellaniella* strain (MT123), showed significant growth in the presence of nitrate on all or most of the carbon sources tested. The carbon sources that supported the highest growth rates or the most increased growth rate in the presence of nitrate were used in designing the growth media for determining their responses to metals and in measuring nitrate and nitrite reductase activities. The parameters for these studies are summarized in Table S2.

Metal resistance of ORR strains. One of the defining characteristics of the contaminated groundwater at ORR near the S-3 ponds is the elevated concentrations of a

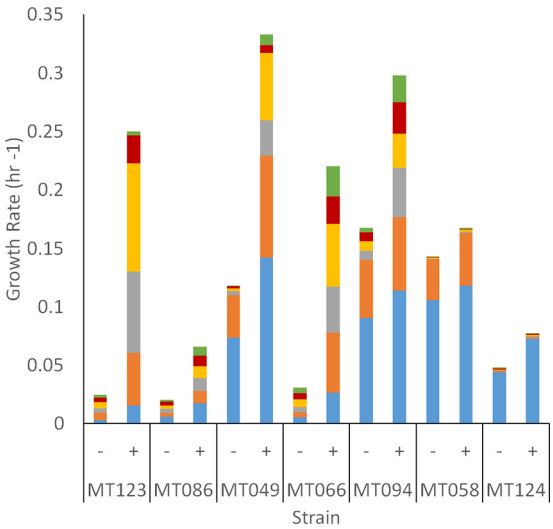

FIG 2 Growth of ORR strains on different carbon sources with and without nitrate. Strains were grown anaerobically with (+) and without (−) 20 mM nitrate on glucose (blue), pyruvate (orange), lactate (gray), fumarate (yellow), ethanol (red), and formate (green). Maximum growth rates on each carbon source were calculated, and the average values are reported. The pH values used for each strain are listed in Table S2, and standard deviations between the biological triplicates are given in Table S4. Strains are MT049 (*Serratia*), MT058 (*Pantoea*), MT066 (*Bacillus*), MT086 (*Paenibacillus*), MT094 (*Bacillus*), MT123 (*Castellaniella*), and MT124 (*Paenibacillus*).

combination of metals that exist in three different ionic forms depending on the metal and the redox state. The groundwater contains elevated concentrations of the cations Al^{3+}, $Fe^{2,3+}$, Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, and Mn^{2+}, the oxyanion CrO_4^{2-}, and the oxycation UO_2^{2+} (12). Dose-response curves for all seven ORR strains were obtained using all of these metals, either individually or with COMM. Al and Fe were not included in this study due to problems of precipitation (Al^{3+} and Fe^{3+} precipitate out of our growth medium at pH values of >5.5 and >3.0, respectively). The sensitivities of all seven strains to nitrate and nitrite were also determined. The dose-response curves were used to calculate half-maximal effective concentration (EC_{50}) values for each ion or for COMM, and these are shown in Table 3. The 95% confidence intervals (95% CI) for the EC_{50} values are given in Table S5.

As expected, all seven strains were able to grow in the presence of high concentrations of nitrate, with EC_{50} values ranging from 167 mM to a remarkable 711 mM. Growth at these concentrations of nitrate exceed concentrations observed in the ORR environment, where the most contaminated well (FW126) contained 188 mM nitrate (Table 1). EC_{50} values for nitrite were lower than those for nitrate and ranged from 9.0 to 77 mM. Three of the four Gram-positive strains (MT066, MT094, and MT124) had significantly higher nitrite EC_{50} values than the Gram-negative strains (MT049, MT058, and MT123). However, there does not appear to be a correlation between nitrate and nitrite resistance.

TABLE 3 EC_{50} values for nitrate, nitrite, individual metals, and COMM of ORR strains[a]

Strain	Nitrate (mM)	Nitrite (mM)	COMM (X)	Cu	Cd	Co	Ni	Mn	U	Cr
MT124	625	58.1	0.938	46.8	157	52.1	128	>350	>200	130
MT058	619	21.2	0.741	15.2	32.4	112	158	>350	>300	40.0
MT094	711	76.6	0.603	16.8	36.0	120	99.1	>300	>300	>500
MT066	167	70.9	0.541	5.61	31.8	33.0	108	>200	>300	19.0
MT049	420	9.76	0.384	14.8	55.0	54.1	78.5	160	>300	308
MT086	254	24.8	0.336	12.4	12.8	39.0	85.0	43.2	>200	42.0
MT123	279	9.01	0.238	65.8	69.7	31.2	140	69.3	6.01	6.51

[a]Individual metal EC_{50} values are given in μM and are colored such that lower values are red and higher values are green. The strains are MT049 (*Serratia*), MT058 (*Pantoea*), MT066 (*Bacillus*), MT086 (*Paenibacillus*), MT094 (*Bacillus*), MT123 (*Castellaniella*), and MT124 (*Paenibacillus*).

In the ORR contaminated groundwater, concentrations of Mn and U as high as 3 mM and 0.6 mM, respectively, were measured (Table 1). In general, EC_{50} values were high for Mn and U, with five of the seven strains having EC_{50} values for Mn of >150 μM (with some of >350 μM), and six of the seven strains have EC_{50} values for U of >200 μM. The exception was the *Castellaniella* strain MT123, which has an EC_{50} for U of only 6.0 μM. The optimal pH for growth of this strain (used to measure EC_{50} values) is lower than that for any other strain (Table S2). Uranium speciation in growth media can dramat-ically change with pH, thereby affecting toxicity (26).

Although Cr is not a major contaminant in all of the ORR contaminated groundwater wells (Table 1), the seven strains exhibited a wide range of EC_{50} values for chromate, from 7.0 μM to >0.5 mM (Table 3). Once again, *Castellaniella* strain MT123 had by far the lowest EC_{50} value, perhaps due to increased toxicity of the metal at the lower growth pH of 5.5. In the ORR environment, Cr was present in only some of the contaminated groundwater samples tested (FW126, FW106, and FW109) (Table 1), and with the exception of MT123, all of the strains had EC_{50} values greater than the highest Cr concentration measured in the ORR groundwater. Interestingly, the higher EC_{50} values did not correspond to exposure of that strain to Cr in the environment. For example, the *Serratia* strain (MT049) had an EC_{50} of 0.3 mM (Table 3), even though it was isolated from a well (GW066) that contained no detectable Cr (Table 1). With only a few counterexamples, the EC_{50} values for each of the strains to one of the three classes of contaminating metals, divalent metal cations, decreased in the order Ni $>$ Co $>$ Cd $>$ Cu (Table 3). This is generally the same pattern as that seen for the concentrations of these elements in the contaminated groundwater (Table 1). EC_{50} values for Ni, Co, Cd, and Cu ranged from 79 to 160 μM, 31 to 120 μM, 13 to 157 μM, and 6 to 66 μM, respectively.

Each ORR strain was also investigated for its response to a combination of metal ions (Cd^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and UO_2^{2+}). This was the same as those in COMM that were used in the enrichments, except that Al was omitted to avoid issues with precipitation. The strains are ordered in Table 3 in terms of their resistance to COMM, with EC_{50} values ranging from 0.9 to 0.2 times the COMM enrichment concentration. The strain most sensitive to COMM was the *Castellaniella* strain MT123 (EC_{50} of 0.24$\times$), and it also had the lowest EC_{50} values for Co, U, Cr (not present in COMM), and nitrite. (Table 3). Interestingly, the *Pantoea* sp. strain MT058, which was one of two strains that were isolated from a noncontaminated source and was the only strain isolated from sediment (the other six were from groundwater), had the second highest EC_{50} value to COMM (0.7$\times$).

Clearly, the properties of the groundwater from which the strains were isolated are not a good indicator of their metal resistance. For example, the two *Paenibacillus* sp. strains, MT086 and MT124, were both isolated from the most highly contaminated well (FW126) but had very different EC_{50} values for COMM (0.3$\times$ versus 0.9$\times$). This result is also supported by their resistance to individual metals, where MT124 was shown to be more resistant than MT086 to Cr, Cd, Cu, Ni, and Mn (Table 3). It is curious that MT124 is highly resistant to these metal cations, as it was enriched in the presence of U (200 μM) rather than COMM. In any event, it is obvious that there is large variation in the metal resistance properties of the seven ORR strains and that this is independent of the source groundwater.

Effects of multiple metals on nitrate reduction rates. A key issue to address was whether a mixture of metals reflecting the contaminated ORR environment affected the ability of any of the ORR strains to reduce nitrate. The seven ORR strains were grown in the presence and absence of COMM under denitrifying conditions with a carbon source that supported increased growth for each strain when nitrate was present (Table S2). Cells were harvested and whole-cell nitrate and nitrite reductase levels, measured by the production or disappearance of nitrite, respectively, were determined. As shown in Fig. 3, there was a 60-fold variation in the specific nitrate-reducing activities of the seven strains (in the absence of COMM). The values ranged from 5.4 $\pm$ 0.7 units/OD_{660}

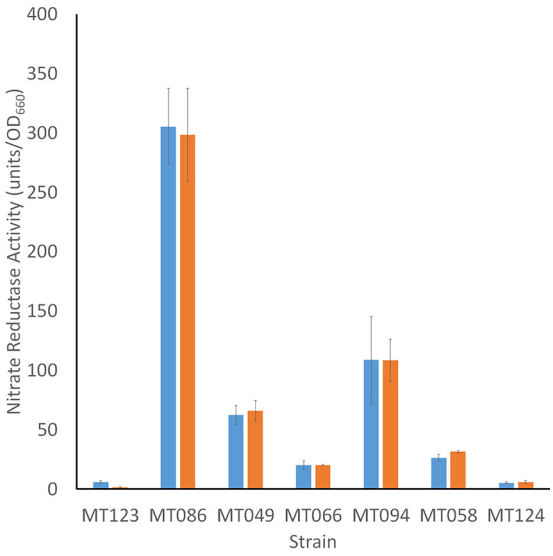

FIG 3 Whole-cell nitrate reductase activities of ORR strains grown in the absence (blue) and presence (orange) of COMM. Strains were grown anaerobically with 20 mM nitrate, harvested, and assayed for whole-cell nitrate reductase activity. Average values and standard deviations between the biological replicates are shown. The values for MT123 are likely underestimated due to the nitrite reductase activity observed for the strain. The carbon sources and pH values used in the growth medium for each strain are listed in Table S2. Strains are MT049 (*Serratia*), MT058 (*Pantoea*), MT066 (*Bacillus*), MT086 (*Paenibacillus*), MT094 (*Bacillus*), MT123 (*Castellaniella*), and MT124 (*Paenibacillus*).

for MT124 to 305 ± 32 units/OD_{660} for MT086, both of which are *Paenibacillus* strains. There does not appear to be any relationship between nitrate reduction rate and nitrate resistance (Table 3 and Fig. 3). When the ORR strains were grown in the presence of COMM, six of the seven strains had nitrate reductase activities similar to those measured when they were grown in its absence (Fig. 3). Only the *Castellaniella* sp. strain MT123, the strain that was most sensitive to COMM (Table 3), showed a significant decrease (from 6.0 ± 1.3 to 1.5 ± 0.6 units/OD_{660}). MT123 was also the only strain to have detectable nitrite reductase activity. All of the other six strains lacked detectable nitrite reductase activity, indicating that these strains reduce nitrate and excrete the nitrite that is generated. When grown in the absence of COMM, the nitrite reductase activity of MT123 was 22.0 ± 12.5 units/OD_{660}, and when grown with the metal mixture it was 38.5 ± 3.6 units/OD_{660}. Since nitrite production is used to determine nitrate reductase activity in this assay, the presence of nitrite reductase activity for MT123 likely causes the nitrate reductase activity of this strain to be underestimated. Conversely, with the exception of MT123, the nitrate-reducing activities of the ORR strains shown in Fig. 3 are likely a true reflection of their ability to reduce nitrate, and clearly there is huge variation between the strains. More importantly, these are largely unaffected by high concentrations of multiple metals.

An inverse linear relationship ($r^2 > 0.99$) between the specific nitrate reductase activity and nitrite resistance (up to 80 mM) for the four Gram-positive strains (MT066, MT086, MT094, and MT124) was observed (Fig. S1). Since none of these strains have nitrite reductase activity, one explanation for this is that the strains with higher rates of nitrite production from nitrate are tuned to survive in electron donor-limited environments where competition for both nitrate and nitrite is high and neither is expected to accumulate.

Matching metal-resistant strains to the ORR environment. In a previous survey of the ORR environment, groundwater samples were taken from 93 contaminated and noncontaminated wells and 16S rRNA gene sequence data for the V4 region were obtained (27). We used these data to search for ESV matches using the V4 region of the 16S rRNA gene sequences of the seven ORR strains described here. Of the seven strains, the two *Paenibacillus* strains (MT086 and MT124) did not have ESV matches in the ORR

TABLE 4 Abundance of ESVs in a survey of ORR wells

Strain	Closest species	Rank	No. of wells	Highest abundance[a] (%)	Well[a]	ESV rank[a]
MT123	*Castellaniella defragrans*	177	32	7.00	FW104	4
MT094	*Bacillus cereus*	412	23	1.40	FW109	10
MT058	*Pantoea ananatis*	2,301	15	0.46	GW621	14
MT049	*Serratia proteamaculans*	3,273	7	0.65	GW537	12
MT066	*Bacillus* sp. strain FJAT-18043	>5,000	1		GW537	

[a]These columns refer to the abundance of each strain in the indicated contaminated well.

survey but the other five did. However, there was great variation in the abundance of these five ESVs. For example, the ESV for *Bacillus* strain MT066 was found in only one of the 93 wells, whereas the *Castellaniella* strain MT123 ESV was present in 32 different wells and was the 177th most common ESV found in the survey (Table 4). In particular, MT123 constituted 7% of the ESV reads in well FW104, a highly contaminated well near the S-3 ponds from which MT123 was isolated. As shown in Fig. 4, the ESV matches of all five ORR strains were found in wells that spanned a wide range of both pH values (from 3.1 to 10.4) (Fig. 4A) and uranium concentrations (0.007 to 143 μM) (Fig. 4B). In fact, for all four strains found in multiple wells (MT049, MT058, MT094, and MT123), each was found in both noncontaminated (pH ~7.0, U < 0.126 μM) and highly contaminated wells (pH <4.0, U > 70 μM).

The ESV abundance at each well location is shown for MT123 (Fig. 5), MT049 (Fig. S2), MT058 (Fig. S3), and MT094 (Fig. S3). As shown in Fig. 5, the wells span a 11-km-by-1-km area within the Bear Creek Valley containing the S-3 ponds. The wells from which the four ORR strains (for which ESV data are available) were isolated are marked with a white pointer, and the S-3 ponds are indicated with a red square (Fig. 5). Two of the strains were isolated from contaminated wells within 10 m of the S-3 ponds (MT123 and MT094), while MT049 was isolated from a noncontaminated well and MT058 was isolated from a pristine sediment sample that was taken 7 km from the S-3 ponds. Three of the strains (MT058, MT094, and MT123) had ESV matches in the samples they were isolated from, and the other (MT049) was matched to a well (GW537) with 0.65% of the ESV reads, which was less than 500 m from the isolation well. In terms of geography, based on the ESV data, all four strains were found in both noncontaminated and contaminated wells. In fact, the two strains isolated from contaminated wells (MT094 and MT123) were both found in at least one pristine well in a defined site with no known contamination sources that is located in the far south of the site, some 7 km from the S-3 ponds. All four strains were also located in a least one highly contaminated well next to the S-3 ponds. For example, the highly contaminated wells include FW106,

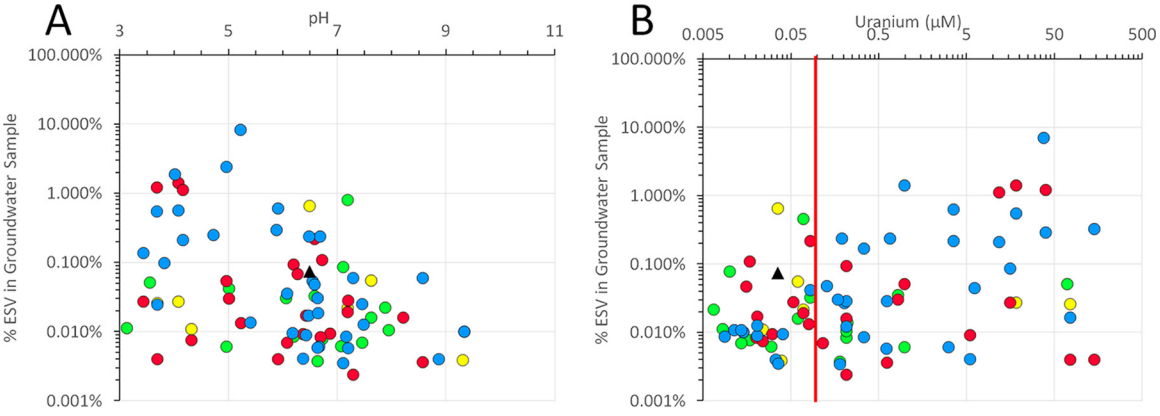

FIG 4 ESV abundance of each ORR strain in ORR wells with different pH values (A) and uranium concentrations (B). The 16S rRNA gene data set from a survey of 93 ORR noncontaminated and contaminated wells was searched for the ESV of each of five ORR strains: MT049 (yellow), MT058 (green), MT066 (black), MT094 (red), and MT123 (blue). The red line in panel B represents the U division between contaminated and uncontaminated at 0.126 μM. % ESV values below 0.010% are not quantitative. Strains are MT049 (*Serratia*), MT058 (*Pantoea*), MT066 (*Bacillus*), MT094 (*Bacillus*), and MT123 (*Castellaniella*).

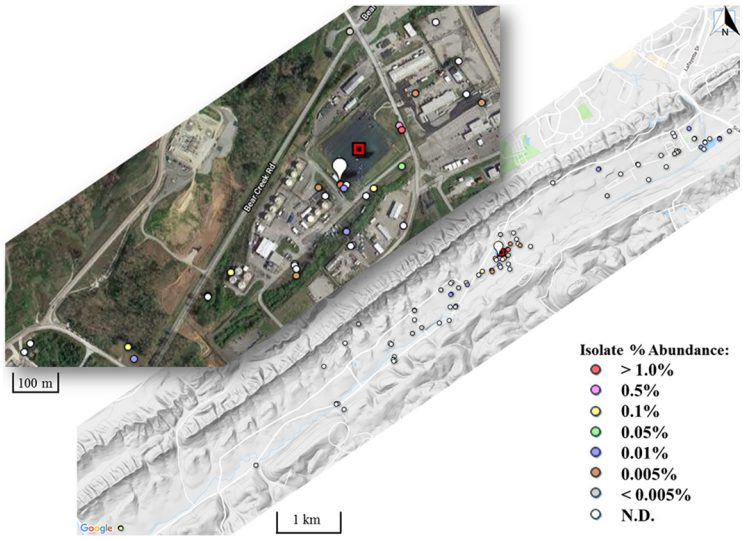

FIG 5 Location of ORR wells with ESV matches to ORR strain MT123 (*Castellaniella*). The 93 wells that were analyzed for ESV matches to MT123 are indicated as dots on a topography map of the ORR site. The S-3 ponds are marked with a red square, and dots are colored based on the percent abundance of the ESV for the indicated strain in a particular well, with white indicating no ESV match detected. Percent ESV values below 0.01% are not quantitative. A white pointer is used to show the isolation source of MT123. Map data are from Google. Zoom map data are from Google Imagery, DigitalGlobe, the U.S. Geological Survey, and the USDA Farm Service Agency.

FW109, and FW104, all within 10 m of the S-3 ponds. MT049 made up 0.026% of the ESV reads in FW106, MT058 made up 0.051% of the reads in FW106, MT094 made up 1.21% of the reads in FW109, and MT123 made up 7.0% of the reads in FW104. Mapping the location of the isolates by ESV back to the ORR environment showed that strains capable of surviving in the contaminated environment were not restricted to this specialized environment. Additionally, enrichment and isolation of nitrate-reducing strains under environmentally relevant contaminating conditions produces metal-resistant strains, even from the noncontaminated environment.

DISCUSSION

The contamination plumes extending from the S-3 ponds at ORR represent an extreme example of a human-made contaminated environment. The site provides a unique opportunity to study the impact of high concentrations of both nitrate and of multiple metals on microbial communities. For example, nitrate concentrations as high as 50 g/liter in the main nitrate-containing plume at the ORR site were estimated using three-dimensional resistivity tomogram measurements (11), and direct chemical analysis of groundwater taken from contaminated wells within the plumes revealed nitrate concentrations as high as 11.6 g/liter (190 mM) (12). For comparison, the nitrate concentrations in the other contaminated environments are about 2 orders of magnitude less. These include the Chesterville Branch Watershed, MD, affected by agricultural fertilizer practices (10 mg/liter) (3), and the Shiraz alluvial aquifer under the city of Shiraz, Iran, which is highly contaminated with nitrate from agricultural and industrial activities and has nitrate concentrations as high as 149 mg/liter (6). The U.S. Environmental Protection Agency's (EPA) maximum contaminant level (MCL) for nitrate in drinking water is 10 mg/liter.

Similarly, in terms of contamination by a mixture of metals, the ORR site has unique properties in terms of their numbers and their concentrations. For example, direct chemical analysis of contaminated wells near the S-3 ponds show elevated concentrations of many metals, including Al (560 mg/liter), Mn (170 mg/liter), U (140 mg/liter), Ni (9.4 mg/liter), Co (1.8 mg/liter), Cd (1.1 mg/liter), Cu (0.95 mg/liter), and Fe (0.55 mg/liter) (12). Comparison with other contaminated sites is difficult, as data are typically

available for effluents prior to their discharge into the environment. For example, metallurgic effluent from stainless steel pickling contains much higher concentrations of Fe (133.2 mg/liter) and Ni (30.3 mg/liter), but these values are prior to discharge into the environment (23). Similarly, in acid mine drainage (AMD) sites, such as the Pikeville AMD site in Kentucky, the effluent before it is released into a local stream has higher concentrations of Fe (2.88 mg/liter) than in the ORR plume but much lower concentrations of other metals, such as Mn (1.25 mg/liter) and Al (0.641 mg/liter) (28). At the Widows Creek AMD site in Tennessee, influent concentrations of Fe (474 mg/liter) are much higher than those at the ORR site, but Mn (9.4 mg/liter), Al (1.8 mg/liter), and Cd (0.04 mg/liter) levels are much lower (29). The Midnite mine in Washington is an inactive open-pit uranium mine, and water samples contained levels of Mn (143 mg/liter) and Co (1.6 mg/liter) similar to those of ORR but lower concentrations of U (24 mg/liter), Ni (2.7 mg/liter), Cu (0.18 mg/liter), Fe (0.18 mg/liter), and Cd (0.05 mg/liter) (30). Sediment samples taken from uranium waste piles in Johanngeorgenstadt, Germany, contained higher concentrations of Fe (760 mg/liter) and Cu (6.6 mg/liter) than ORR but similar concentrations of Al (680 mg/liter), Ni (5.0 mg/liter), and Co (2.4 mg/liter), with lower Mn (50 mg/liter) and U (3.0 mg/liter) concentrations (31).

The seven metal-resistant strains characterized in this study were all isolated from ORR groundwater or sediment using enrichments that mirrored the metal concentrations in the contaminated environment. It is important to emphasize that the various metals can be subdivided into different categories and that these differ dramatically in their chemical properties. For example, most of the metals exist in the neutral-acidic pH groundwater as soluble divalent cations, including Cu^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Mn^{2+}. In contrast, some, such as Al^{3+} and Fe^{3+}, are trivalent cations, and these form insoluble hydroxides at neutral pH and are mostly soluble in groundwater below pH 5.5 and 3.0, respectively. In further contrast, uranium is present as the soluble oxycation UO_2^{2+}, while chromium is in the form of the soluble oxyanion CrO_4^{2-}. The sensitivities of the seven new strains to these different metals are shown in Table 3, where the metals are grouped in terms of their chemical properties. Of the divalent cations, in general all seven strains had similar EC_{50} values with increasing resistance in the order Cu < Cd < Co < Ni < Mn. For the oxycation UO_2^{2+}, all strains except for the *Castellaniella* sp. strain MT123 were highly resistant, but EC_{50} values for the oxyanion CrO_4^{2-} spanned a large range. Interestingly, MT123 was highly sensitive to the oxyca-tion (UO_2^{2+}) and the oxyanion (CrO_4^{2-}) but was more resistant than most of the other strains to the two more highly toxic divalent cations, Cu^{2+} and Cd^{2+}. The optimal growth pH of MT123 in which the metal resistance growth studies were conducted was lower than that of the other strains (pH 5.5 versus pH 6.0 to 7.0). The toxicity of U and Cr can vary greatly with pH in complex organic media as a result of changing speciation in forming different phosphate, sulfate, and hydroxide ions (26, 32).

Five of the strains were obtained from contaminated groundwater, but two, the *Pantoea* sp. strain MT058 and the *Serratia* sp. strain MT049, were isolated from noncontaminated sediment and groundwater, respectively, thereby demonstrating that nitrate- and metal-resistant strains are present in noncontaminated environments. Interestingly, one of these strains, MT058, was the second most resistant strain to COMM (EC_{50} of 0.7×). The ESV for MT058 was found not only in several noncontaminated wells but also in four contaminated wells containing over 5 μM uranium, with one having 50 μM uranium (27). In fact, the ESVs of four of the seven strains we isolated (MT049, MT058, MT094, and MT0123) were found in multiple ORR wells, and all four were found in both noncontaminated wells and at least one well contaminated with over 15 μM uranium. Therefore, the method used here for enriching metal-resistant bacteria under environmentally relevant metal concentrations was successful even when starting with noncontaminated environmental samples.

The wide pH range for growth of the metal-resistant strains mirrored the pH values of wells giving rise to ESV matches (Fig. 4A). For example, MT123 had a pH optimum of 5.5 and constituted 7% of the ESV matches in a groundwater well that had a pH of 5.2 (FW104). The same strain (MT123) was also present at lower levels in wells that

ranged in pH from 3.4 to 9.8. On the other hand, two *Paenibacillus* sp. strains (MT086 and MT124) did not have any ESV matches in the ORR groundwater survey. It is possible that *Paenibacillus* DNA was not detected in the 16S rRNA gene sequence data, as *Paenibacillus* species are spore-forming organisms. Additionally, these two strains were isolated from well FW126, which has one of the lowest pH values (3.0) and highest nitrate and metal concentrations of all of the ORR-contaminated wells (Table 1). Such conditions could promote spore formation and/or complicate DNA extraction from the groundwater samples (33), although various *Paenibacillus* species have previously been detected by 16S rRNA gene sequence in ORR contaminated sediment (34). Future groundwater 16S rRNA surveys at ORR could benefit from the use of an internal spore control to determine the efficiency of DNA extraction from spores in the highly variable groundwater matrixes.

One of the unique aspects of this study is that it investigates nitrate reduction at ORR in combination with multiple-metal resistance rather than focusing on nitrate reduction or resistance to a single or small number of metals. A review studying nitrate-reducing bacteria at ORR combined microbial community data from 24 different studies (25). The review identified 32 potential nitrate-reducing genera that significantly overlapped the isolates from this study (25). In fact, nitrate-reducing *Castellaniella* and *Paenibacillus* were both identified in studies in which reverse-transcribed total RNA was amplified to specifically look at the active microbial community at ORR (25, 35, 36). A *Castellaniella* species was also identified in an ethanol biostimulation experiment on a nitrate-contaminated well as potentially important for *in situ* nitrate removal at ORR (37). Several *Bacillus* and other species were isolated from contaminated sediment at ORR that were subsequently screened for resistance to Pb, Hg, Cr, Cd, and U. However, this study was different from the current study in that the strains were not characterized under nitrate-reducing conditions, and the enrichments were not per-formed in the presence of metal concentrations reflecting the contaminated environ-ment (38).

In our study, seven new strains were obtained from the ORR environment that were able to obtain energy for growth by reducing nitrate in the presence of multiple metals, namely, Al, Mn, Fe, Co, Ni, Cu, Cd, and U, at concentrations that approximate those found at the ORR contaminated site. In fact, nitrate reduction rates were similar for the seven isolates when grown with or without the COMM metal mixture (Fig. 3). Various metal ions at high enough concentrations are known to disrupt oxidoreductase-type enzymes, such as nitrate reductase, and respiratory pathways, such as denitrification. For example, in a study of denitrification rates in saltmarsh sediments, it was reported that initial denitrification rates were inhibited by several metals at 1 g/liter, including Pb, Ni, Cr, Zn, Cu, Fe, and Cd (24). Similarly, whole-genome fitness assays conducted on a model denitrifying organism, *Pseudomonas stutzeri* RCH2, grown under denitrifying conditions, revealed that disruptions to nitrate reductase and denitrification-related genes resulted in decreased fitness of the organism when grown in the presence of elevated concentrations of several different metals, including Cu, Zn, Cr, and U (39, 40). The ORR organisms that we isolated must therefore have molecular and physical mechanisms of metal resistance that allow them to survive in the extreme ORR environ-ment, and the nature of those mechanisms is currently under study.

Overall, the nitrate- and metal-contaminated ORR site surrounding the S-3 ponds is an extreme environment from which we were able to isolate seven new nitrate-reducing bacteria that are individually and simultaneously resistant to a combination of metals, including divalent cations, trivalent cations, oxycations, and oxyanions. These strains exhibited diverse properties in terms of pH optima and pH range for growth, carbon source preference, and degree of metal tolerance. Several of these strains were also detected by 16S rRNA gene sequences in a variety of noncontaminated and contaminated ORR wells. However, all of the strains retained nitrate reductase activity when grown in the presence of multiple metals at environmentally relevant concentrations. Future studies on the molecular mechanisms of metal resistance these strains

contain will likely uncover unique properties that will further our understanding of microbial communities in other nitrate- and metal-contaminated sites.

MATERIALS AND METHODS

Enrichment cultures. High-throughput enrichment cultures (1.75 ml in volume) were set up in autoclave-sterilized 2-ml Fisherbrand 96-well DeepWell polypropylene microplates (Waltham, MA). Base medium contained 0.25 g/liter NH_4Cl, 0.1 g/liter KCl, 0.5 g/liter $MgSO_4 \cdot 7H_2O$, 0.37 g/liter $CaCl_2 \cdot 2H_2O$, 0.25 g/liter NaCl, 2.5 g/liter $NaHCO_3$, and 0.7 g/liter $NaH_2PO_4 \cdot H_2O$, as well as vitamins and trace elements prepared as described by Widdel and Bak (41). The pH of the base medium was adjusted using HCl or NaOH to the indicated pH before filter sterilization. Medium that was adjusted to pH 5.5 also contained 10 mM morpholineethanesulfonic acid buffer. Where indicated, the base medium also contained 1 g/liter yeast extract. The various carbon sources used in the enrichment cultures were added as sterile stock solutions to the bottom of the microplate wells, resulting in final concentrations of 10 mM glucose, 10 mM cellulose, 20 mM lactate, 20 mM fumarate, 20 mM pyruvate, 30 mM ethanol, 30 mM acetate, 100 mM formate, or 0.5%, vol/vol, compost tea. A mixture of 10 mM lactate and 10 mM fumarate was also used for some enrichments. Electron acceptors $NaNO_3$ and $MgSO_4 \cdot 7H_2O$ were also added as sterile stock solutions to the bottom of the microplate wells where indicated, resulting in the given final concentra-tions. Environmentally relevant metals (COMM) were also added to the bottom of the microplate wells using a 10 mM sterile solution of uranyl acetate and a sterile 100× metal mix solution (described below). For enrichments containing COMM, $AlK(SO_4)_2 \cdot 12H_2O$ powder was suspended in the medium at the desired concentration before adding base medium to the microplate wells. Depending on the pH of the base medium, the Al did not always dissolve completely. Once the base medium was added to the microplate wells containing the various additions, each well was inoculated with 50 to 150 μl of the indicated ORR groundwater sample or 0.1 g of the indicated ORR sediment sample. The microplates were then covered with PHENIX sterile microporous sealing films (Candler, NC) and incubated in the dark at 22°C. Anaerobic enrichment incubations were carried out in sealed anaerobic jars under an atmosphere of 20% CO_2 and 80% N_2, while microaerophilic enrichments where similarly incubated but with approximately 3% O_2 in the gas phase, which was exchanged every 24 h. The enrichments were routinely checked for growth over the course of 3 months. Isolates were obtained from enrichments displaying growth by streak plating on solid media similar to the composition of the enrichment culture or using a rich LB medium and culturing isolated colonies.

The 1× COMM is defined as containing 1 mM $AlK(SO_4)_2 \cdot 12H_2O$, 100 μM $UO_2(CH_3COO)_2$, 100 μM $MnCl_2 \cdot 4H_2O$, 100 μM $NiSO_4 \cdot 6H_2O$, 30 μM $CoCl_2 \cdot 6H_2O$, 10 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 10 μM $CuCl_2 \cdot 2H_2O$, and 5 μM $Cd(CH_3COO)_2 \cdot 2H_2O$. The Al and U in the COMM were added to cultures separately as a dry powder and a 10 mM filter-sterilized stock solution, respectively. The remaining metals were added to cultures using a 100× filter-sterilized stock solution. This solution was either prepared fresh or stored in one-use aliquots at − 80°C. As indicated, permutations of the COMM were used in some experiments in which Al and/or U were removed from the mix or were added at lower concentra-tions.

The compost tea was prepared from finished compost obtained from the ACC Commercial Composting Facility (Athens, GA). Finished compost (1.0 kg) was mixed with 5 liters of distilled H_2O and allowed to steep for 4 days at room temperature in a sealed glass container. The resulting compost tea was filtered through cheesecloth before filter sterilization with a Millipore express 0.22-μm filter (Burlington, MA).

Growth curves. High-throughput growth curves (400 μl in volume) were conducted using a Thermolab Scientific Equipments Bioscreen C automated microbiology growth curve analysis system (Maharashtra, India). For anaerobic growth, the Bioscreen C was placed in a Plas Labs (Lansing, MI) hard glove box with an atmosphere of 5% H_2 and 95% Ar. Growth curves were conducted at 22°C with normal speed shaking for 15-s intervals with 10 s between shaking. Growth was monitored at an optical density of 600 nm (OD_{600}) every hour. The same base medium used in the enrichment experiments was used for the growth curves, with additions including carbon sources, electron acceptors, and metals being handled in the same fashion. The carbon source and pH conditions of the media used for each strain are described in Table S2 in the supplemental material. All growth curves containing COMM at the indicated concentrations lacked Al, as there were precipitation issues that interfered with OD readings. Growth data were analyzed using the grofit package (42). The model-free spline fit was used to determine growth rates for each curve, which were then used to fit dose-response curves and calculate EC_{50} values when applicable. The 95% CI were determined using a bootstrap value of 100. No yeast extract was added to growth curve experiments.

Nitrate and nitrite reductase assays. Whole-cell nitrate and nitrite reductase assays were per-formed on cells grown in the presence and absence of 1× COMM (43). In the case of strain MT123 grown with COMM, the concentration of U was decreased to 10 μM instead of 100 μM due to the sensitivity of MT123 to U. For the assays, cells were grown anaerobically in 50-ml cultures at room temperature without shaking on base medium containing 20 mM nitrate and 1 g/liter yeast extract to late-log phase. The carbon source and pH conditions of the media used for each strain are given in Table S2. Cells were harvested at 10,500 × *g* for 10 min before washing three times with 5 ml of assay buffer (50 mM potassium phosphate buffer, pH 7.2) and suspending in assay buffer to give a final OD_{600} value between 0.1 and 0.4. In a 15-ml Falcon tube, 200 μl of the suspended cells was combined using gentle mixing with 25 μl of freshly prepared 0.5 mg/ml methyl viologen and 75 μl of a solution containing 4 mg/ml sodium dithionite, 4 mg/ml sodium bicarbonate, and either 100 mM $NaNO_3$ for nitrate reductase assays or 1.5 mM $NaNO_2$ for nitrite reductase assays. The assay mixture was incubated at room temperature for 5

to 10 min before vortexing in air until the solution became clear to stop the reaction. One ml of 1% sulfanilic acid in 20% HCl was then added with vortexing, followed by 1 ml of a 1.3 mg/ml *N*-(1-naphthyl) ethylenediamine-HCl solution, forming a nitrite-dependent azo dye that is red in color. The OD_{540} and OD_{420} of the assay solution were taken to measure the azo dye and absorbance due to light scattering, respectively. Nitrate reductase activity is reported in units/OD_{660}, where units are calculated using the formula $100 \times [OD_{540} - (0.72 \times OD_{420})]/(T \times V)$, where T is time in minutes and V is reaction volume in milliliters (43, 44). Nitrite reductase activity is also reported in arbitrary units using the same equation, except the change in OD_{540} between the final assay solutions and a no-cell control is used in place of the OD_{540}. Errors are reported as the standard deviations between three biological replicates.

16S rRNA gene sequencing. Genomic DNA was isolated from each ORR strain using the ZymoBead genomic DNA kit (Irvine, CA). The 16S rRNA gene for each strain was amplified using EmeraldAmp GT PCR master mix (Kusatsu, Shiga Prefecture, Japan) with primers 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT). Amplicons were sequenced by GENEWIZ (South Plainfield, NJ), and the sequences were analyzed for closest 16S rRNA gene match using nucleotide BLAST.

Matching 16S rRNA gene sequences to ORR groundwater survey samples. The 16S rRNA gene sequences were taken from a previous groundwater survey of 93 different noncontaminated and contaminated ORR wells (27). Read-paired merged rRNA sequences were downloaded from MG-RAST (mgm4554509.3). Low-quality sequences (>1 expected error) were eliminated using usearch-fastq_filter, and the reads were trimmed to cover the region between the V4 primers with a custom Perl script. Usearch-fastx_uniques was used to count the number of times each ESV occurred in each sample. The ESVs were then ranked by total abundance across the samples, and only the top 5,000 ESVs were retained to reduce contamination. Usearch-search_exact was used to find exact matches between the isolate 16S rRNA sequences and the 100-well V4 regions. In many cases isolates matched to multiple ESVs in each sample, so the matching ESV with the most reads per sample from a given well was selected. The highest percent abundance calculation is based on the ESV reads per sample divided by the total number of sample reads. Only ESVs with >1 read per sample were used for calculations and plots; however, all ESVs were used for maps. These aggregated data were combined with the longitude and latitude of each well into a Google Fusion Table (Mountain View, CA) for geospatial maps. Abundances on maps below 0.01% are not quantitative and are shown for illustrative purposes.

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