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Physiological and Genomic Characterization of Two Novel Bacteroidota Strains *Asinibacterium* spp. OR43 and OR53

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Abstract: *Asinibacterium* spp. (Family Chitinophagaceae, Phylum Bacteroidota) are abundant in environments contaminated with heavy metals. We characterized the physiology and genome of two *Asinibacterium* species to elucidate their ability to survive and grow at ambient conditions in the uranium-contaminated environments. Both strains were able to grow at pH 4.5 or 50 mM nitrate under aerobic conditions and did not grow with alternative electron acceptors under anaerobic conditions. *Asinibacterium* sp. OR53 grew in medium with uranium concentrations up to 300 µM uranium while *Asinibacterium* sp. OR43 could not grow at uranium concentrations > 200 µM. Elemental mapping using energy dispersive X-ray spectroscopy indicate that uranium co-localized with phosphorus-containing compounds on the cell surface. Genes potentially encoding resistance mechanisms to a variety of heavy metals were detected in the genomes of both strains. The localization of uranium and missing acidic and alkaline phosphatase genes in the genome suggest that biosorption of uranium to the lipopolysaccharide layer might be the mechanism of uranium resistance. In summary, *Asinibacterium* spp. OR43 and OR53 are physiologically similar to closely related strains within the Chitinophagaceae family but are uniquely acclimated to the presence of uranium and other heavy metals prevalent in the subsurface at Oak Ridge, Tennessee.

Keywords: uranium resistance; Asinibacterium; genome

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1. Introduction

The family Chitinophagaceae in the phylum Bacteroidota contains among many other genera, the genera *Sediminibacterium*, *Hydrotalea*, and *Asinibacterium* [1–4]. Described strains belonging to these genera have been found in freshwater (*Sediminibacterium* and *Hydrotalea*), sediments (*Sediminibacterium* and *Hydrotalea*), soil (*Sediminibacterium*), and Donkey milk powder (*Asinibacterium*) [1–6]. *Sediminibacterium* spp. and *Hydrotalea* spp. are the closest relatives to *Asinibacterium lactis* [2]. Sequences closely related to *Sediminibacterium* spp. were detected in freshwater biofilms, eutrophic lakes, salt water, wastewater, and the gut of beetles and cows [7–15]. In addition, many sequences related to *Sediminibacterium* spp. were found in contaminated environments with high concentrations of organic compounds and/or heavy metals [16–20].

Asinibacterium spp. OR43 and OR53 (formerly called *Sediminibacterium* spp. OR43 and OR53) from the family Chitinophagaceae were isolated from the highly contaminated subsurface sediment at the Integrated Field Research Challenge (IFRC) in Oak Ridge, TN, USA [21]. The prevalence of members of the genera *Sediminibacterium* and *Asinibacterium* in contaminated environments suggests these microbes could have physiological and genomic characteristics that enable them to function in the presence of contaminants such as heavy metals. The ground water at the sampling site is contaminated with heavy metals such as cadmium, chromium, cobalt, nickel, and uranium, has a low pH (pH 3–3.5), and a high nitrate concentration (http://www.esd.ornl.gov/orifrc, accessed on 20 December 2021).

Many heavy metals are essential for metabolism in low concentrations, but can have damaging effects on the physiology of microbes in high concentrations. Effects include disrupting metabolic pathways by inhibiting protein enzymatic activity, displacing native metals from their binding sites, and oxidative stress caused by hydrogen peroxide (H_2O_2) production [22,23]. Microbes have developed mechanisms to decrease intracellular heavy metal concentrations and thereby avoid the damaging effects of heavy metals. The most common mechanisms to reduce the concentration of heavy metals are active transporters and efflux pumps [22–24]. Uranium is a heavy metal without a known biological function for the cell [25]. Thus far, no efflux pumps or transporters for the detoxification of uranium have been discovered. Therefore, microorganisms are likely to use alternative tolerance/detoxification mechanisms such as uranium as an electron acceptor (bioreduction), passive sorption to anionic groups in the cell wall (biosorption), and accumulation inside of the cell (bioaccumulation) or active precipitation outside of the cell (biomineralization) [25].

Here we present a study of the physiological and genomic attributes of *Asinibacterium* spp. OR43 and OR53, two strains isolated from the uranium-contaminated subsurface sediment at Oak Ridge, Tennessee [21]. The growth of both strains was tested under a variety of environmentally relevant conditions such as pH, nitrate, and uranium. The closed genome of *Asinibacterium* sp. OR53 and the draft genome of *Asinibacterium* sp. OR43 were analyzed and compared with an emphasis on the genes potentially related to the resistance of uranium and other heavy metals.

2. Results and Discussion

Phylogenetically *Asinibacterium* spp. OR43 and OR53 were most closely related to *Asinibacterium lactis* a strain isolated from Donkey milk [2] (Figure 1). Other close relatives include *Vibrionimonas magnilacihabitans* and *Hydrotalea* spp. isolated from freshwater and *Sediminibacterium* spp. isolated from soils and sediments [1,3–5,26]. Additionally, 16S rRNA gene sequences closely related to *Sediminibacterium* spp. were detected in contaminated soil and sediment environments [7–15,17–21,27–30]. In summary, most 16S rRNA sequences and closely related isolates to *Asinibacterium* spp. OR43 and OR53 were found in soils and sediments indicating that soils and sediments are their primary habitat.

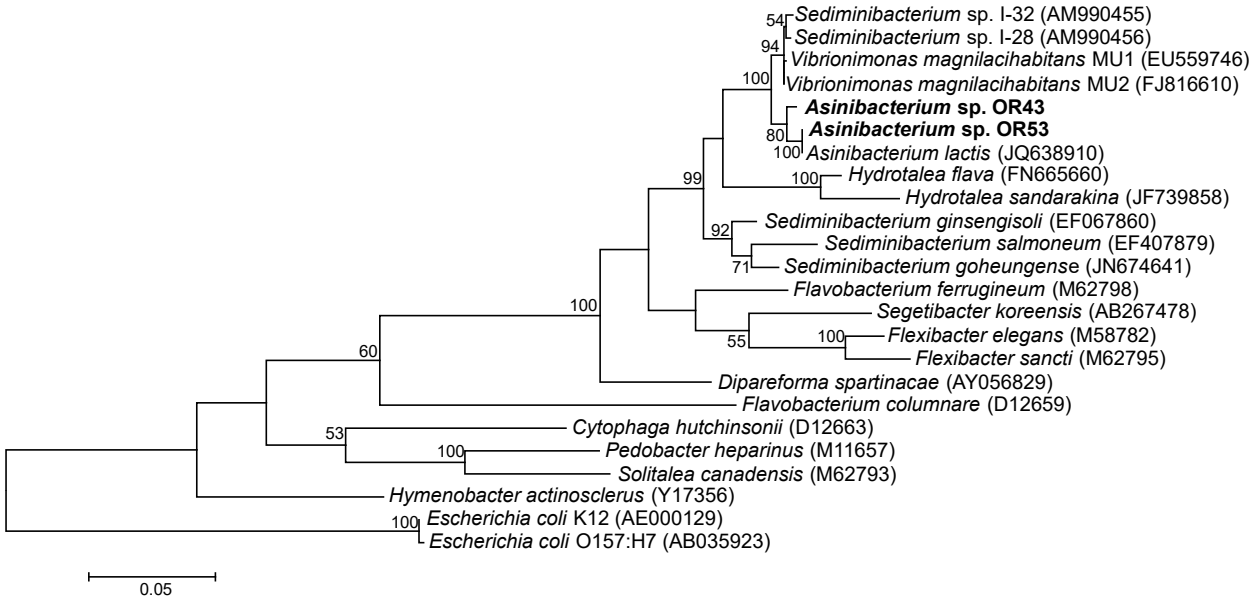

Figure 1. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic position of *Asinibacterium* sp OR43 and OR53. Numbers at the nodes represent bootstrap values > 50% after 100 replications.

2.1. Physiological Characterization

Physiologically, *Asinibacterium* spp. OR43 and OR53 were very similar to each other and to related cultured strains (Tables S1 and S2). Both strains were aerobic, did not use alternative electron acceptors under anaerobic conditions, had a low salt tolerance, and grew optimally at pH 5.5 to 7 and at temperatures between 27 and 30 °C (Table S1). Both strains utilized many different sugars as carbon sources (Table S1). *iso*-$C_{15:0}$, *iso*-$C_{15:1}$ G, and *iso*-$C_{17:0}$ 3-OH were the dominant fatty acids in both strains (Table S2). The physiological characterization shows that *Asinibacterium* spp. OR43 and OR53 behave very similarly to their closest cultured relatives (Tables S1 and S2) [1,3–5,26].

2.2. Growth in the Presence of Stress Factors

Asinibacterium spp. OR43 and OR53 were isolated from the IFRC area in Oak Ridge, TN [21]. The nitrate concentration in the ground water around the borehole near where *Asinibacterium* spp. OR43 and OR53 were isolated was 1541–1575 mg/L (24.9–25.4 mM), with a uranium concentration and pH between 15.97 and 17.03 mg/L (67.1 and 71.5 µM) and 2.99 and 3.52, respectively (http://www.esd.ornl.gov/orifrc, accessed on 20 December 2021). Growth experiments were conducted to evaluate the growth of the isolates under conditions similar to the original environment. Under aerobic conditions both strains grew with the highest growth rate at pH 5–7 (Figure 2A). The growth rates decreased at pH 4.5 and pH 8 and no growth was observed at pH lower than 4.5. In the presence of uranium, the growth rates decreased with increasing uranium concentrations (Figure 2B). *Asinibacterium* sp. OR43 grew in the presence of up to 200 µM uranium, and *Asinibacterium* sp. OR53 grew in the presence of up to 300 µM uranium (Figure 2B). Both strains grew in the presence of up to 50 mM nitrate (Figure 2C). These results indicate that *Asinibacterium* spp. OR43 and OR53 are able to withstand uranium and nitrate concentrations comparable to the concentrations in their original environment but are inhibited by environmentally relevant pH values.

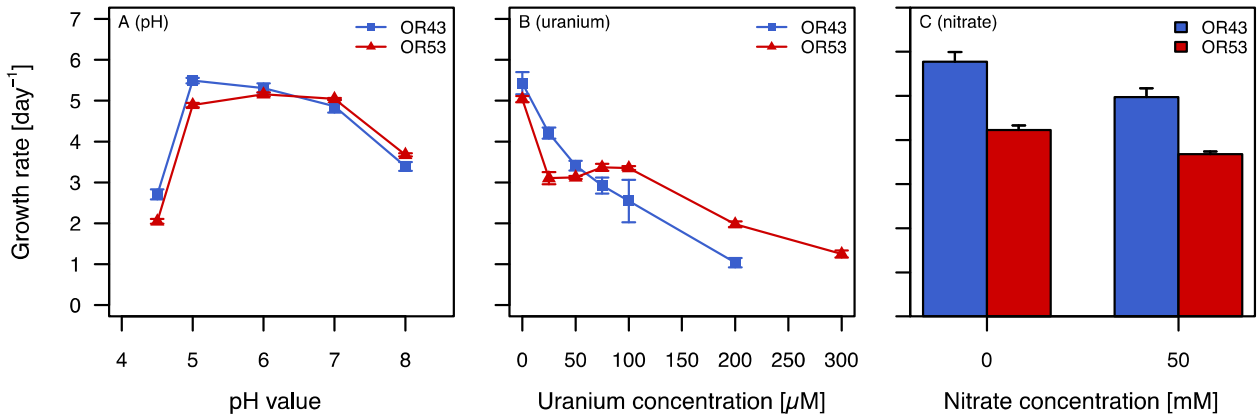

Figure 2. Growth rate (day $^{-1}$) of *Asinibacterium* spp. OR43 and OR53 in the presence of different pH values (**A**); uranium (**B**); and nitrate (**C**) concentrations (mean ± SD; n = 3–6). No growth was observed at pH < 4.5, at uranium concentrations > 200 µM (OR 43) and > 300 µM (OR 53), and at nitrate concentrations > 50 mM.

2.3. Growth of Asinibacterium sp. OR53 in the Presence of Uranium

Additional experiments were conducted to characterize the response of *Asinibacterium* sp. OR53 to elevated levels of uranium. *Asinibacterium* sp. OR53 was used due to its ability to grow at higher uranium concentrations than *Asinibacterium* sp. OR43 (Figure 2B). In addition, the strain was also an abundant member of an artificial consortium incubated in the presence of 200 µM uranium for 300 generations [31]. During growth experiments, a trend was discovered between the measured uranium concentration in the medium and the growth of *Asinibacterium* sp. OR53 over time (Figure 3). In the absence of uranium,

growth of *Asinibacterium* sp. OR53 started within a few hours after inoculation (Figure 3). In the presence of 300 µM uranium the culture had a lag phase of around 3 days before exponential growth started. The uranium (VI) concentration in the medium decreased from 220 µM to around 70 µM within four days and stayed at that level until the end of the experiment (day 7). The uranium concentration decreased also in medium controls without *Asinibacterium* sp. OR53, but the decrease was much slower, showing that the presence of *Asinibacterium* sp. OR53 accelerated the decrease in uranium (Figure S1). The results showed that the uranium concentration must decrease to around 90 µM before *Asinibacterium* sp. OR53 can grow (Figure 3).

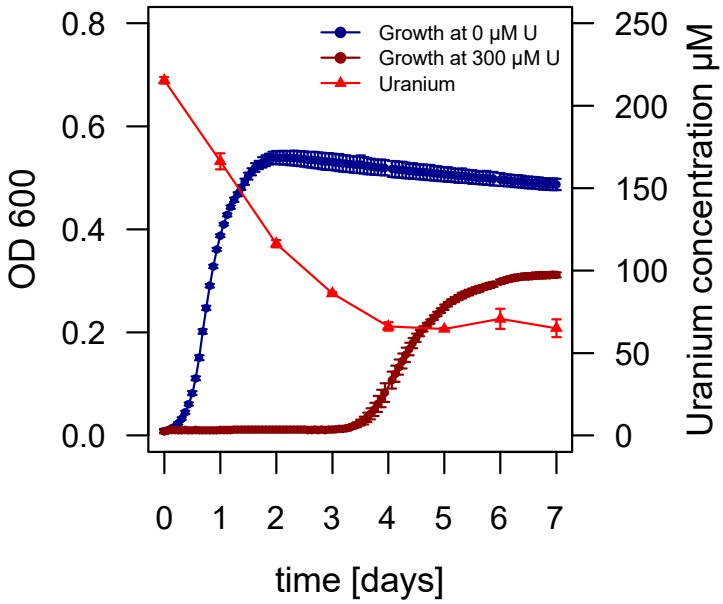

Figure 3. OD_{600} of *Asinibacterium* sp. OR53 grown in the absence (0 µM) and presence (300 µM) of uranium and the measured uranium concentration in the medium over time (mean ± SD; n = 3).

TEM analysis of unstained, whole mounted *Asinibacterium* sp. OR53 cells showed a higher signal contrast (Z-contrast) when grown in the presence of uranium (Figure 4B) than in the absence of uranium (Figure 4A) indicating an association of uranium with the cells. STEM and EDS were used on uranium-exposed cells to visualize uranium localization through elemental mapping. Bright-field STEM imaging revealed lighter, low signal contrast cells surrounding one distinctly higher signal contrasted cell (Figure 5A). EDS analysis showed that the distribution of uranium (Figure 5C) matched closely with the dark plaques present on the higher signal contrasted cell (Figure 5A). In addition, phosphorus localized with all cells, while uranium was only detected in the darkly contrasted cell (Figure 5A–C). Overlays of phosphorus and uranium resulted in bright co-localization of the two elements along cell peripheries (Figure 5D) suggesting that both elements were distributed across the cell surface. The distribution of the two elements across the cell and the bright co-localization of both elements at the periphery of *Asinibacterium* sp. OR53 cells suggest that this strain could use biomineralization or biosorption as a mechanism of uranium resistance. Biomineralization is an active mechanism that uses phosphate from phosphatases to precipitate uranium [25]. In contrast, biosorption is a passive mechanism where uranium binds to extracellular structures such as the LPS layer. In the LPS layer, uranium can be bound by different ligands in the cellwall such as carboxyl, amine, hydroxyl, and phosphoryl groups [25]. In *Pseudomonas aeruginosa* most of uranium is bound to phosphoryl groups while small amounts are bound to carboxyl groups in the LPS layer [32].

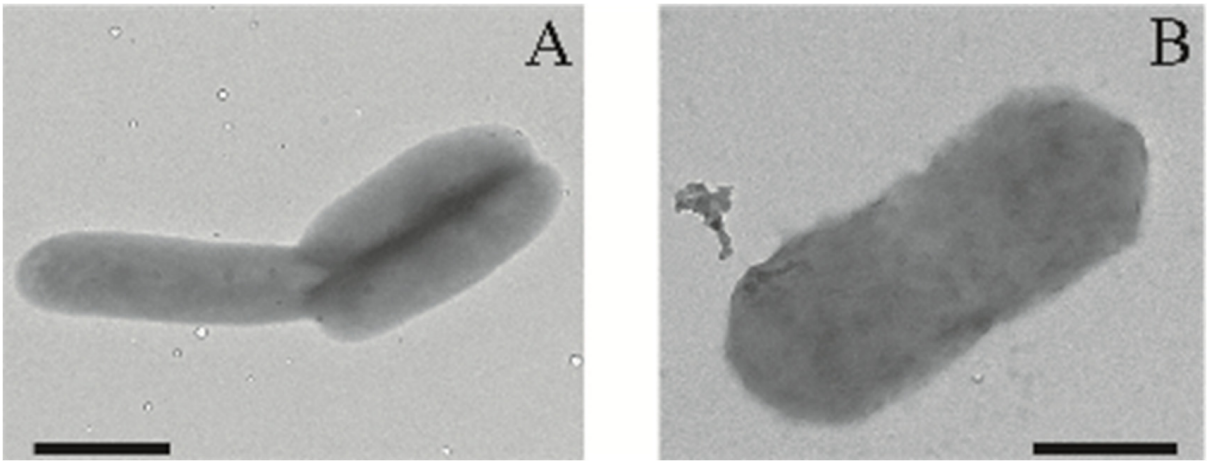

Figure 4. TEM micrographs of unstained, whole-mount cells of *Asinibacterium* sp. OR53 grown in the absence (**A**) and presence (**B**) of 300 μM uranium. Scale bars = 1.0 μm.

Interestingly, TEM micrographs and EDS analysis indicate that some cells did not have detectable levels of uranium (Figure 5). It is possible that *Asinibacterium* sp. OR53 cells that bind uranium above a certain level are no longer metabolically active or are severely debilitated. Healthy cells or cells with very low concentrations of bound uranium may initiate growth when the uranium concentration decreases below a certain threshold (Figure 3). Below the threshold *Asinibacterium* sp. OR53 may have additional mechanisms to withstand the toxic effects of uranium so that the cells do not need to bind uranium anymore. However, the cells still grow slower and produce less biomass in the presence of uranium than in the absence (Figure 3), indicating that withstanding the toxic effects of uranium negatively impacts their growth.

2.4. Genomes of Asinibacterium spp. OR43 and OR53

The genomes of *Asinibacterium* spp. OR43 and OR53 [33] were analyzed with the focus on genes involved in processes important at the IFRC: nitrate metabolism, pH homeostasis, and heavy metal resistance genes. General information about the genomes can be found in Tables S3–S5. The average nucleotide identity (ANI) calculated by the IMG database resulted in an identity between the two genomes of 96.4% indicating that the genomes belonged to the same species [34,35].

Genes involved in the nitrate metabolism: Denitrification is the reduction of nitrate to molecular nitrogen via the intermediates nitrite, nitric oxide, and nitrous oxide [36]. During dissimilatory nitrate reduction to ammonium (DNRA) nitrate is reduced to nitrite and then to ammonium [36]. Both processes are important processes in the global nitrogen cycle. *Asinibacterium* spp. OR43 and OR53 were missing the nitrate reductase (*napAB*) necessary to use nitrate as a terminal electron acceptor during denitrification and/or the DNRA pathway (Tables 1 and S6). Nitrite is reduced by the copper-containing nitrite reductase (*nirK*) [36], which was detected in both genomes. Both strains encoded the genes for the nitric oxide reductase (qNOR). qNOR in contrast to other nitric oxide reductases is not part of an operon and has been shown to be functional in pathogens and other microorganisms in the absence of the other enzymes of the denitrification pathway [37,38]. The final step of denitrification is catalyzed by the nitrous oxide reductase, a multi subunit enzyme. The nitrous oxide reductase (*nosZ*) is encoded in both genomes together with the *nosLDFY* cluster that encodes the copper sulfide center (CuZ center) and is important in electron transfer (Table S6) [39]. The detected *nos* genes are often found together with the *nosR* gene, a gene involved in the regulation of the nitrous oxide reductase activity [39], which was not detected in the *Asinibacterium* spp. genomes. Based on the genes present neither strain can use nitrate. The absence of potential genes for nitrate reduction was

supported by the inability of both strains to grow under anaerobic conditions with nitrate as a terminal electron acceptor (Table S1). *Asinibacterium* spp. OR43 and OR53 were isolated from an environment with a relatively low nitrate concentration and both strains were capable of withstanding environmental nitrate concentrations under laboratory conditions (Figure 2C) [21]. However, strains closely related to *Asinibacterium* spp. OR43 and OR53 were detected with molecular methods in areas with nitrate concentrations higher than 700 mM [16]. These strains might have a higher tolerance than *Asinibacterium* spp. OR43 and OR53 to nitrate or live in association with nitrate-reducers that keep nitrate concentrations low for surrounding microorganisms.

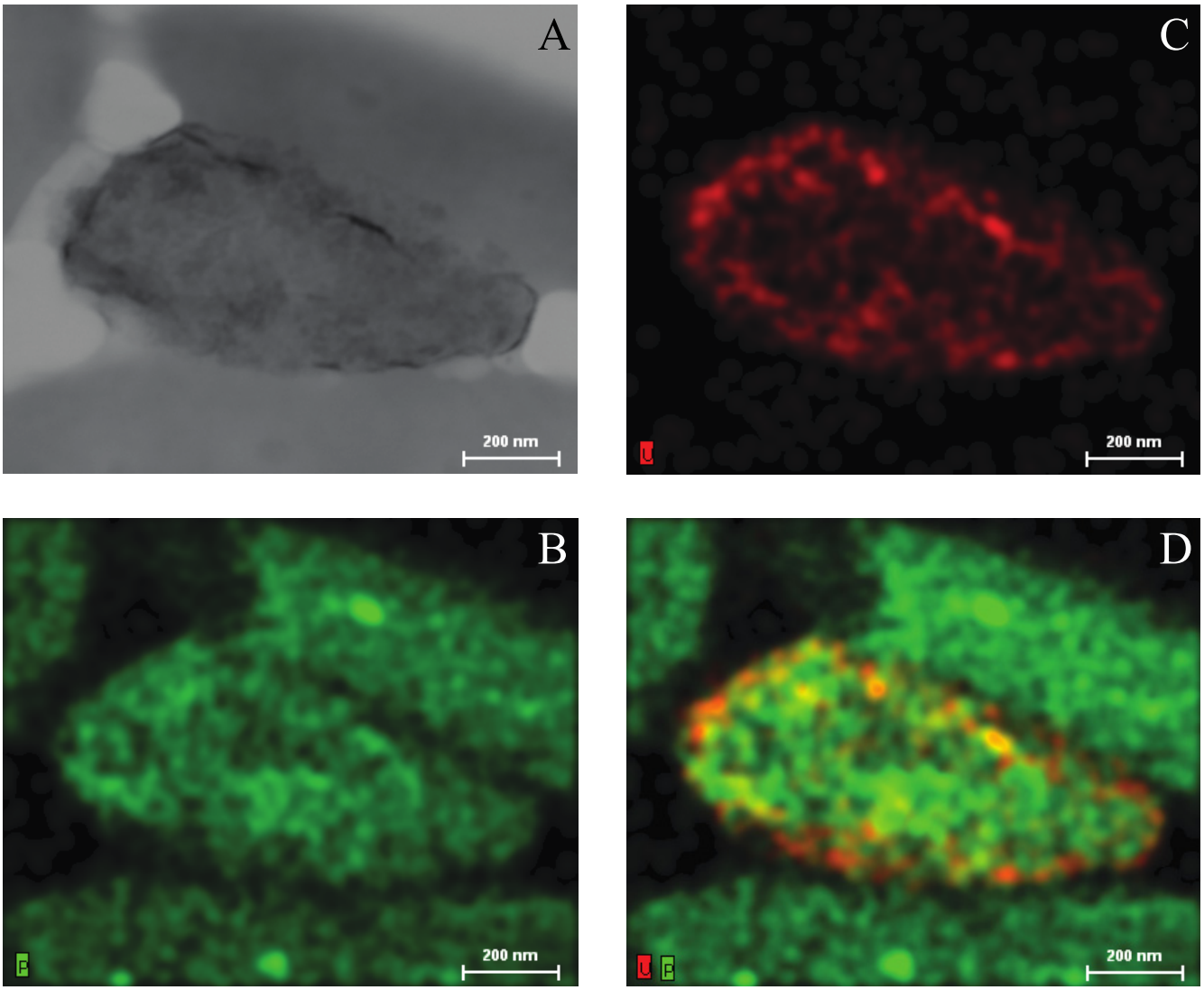

Figure 5. Elemental analysis of whole-mounted cells of *Asinibacterium* sp. OR53 grown in the presence of 300 µM uranium. Bright-field STEM micrograph of *Asinibacterium* sp. OR53 cells (**A**); an EDS mapping of the distribution of elemental phosphorus (green) (**B**); the distribution of elemental uranium (red) (**C**); and the distribution of elemental phosphorus and uranium (**D**). Scale bars = 0.2 µm.

Table 1. Presence (+) and absence (-) of genes involved in nitrate metabolism in *Asinibacterium* spp. OR43 and OR53 genomes.

Gene Product Name	Gene Symbol	OR43	OR53
Nitrate/nitrite transporter			
Nitrite/nitrate transporter	*narK*	+	+
ABC transporter	*nrtABC*	+	-
Assimilatory nitrate reduction			
Ferredoxin nitrate reductase	*narB*	+	+
Dissimilatory nitrate reduction			
Nitrite reductase	*nirBD*	+	+
Denitrification			
Nitrate reductase	*napAB*	-	-
Nitrite reductase (copper containing)	*nirK*	+	+
Nitric oxide reductase (qNOR)	*norC*	+	+
Nitrous oxide reductase	*nosZLDFY*	+	+

Genes potentially involved in pH homeostasis at low pH: F-type ATPases (Atp) are capable of proton export and were shown to be important for *Listeria monocytogenes* to persist in acidic environments [40,41]. F-type ATPases often work concurrently with potassium transporters (Kup) to balance membrane potential during proton extrusion [42–44]. *Asinibacterium* spp. OR43 and OR53 had all genes necessary for a functional F-type ATPase (*atpABEFH*, *atpC*, *atpD*, and *atpG)* in addition to one copy of the high affinity potassium transporter (*kdpABC*) and two copies of the low affinity potassium transporter (*kup*) (Tables 2 and S7). Both strains were able to grow at pH values as low as 4.5 (Figure 2A). The ability to tolerate pH 4.5 is very likely due to the presence of the F-type ATPase (Atp) and the low affinity potassium transporter (Kup). Bacteria that are able to withstand pH < 3 typically have genes that encode acid resistance such as lysine-, glutamine-, or arginine-decarboxylases [45], which are not present in the genomes of *Asinibacterium* spp. OR43 and OR53. These results indicate that *Asinibacterium* spp. OR43 and OR53 are neutrophilic bacteria, which is consistent with previously cultured relatives [1–5,26]. In the acidic environment at Oak Ridge, *Asinibacterium* spp. OR43 and OR53 could thrive in niches with a higher pH or in consortia with bacteria that can increase the local pH.

Table 2. Number of genes potentially involved in pH homeostasis at low pH in *Asinibacterium* spp. OR 43 and OR53.

Gene Product Name	Gene Symbol	OR43	OR53
F-type proton translocating ATPase	*atpG*	1	1
	atpCD	1	1
	atpBEFHA	1	1
High affinity potassium transporter	*kdpABCD*	1	1
Low affinity potassium transporter	*kup*	2	2

Genes Potentially involved in Uranium Resistance: Phosphatases cleave organo- and polyphosphate compounds [46,47] to release inorganic phosphate. The activity of acidic and alkaline phosphatase has been shown to correlate with the formation of extracellular or cell-associated uranyl phosphate precipitates during biomineralization [46–50]. The *Asinibacterium* spp. OR43 and OR53 genomes contained 34 and 36 phosphatases, respectively (data not shown). However, no acidic or alkaline phosphatases were identified. The absence of acidic or alkaline phosphatase in the genome indicates that *Asinibacterium* sp. OR53 uses biosorption and not biomineralization as a mechanism of uranium resistance (Figures 4 and 5). During biosorption, uranium can bind to functional groups associated with the cell wall or extracellular structures. The LPS layer is distributed across the cell surface of Gram-negative bacteria and contains phosphate rich regions that support uranium

binding [25]. Biosorption of uranium has been described for a variety of microorganisms: *Sphingomonas* spp., *Microbacterium* spp., *Myxococcus xanthus, Streptomyces sporoverrucosus*, *Pseudomonas* spp., and *Acidovorax facilis* [32,51–56].

Based on the genomic similarities between *Asinibacterium* sp. OR53 and *Asinibacterium* sp. OR43, it is likely that *Asinibacterium* sp. OR43 also uses biosorption as a mechanism to resist high concentrations of uranium too. In summary, the absence of relevant phosphatases in both genomes and the manner in which uranium co-localizes with phosphorus during EDS mapping with *Asinibacterium* sp. OR53 cells indicates that biosorption of uranium to phosphate groups in the LPS layer is the mechanism of uranium resistance for *Asinibacterium* spp. OR43 and OR53.

Genes involved in heavy metal metabolism/resistance: We determined genes potentially involved in heavy metal resistance and metabolism by (1) analyzing and comparing the genomes annotated in the JGI IMG/ER database [57] and (2) blasting the complete genome of *Asinibacterium* sp. OR53 and selected genes of *Asinibacterium* sp. OR43 against the Antibacterial biocide and metal resistance genes database (BacMet) [58]. In the genomes of *Asinibacterium* spp. OR43 and OR53, genes potentially involved in the resistance to arsenic, copper, cadmium, cobalt, zinc, mercury, and chromium were identified (Tables 3 and S8).

Table 3. Heavy metal resistance genes in the genomes of *Asinibacterium* spp. OR43 and OR53.

Gene Product Name	Gene Symbol	OR43	OR53
Arsenic			
Arsenic resistance operon	*arsBCR*	0	1
Arsenate reductase (protein-tyrosine phosphatase)	*arsC*	1	1
Copper and silver			
Copper exporting P-type ATPase (Cu^{2+})	*copAB*	1	1
Copper exporting P-type ATPase (Cu^{2+})	*copA*	2	2
Copper exporting P-type ATPase (Cu^{2+})	*copB*	2	2
Copper chaperone	*copZ*	1	1
Copper homeostasis protein	*cutC*	1	1
Cu(I)/Ag(I) efflux system operon	*cusAB*, *silAB*	1	3
Copper two component regulatory systems	*copRS*, *cusRS*	4	4
Cobalt, zinc, cadmium			
Cobalt–zinc–cadmium resistance operon	*czcCBA*	2	1
Heavy metal efflux pump	*czcA*	3	3
Membrane fusion protein	*czcB*	2	2
Cation diffusion facilitator	*czcD*	2	2
Cd^{2+}/Zn^{2+} exporting ATPase	*zntA*	1	1
Mercury			
Possible transcriptional regulator	*merR*	2	2
Putative mercury transport protein	*merT-P*	2	2
Mercury reductase	*merA*	1	1
Mercury resistance protein	*merC*	1	1
Zinc			
Cd^{2+}/Zn^{2+} exporting ATPase	*zntA*	1	1
Nickel			
Nickel transport operon	*nikABCE*	1	1
Chromium			
Chromate transporter	*chrA*	1	1

Arsenic: Arsenic resistance is encoded within an operon containing three genes: a transporter (*arsB*), reductase (*arsC*), and a transcriptional regulator (*arsR*). All genes within the *arsRBC* operon are necessary for conferring arsenic resistance [59,60]. It is likely that only *Asinibacterium* sp. OR53 would be resistant to arsenic since the *Asinibacterium* sp.

OR53 genome contained all genes within the operon, while only *arsC* was identified in the genome of *Asinibacterium* sp. OR43 (Tables 3 and S8).

Copper: Two different copper resistance mechanisms were detected in the genomes of *Asinibacterium* spp. OR43 and OR53: (1) copper-transporting ATPases (*copA* and *copB*); and (2) proton driven transporters (*cusBA*) (Tables 3 and S8). *copA* encodes a copper exporting ATPase, which transports copper from the cytoplasm to the periplasm when copper is present in excess [61,62]. However, the role of *copB* in copper resistance has not been elucidated for Gram-negative organisms [62]. *cusA* encodes a RND protein and *cusB* encodes a membrane fusion protein of a proton driven transporter. Both genes are present in both genomes, while *cusC*, the gene for the outer membrane protein, is missing (Tables 3 and S8). Instead, the gene for a putative TolC protein was detected between *cusA* and *cusB*. TolC is an outer membrane protein typically associated with RND transporters [63,64]. It is likely the potential *tolC* gene product encoded between *cusA* and *cusB* functions as the outer membrane protein in the absence of *cusC* [64]. In addition, the gene for the copper binding chaperone *copZ* and four copies of a copper responsive two-component regulatory system (*cusRS*) were detected in both strains (Tables 3 and S8). In summary, both strains are very likely resistant to elevated levels of copper.

Cadmium, zinc, and cobalt: The genomes of *Asinibacterium* spp. OR43 and OR53 contained at least one copy of the full *czcCBA* operon and two additional copies of *czcBA* (Tables 3 and S8). The genes in the operon encode a cobalt, zinc, and cadmium exporting RND protein (CzcA), a membrane fusion protein (CzcB), and an outer membrane protein (CzcC) [24,65]. It is likely that genes located elsewhere in the genome and encoding for non-specific outer membrane proteins, such as TolC, could allow for heavy metal extrusion in the absence of CzcC [24]. Both genomes also contained genes for the cation diffusion facilitator *czcD* (Tables 3 and S8), which encodes a membrane spanning protein that functions as a metal/proton antiporter for various divalent metals [66]. Previous growth experiments showed that both strains grew at elevated cobalt concentrations and were inhibited by low concentrations of cadmium [21]. These observations are in contrast with the identification of *czc* genes in the genomes (Tables 3 and S8). While the efflux pump very likely could contribute to cobalt resistance it may not successfully extrude cadmium.

Nickel: The *Asinibacterium* spp. OR43 and OR53 genomes did not contain any gene products for the extrusion of nickel (Tables 3 and S8). The genes *nikABCDE* encode a high affinity ABC nickel transporter that imports nickel into the cell and the gene *nikR* encodes a repressor that suppresses transcription of *nik* genes when nickel is in excess [67]. The genes *nikA*, *nikB*, *nikC,* and *nikE* were found in the genomes of *Asinibacterium* spp. OR43 and OR53, but not as an operon (Tables 3 and S8). Additionally, the genes encoding NikD, one of the ATP binding proteins, and NikR, the regulatory protein of the nickel transporter were missing from both genomes. In the absence of a nickel exporter and genes necessary for a complete ABC transporter, it would be expected that *Asinibacterium* spp. OR43 and OR53 are sensitive to the presence of nickel. However, both strains were shown to be resistant to elevated concentrations of nickel [21]. These results suggest that other unidentified transporters may contribute to nickel resistance in *Asinibacterium* spp. OR43 and OR53.

Mercury: Both *Asinibacterium* spp. OR43 and OR53 genomes contained two copies of genes encoding a mercury binding protein (*merP*), mercury transporter (*merT*), and the transcriptional regulator (*merR*) as well as one copy of the mercury reductase (*merA*) and the mercury resistance gene (*merC*) (Tables 3 and S8). MerP binds toxic Hg^{2+} in the periplasm and MerC and MerT transport Hg^{2+} from the periplasm to the cytoplasm. In the cytoplasm, MerA reduces Hg^{2+} to a less toxic form (Hg^{0}), which diffuses out of the cell [22,68]. These results suggest both strains would be resistant to mercury.

Chromium: The gene *chrA* encodes an efflux pump that was responsible for chromate resistance in *P. aeruginosa* and *Shewanella* sp. ANA3 [69,70] that was identified in both genomes (Tables 3 and S8), indicating that *Asinibacterium* spp. OR43 and OR53 can extrude chromium.

3. Material and Methods

Media: All growth experiments were conducted in mineral salts (MS) medium unless otherwise noted. The MS medium consisted of: 5 mM NH_4Cl, 1 mM NaCl, 0.25 mM $MgSO_4 \bullet 7H_2O$, 5 µM $CaCl_2 \bullet 2H_2O$, 5 µM KH_2PO_4, 10 mM HEPES, and 1 mL/L trace elements [71]. The pH was adjusted with NaOH to 7.0. After autoclaving, sterile solutions of glucose, tryptone, and yeast extract were added to obtain a final concentration of 0.5 g/L in the MS medium. DIFCO Bacto agar (15 g/L) was added for agar plates.

To test the growth in the presence of different stress factors nitrate (0–500 mM) as potassium nitrate or uranium (0–400 µM) as uranyl acetate was added to the MS medium. Growth at different pH values was tested in MS medium with 10 mM HEPES as the buffer at pH 5–8 or with 10 mM HOMOPIPES as the buffer at pH 3.5–4.5. Growth under anaerobic conditions was tested in MS medium with nitrate (200 mM KNO_3), uranium (100 µM uranyl acetate), or iron (400 mM $FeCl_3$) as terminal electron acceptors.

R2A was used for growing biomass for fatty acid analysis and for the temperature profile [72].

Strains and culture conditions: *Asinibacterium* spp. OR43 and OR53 (formerly *Sediminibacterium* spp. OR43 and OR53) were isolated from the highly contaminated subsurface sediment at Oak Ridge, TN [21]. The strains were maintained on MS medium agar plates. If liquid cultures were needed as inoculum for experiments, each strain was inoculated into 5 mL of liquid MS medium and incubated for 2 days at 27 °C.

Growth in the presence of different stress factors: Pre-cultivated bacterial cultures (15 µL = 1:10 dilution or 1.5 µL = 1:100 dilution) were inoculated into 150 µL MS medium in 96-well plates with the different stress factors (pH, uranium, or nitrate). The plates were incubated at 27 °C in a plate reader (BIOTEK Synergy HT, Biotek, Winooski, VT, USA) for several days depending on the experiment. The optical density was measured every 10 min at 600 nm (OD_{600}) after shaking the plate for 10 s. The growth rates were calculated from the slope of the natural log-transformed OD_{600} data plotted against time.

Carbon utilization: Bacteria were grown to the end of the logarithmic phase in MS medium, centrifuged, washed twice, and re-suspended in MS medium without carbon sources. The OD_{600} of the culture was adjusted to approximately 0.3. The cells were inoculated into GN2 MicroPlate™ (BIOLOG, Hayward, CA, USA). The plates were incubated in the dark at 27 °C for 5 days and the absorbance was measured once a day in the plate reader (BIOTEK Synergy HT, Biotek, Winooski, VT, USA) at 590 nm. If the the absorbance at 590 nm was $\geq$0.5 the strain was considered to be positive for the utilization of each carbon source.

Growth under anaerobic conditions: Pre-cultivated bacteria were inoculated at a 1:10 dilution in each type of medium and observed for 5–9 days for growth. Denitrification was tested in MS medium supplemented with nitrate, iron reduction in MS medium with iron, uranium reduction in MS medium with uranium, and fermentation in MS medium without any additional supplements. All cultures were incubated under anaerobic conditions in an anaerobic chamber (5% hydrogen, 5% carbon dioxide, and 90% nitrogen).

Motility: Different modes of motility were tested: swimming, swarming, twitching [73], and gliding [74].

Temperature: The temperature range was determined in a custom-made temperature gradient block that was controlled by a water bath and a heating block. The temperatures were adjusted to values between 4 °C and 42 °C. The cultures were inoculated into liquid R2A medium in test tubes and incubated in the temperature block. Samples were taken in regular intervals to measure the OD_{600}. The growth rates were calculated as described above.

Biochemical characteristics: Catalase activity was observed as bubble formation after addition of 3% (*v/v*) H_2O_2. Oxidase activity was determined by the color change of 1% *N*,*N*,*N*,*N*-tetramethyl-p-phenylenediamine hydrochloride. H_2S production was determined in triple sugar iron (TSI) agar.

Fatty acid methyl ester analysis was carried out by the identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (GmbH, Braunschweig, Germany). The bacteria were cultivated on R2A plates for 4 days at 28 °C. Fatty acid methyl esters were analyzed using the Sherlock Microbial Identification System (MIDI) [75–77]. The peaks were identified using the library TSBA40 4.10.

Uranium (VI) assay: Cells were centrifuged in a 1.5 mL microcentrifuge tube at 28,000× *g* for 10 min. The uranium concentration in the supernatant was determined using a colorimetric assay [78].

Transmission Electron Microscopy (TEM): Copper mesh grids with a single layer of 3% collodion (nitrocellulose) were lightly coated with 20 nm of carbon. *Asinibacterium* sp. OR53 was grown to mid-to-late log phase in MS medium with or without 300 µM uranium. Cells (1–3 mL) were centrifuged at 9500× *g* for 3 min, washed twice with MS medium without carbon sources or phosphate and re-suspended in 0.5–1 mL of medium. Cells (10 µL) were added to the carbon-coated grids and allowed to sit for 2 min. Excess medium and cells were removed with filter paper. Grids were viewed in the JEM-1200 EX II (JEOL, Tokyo, Japan) transmission electron microscope at 100 keV.

Scanning Transmission Electron Microscopy (STEM) Elemental Analysis: Copper mesh grids previously prepared for TEM analysis were analyzed under the JEM-2100 (JEOL, Tokyo, Japan) scanning transmission electron microscope (STEM) fitted with a Bruker Quantax energy dispersive X-ray spectroscopy (EDS) system with a XFlash silicon drift detector (SDD) (Bruker, Billerica, MA, USA) at 200 keV. Quantax Esprit software (Bruker, Billerica, MA, USA) was used to convert X-ray data into elemental spectrums or maps.

Phylogenetic analysis: DNA was isolated using the Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' recommendations. The DNA was amplified with the primers 27F and 1492R [79]. The PCR products were purified and sequenced using the BigDye Terminator V3.1 cycle sequencing kit (Life Technology Corporation, Carlsbad, CA, USA) on an Applied Biosystems 3730 × 1 DNA analyzer (Life Technology Corporation, Carlsbad, CA, USA) at the Center for Bioinformatics and Functional Genomics at Miami University using the 3 internal primers 357F, 518R, and 518F [80]. All sequences were edited with 4Peaks (A. Griekspoor and T. Groothuis, The Netherlands Cancer Institute; http://nucleobytes.com/index.php/4peaks, accessed on 20 December 2021) and aligned using the program ARB version 5.5 [81]. The phylogenetic tree was constructed in MEGA (version 5.2.2) using the maximum likelihood algorithm.

Genome analysis: The draft genomes of *Asinibacterium* spp. OR43 and OR53 were generated and annotated at the DOE Joint Genome Institute (Walnut Creek, CA, USA) [33]. Further analysis of the genomes was conducted using the JGI-IMG database [57]. Heavy metal resistance genes were identified in the genomes of both strains using the Bac-Met–Antibacterial Biocide and Metal Resistance Genes Database (bacmet.biomedicine.gu.se, accessed on 20 December 2021) [58].

4. Conclusions

Asinibacterium spp. OR43 and OR53 were isolated from the contaminated subsurface sediment in Oak Ridge, TN. Physiologically, *Asinibacterium* spp. OR43 and OR53 are similar to other cultured relatives (Tables S1 and S2) and are resistant to elevated concentrations of heavy metals such as uranium (Figure 3), and cobalt and nickel [21]. The resistance of *Asinibacterium* spp. OR43 and OR53 to uranium, cobalt, and nickel did not necessarily correspond with heavy metal genes identified in the genome. It is likely that uranium resistance in both strains is attributed to biosorption of uranium to the LPS layer. While genes for a cobalt exporter were present in the genome, no genes were identified to explain the resistance of *Asinibacterium* spp. OR43 and OR53 to nickel, which suggests that these strains might have alternative nickel resistance mechanisms. Additional studies are needed to verify that genes for arsenic, mercury, copper, chromium, and zinc transporters identified in the genome(s) are actually capable of conferring resistance in *Asinibacterium* spp. OR43

and/or OR53. Overall, this study showed that *Asinibacterium* spp. OR43 and OR53 were able to withstand the uranium and nitrate concentrations, but not the low pH level at the field site in Oak Ridge. Closely related strains are found in significant numbers in the Oak Ridge environment [16] and might have a role in reducing local heavy metal concentrations. To cope with the low pH levels or other environmental stresses, *Asinibacterium* spp. OR43 and OR53 might live in consortia with bacteria capable of modifying the local environment so that conditions are favorable for all co-existing microbes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/bacteria1010004/s1, Table S1: Differential physiological characteristics of strains *Asinibacterium* spp. OR43 and OR53 in comparison with type strains of closely related species. Table S2: Fatty acid profiles of the strains *Asinibacterium* spp. OR43 and OR53 in comparison with type strains of closely related species. Table S3: Genome sequencing project information for *Asinibacterium* spp. OR43 and OR53. Table S4: Genome properties of *Asinibacterium* spp. OR43 and OR53. Values in parentheses represent the percent (%) of total genes. Table S5: Number of genes associated with the general COG functional categories in the genomes of *Asinibacterium* spp. OR43 and OR53. Table S6: Locus information of genes involved in nitrate metabolism in *Asinibacterium* spp. OR43 and OR53 genomes. Table S7: Locus information of genes involved in pH homeostasis in the genomes of *Asinibacterium* spp. OR43 and OR53. Table S8: Potential heavy metal resistance genes in the genome of *Asinibacterium* spp. OR43 and OR53. The genes potentially involved in heavy metal resistance have been compared to the BacMet–Antibacterial Biocide and Metal Resistance genes database (http://bacmet.biomedicine.gu.se, accessed on 20 December 2021). The identity values (%) represent the identities of the genes compared to the experimentally verified and predicted database (xx/xx). Figure S1: Uranium concentration in the medium in the presence (with OR53) and absence (without culture) of *Asinibacterium* sp. OR53 over time (mean ± SD; n = 3).

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Data Availability Statement: The genomes of *Asinibacterium* sp. OR43 and OR53 have been deposited at Genbank under the accession numbers ATYE00000000 (*Asinibacterium* sp. OR43) and AZXP00000000 (*Asinibacterium* sp. OR53). The raw reads have been deposited in the SRA under SRP078705 (Asinibacterium sp. OR53) and SRP078706 (*Asinibacterium* sp. OR43). Both genomes can also be accessed via the Integrated Microbial Genomes and Microbiomes database of the US Department of Energy—Joint Genome Institute (JGI-IMG) with the IMG genome ID 2509887033 (*Asinibacterium* sp. OR43) and 2516143025 (*Asinibacterium* sp. OR53).

Conflicts of Interest: The authors declare no conflict of interest.

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