

Uranium extremophily is an adaptive, rather than intrinsic, feature for extremely thermoacidophilic *Metallosphaera* species

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Thermoacidophilic archaea are found in heavy metal-rich environments, and, in some cases, these microorganisms are causative agents of metal mobilization through cellular processes related to their bioenergetics. Given the nature of their habitats, these microorganisms must deal with the potentially toxic effect of heavy metals. Here, we show that two thermoacidophilic *Metallosphaera* species with nearly identical (99.99%) genomes differed significantly in their sensitivity and reactivity to uranium (U). *Metallosphaera prunae*, isolated from a smoldering heap on a uranium mine in Thüringen, Germany, could be viewed as a "spontaneous mutant" of *Metallosphaera sedula*, an isolate from Pisciarelli Solfataria near Naples. *Metallosphaera prunae* tolerated triuranium octaoxide (U₃O₈) and soluble uranium [U(VI)] to a much greater extent than *M. sedula*. Within 15 min following exposure to "U(VI) shock," *M. sedula*, and not *M. prunae*, exhibited transcriptomic features associated with severe stress response. Furthermore, within 15 min post-U(VI) shock, *M. prunae*, and not *M. sedula*, showed evidence of substantial degradation of cellular RNA, suggesting that transcriptional and translational processes were aborted as a dynamic mechanism for resisting U toxicity; by 60 min post-U(VI) shock, RNA integrity in *M. prunae* recovered, and known modes for heavy metal resistance were activated. In addition, *M. sedula* rapidly oxidized solid U₃O₈ to soluble U(VI) for bioenergetic purposes, a chemolithoautotrophic feature not previously reported. *M. prunae*, however, did not solubilize solid U₃O₈ to any significant extent, thereby not exacerbating U(VI) toxicity. These results point to uranium extremophily as an adaptive, rather than intrinsic, feature for *Metallosphaera* species, driven by environmental factors.

uranium oxidation | uranium resistance | chemolithotrophy | terminal oxidases

Uranium (U) is a naturally occurring element, so it is not surprising that many environmental microorganisms coexist with, and even carry out biotransformations involving, this metal. U has a high binding affinity for phosphoryl and carboxyl groups, such that the metal can associate with cell wall components (1), lipopolysaccharides (2), and peptidoglycan (3), in particular. Dissimilatory, iron-reducing bacteria can reduce soluble U(VI) to insoluble U(IV) as a detoxification step, mediated by membrane-bound c-type cytochromes (4–6). In addition to limiting uranium toxicity by converting soluble U(VI) to insoluble U(IV), membrane-bound acid and alkaline phosphatases can respond dynamically to external conditions to release phosphate for metal ion binding (7, 8). It has been shown that the conductive pili of *Geobacter* function as sites of U(VI) reduction and prevent its accumulation in the periplasm (9). Bioprocesses have been considered for U recovery. *Acidithiobacillus ferrooxidans*, a mesophilic extreme acidophile, was able to extract 87% of bound uranium from low-grade uranium ore by catalyzing the formation of Fe(III) from Fe(II), which then in turn chemically oxidized U(IV) in uraninite to soluble U(VI) (10). In fact, even in the case of nonpyritic U ores, Fe(III) can be supplied to drive U solubilization (11). Direct anaerobic oxidation of uraninite, U(IV) to U(VI), has been observed with nitrate-reducing *Thiobacillus denitrificans* using c-type cytochromes

(12, 13). Oxidation of soluble uranous to uranyl ions coupled to CO₂ fixation has been demonstrated for *A. ferrooxidans* (14).

The genus *Metallosphaera* is comprised of extremely thermoacidophilic archaea that have been isolated from hot, acidic environments worldwide (15–17). Like *A. ferrooxidans*, *Metallosphaera* species are capable of mobilizing heavy metals from pyritic ores, based on their ability to oxidize iron and sulfur, mediated by an array of terminal oxidases associated with their cell membranes (18, 19). Of the *Metallosphaera* species isolated to date, *Metallosphaera sedula* and *Metallosphaera prunae* have the highest optimum temperature for growth (70–75 °C) and lowest pH optimum (2.0). *M. sedula* was initially isolated from Pisciarelli Solfataria near Naples (16), and *M. prunae* was isolated from an abandoned uranium mine in Thüringen, Germany (15), which begs the question of whether the U-laden growth environment of *M. prunae* impacts its sensitivity and reactivity to this heavy metal. This issue was examined by comparing the genome sequences of these two archaea, as well as by examining their microbiological and transcriptional response to solid and soluble forms of uranium.

Results

***M. prunae* Is a U-Resistant "Spontaneous Mutant" of *M. sedula* That Is Supported by Microbiological Data and Genome Sequencing.** Fig. 1A shows that when exposed to solid, spent (i.e., nonradioactive) triuranium octaoxide (U₃O₈) from a nuclear power plant, *M. prunae* grew to densities in excess of 6×10^8 cells/mL, whereas no net increase in *M. sedula* cell densities was observed. Furthermore, U(VI) concentrations increased in the case of *M. sedula*, but decreased in the case of *M. prunae*. Abiotic controls behaved similarly to the *M. prunae* case (Fig. 1A).

Sequencing results indicated that the *M. sedula* and *M. prunae* genomes were nearly identical, but different from the genome of another member of this genus, *Metallosphaera cuprina* (17, 20). In fact, only 220 nt of 2.2 Mb were different in *M. prunae* compared with *M. sedula* (21); in coding regions, 79 single mutations corresponded to 38 frameshifts and 23 in-frame amino acid substitutions (Fig. 1B and Table S1). The highest number of mutations occurred in a pseudogene, Msed_1426 (17 mutations leading to several amino acid substitutions). All ORFs in *M. prunae* mapped to *M. sedula* and vice versa, such that *M. prunae* might be considered as a spontaneous mutant of *M. sedula* rather than a separate species.

The 38 frameshifted ORFs created translational truncations of the associated encoded genes (Fig. 1B and Table S1). In most cases, no direct connection between most of the 38 proteins

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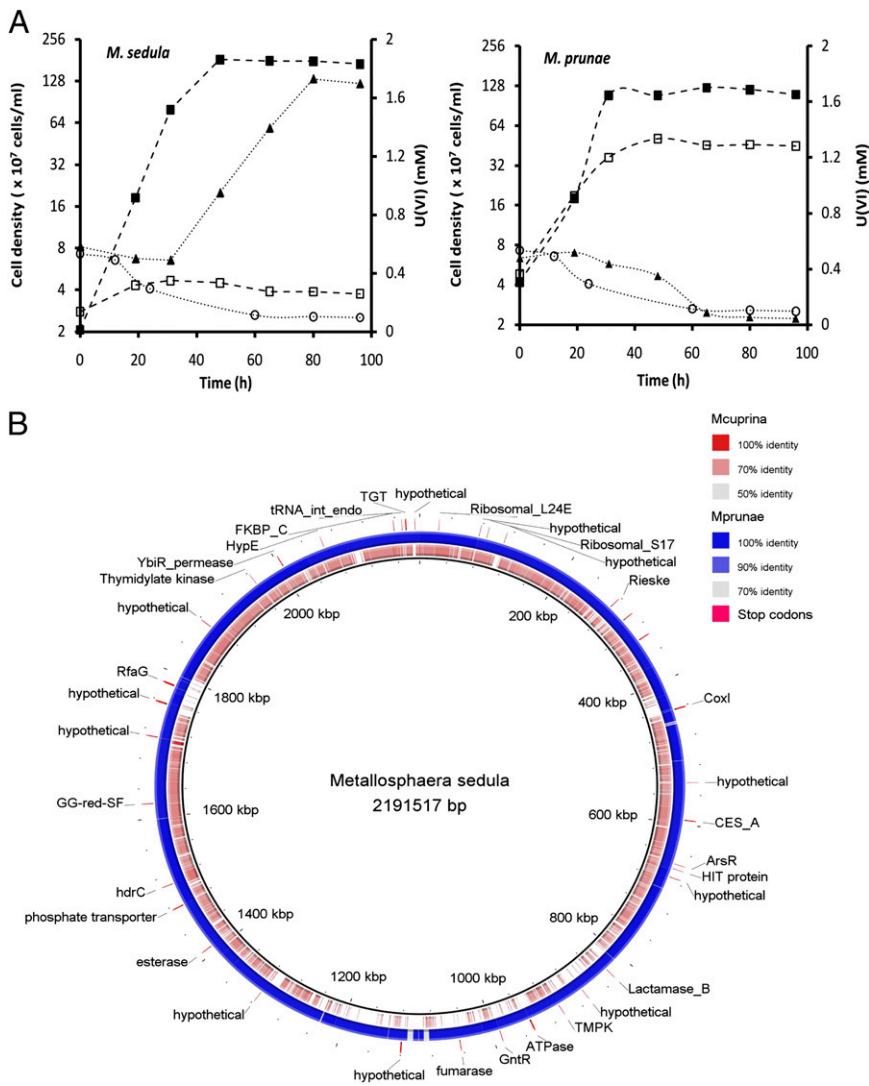


Fig. 1. Phenotype and genotype comparison between *M. sedula* and *M. prunae*. (A) Growth on heterotrophic media in the presence uranium octoxide [U_3O_8 ; 0.125 % (wt/vol)] (\square) and under normal conditions (\blacksquare) for *M. sedula* (Left) and *M. prunae* (Right). Cell densities have been plotted on \log_2 scale. Note that no significant growth of *M. sedula* was observed in the presence of the heavy metal. U(VI) concentration (\blacktriangle , biotic; \circ , abiotic control) in the media increases with time for *M. sedula*, whereas it decreases for *M. prunae* and control. No growth was observed for either microorganism in the presence of 0.25 % (wt/vol) U_3O_8 . (B) *M. sedula* genome is shown as the innermost ring (2,191,517 bp) along with the degree of similarity to *M. cuprina* (red ring) and *M. prunae* (blue ring). The mutations in *M. prunae* genome that lead to truncation of proteins have been indicated.

associated with these frameshifts and metal transformations was evident. However, potential roles in heavy metal oxidation could be the case for Msed_0322 (Rieseke 2Fe-2S protein) and Msed_0485 (cytochrome *c* oxidase, subunit I); these belong to the two iron oxidation clusters in *M. sedula* genome (18). Other truncated ORFs with possible relationships to heavy metal resistance included several major facilitator transporters (Msed_0310, Msed_0420, and Msed_0972) and a phosphate transporter (Msed_1512). Msed_1512 is related to Pho84 in *Saccharomyces cerevisiae*, which is believed to function as a metal-phosphate importer. Msed_2086, which is related to arsenite permease/transporter (ArsB) in *Sulfolobus solfataricus*, was also truncated by a frameshifting mutation.

Transcriptional Response of *Metallosphaera* Species to “U(VI) Shock” Reveals Differentiating Physiological Features. The U toxicity resulting from growth in the presence of solid U_3O_8 likely arose, to some extent, from high levels of soluble U(VI) that were generated in the case of *M. sedula*. As such, both species were subjected to U(VI) shock, where 1.0 mM uranyl acetate was added to the culture after 13 h of growth (cell density, 1×10^8 cells/mL) when cells were in early exponential phase (Fig. 2A). Cell densities in both cases declined immediately post-U(VI) shock and eventually recovered such that growth was initiated at $\sim t = 38$ h for *M. prunae* (25 h post-U shock) and $t = 58$ h for *M. sedula* (45 h post-U shock). Recovery appeared to coincide with declining U(VI) concentrations, likely the result of coprecipitation of U(VI) with medium components, as

noted in abiotic controls (Fig. 2A). Transcriptomes were compared for samples taken 180 min postshock (U180; Fig. 2B, Table S2, and Fig. S1A). *M. prunae* had 128 genes ($\sim 5\%$ of genome) showing twofold change [normal vs. U(VI) shock], compared with 307 genes ($\sim 15\%$ of genome) for *M. sedula*. Many genes implicated in DNA repair, recombination, and stress response were triggered in *M. sedula*, but not in *M. prunae* (Table S2). Five *vapBC* toxin–anti-toxin genes were up-regulated in *M. sedula* as a result of the shock, and only one for *M. prunae* (Table S2). *vapBC* TA loci have been implicated in stress response for a related thermoacidophile, *Sulfolobus solfataricus* (22, 23). In *M. sedula*, a histidine biosynthesis operon (Msed_1943–Msed_1950) was up-regulated, suggesting that the metal-binding capacity of His could help to reduce intracellular uranium toxicity. Many transcriptional regulators responded in *M. sedula*, including Msed_0192, which was down-regulated in *M. sedula* 14-fold, but unaffected in *M. prunae*. Global transcriptional regulators *uspA* (Msed_0254) and *fur* (Msed_0767) were significantly up-regulated in *M. sedula* on uranium shock, along with transcriptional regulators belonging to AsnC, XRE, and GntR families. Though the specific roles of these regulators is not clear, the U180 transcriptome points to a more pronounced effect of uranium on *M. sedula* metabolism and physiology compared with *M. prunae*.

To further pursue the relationship between transcriptomes and U(VI) exposure so to glean insights about resistance mechanisms, shorter time frames postshock were examined (Tables S3 and S4). Transcriptomes from normal growth (N) of the two species were

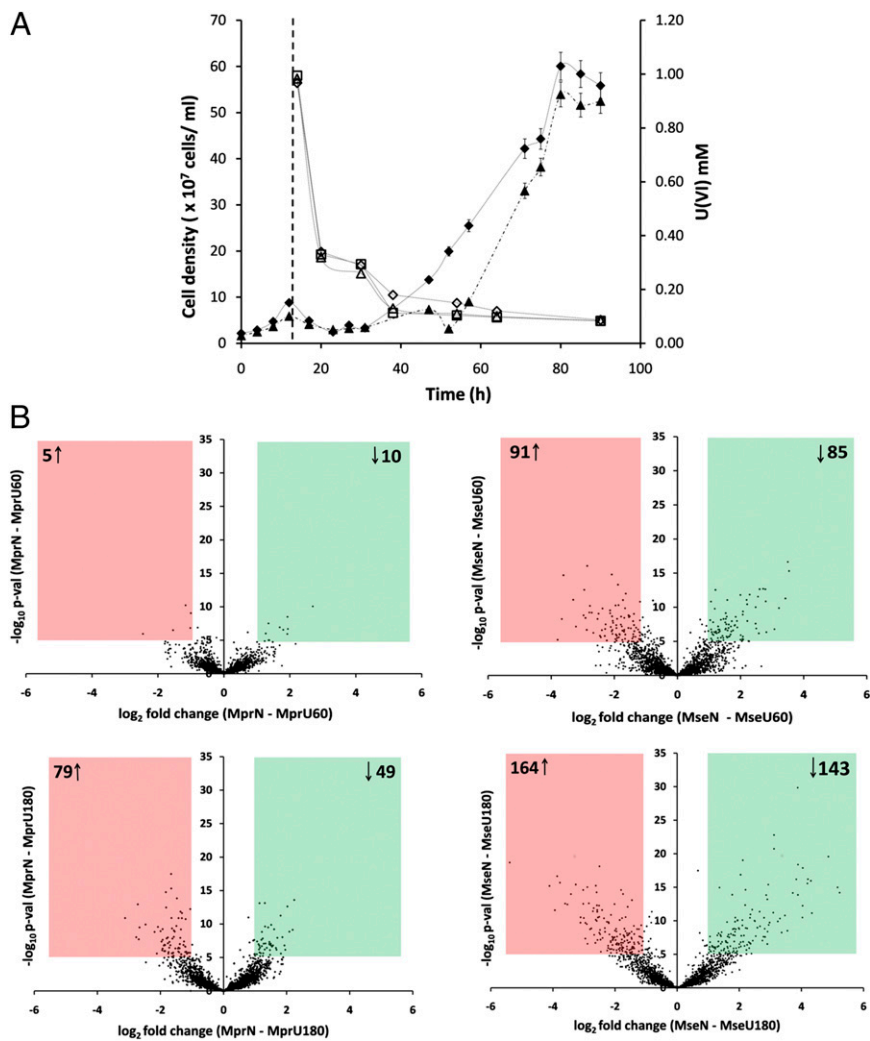


Fig. 2. *M. sedula* and *M. prunae* growth at pH 2.0, 70 °C subjected to U(VI) shock and transcriptional response comparing normal growth to U shock. (A) At 13 h following inoculation (dotted line), 1.0 mM uranyl acetate was added to the *M. prunae* (◆) and *M. sedula* (▲) cultures and then cells were harvested at 15, 60, and 180 min postshock for transcriptional response analysis. U(VI) concentration in the media is shown for *M. prunae* (◇) and *M. sedula* (△) and abiotic control (□). (B) Volcano plots showing the number of genes being up/down-regulated at 60 min (Upper) and 180 min (Lower) following U(VI) shock. The contrast here is intraspecies, before and after U(VI) addition. The highlighted area (red and green) corresponds to statistically significant results for biological repeats based on twofold change and Bonferroni correction of 5. At 60 min, for *M. sedula* (Right), 176 ORFs were differentially transcribed (91 up/85 down) as a result of U(VI) addition in contrast to 15 ORFs (5 up/10 down) for *M. prunae*. At 180 min, for *M. sedula* (Right), 307 ORFs were differentially transcribed (164 up/143 down) as a result of U(VI) addition in contrast to 128 ORFs (79 up/49 down) for *M. prunae*.

compared with U(VI)-shocked cultures sampled at 60 min (U60) after 1 mM uranyl acetate addition. For the shorter time (U60), *M. prunae* showed minimal response, with only 15 genes differentially transcribed twofold or more (5 up/10 down) compared with 176 for *M. sedula*. Most of the differentially transcribed ORFs at U60 in both species also were noted in the U180 transcriptomes (Fig. S2 and Tables S3–S5). At U60, a copper-transporting ATPase (Msed_0490, CopA) and a metallochaperone (Msed_0491, CopM), previously implicated in Cu resistance (24), were up-regulated for both species, but much more so in *M. sedula* (10-fold and 12-fold, respectively) than *M. prunae* (twofold and twofold, respectively; Table S6).

An attempt was made to determine the transcriptomes at 15 min post-U(VI) shock (U15). Here, an interesting observation was made during the RNA quality check done before running the microarrays: RNA for *M. sedula* for the U15 samples retained integrity when viewed on an agarose gel. However, the U15 *M. prunae* RNA sample showed extensive degradation (Fig. 3A). Three biological repeats confirmed this result. The possibility that heightened ribonuclease levels were responsible for RNA degradation was considered. Total ribonuclease activities in cell extracts increased significantly at U15 for both species, with levels in *M. prunae* significantly higher than in *M. sedula*; levels continue to increase for both species at U60 (Fig. 3B). Transcriptomes for *M. sedula* (comparing N, U15, and U60) and *M. prunae* (comparing N and U60; RNA was not suitable for the U15 sample) separately showed up-regulation of genes encoding endo- and exonucleases for both species in the U60 samples (Fig.

3C). At U60, *M. sedula* exhibited up-regulation of several genes associated with the exosome complex, as well as several RNases and putative *vapC* toxins (Msed_0899 and Msed_1385). *M. prunae* showed up-regulation for two putative *vapC* toxins (Msed_0899 and Msed_1307). 2D gel electrophoresis of the U15 and N cell extracts revealed no significant insights that correlated to the increase in ribonuclease activity (Fig. S3). Cell extracts from samples taken 15 h post-U(VI) shock, as growth restarted, showed that *M. prunae* ribonuclease activity dropped significantly from U60 levels [112 relative fluorescence units (RFU)/min or 1.6 \times the normal growth RNase activity], whereas *M. sedula* RNase activity continued to increase (230 RFU/min or 3 \times the normal growth RNase activity).

Taken together, the U(VI) shock results suggest that *M. prunae* resists U toxicity to a much greater extent than *M. sedula*. At least in part, this is accomplished by essentially aborting transcription; attempts to reverse-transcribe RNA from the U15 *M. prunae* samples were unsuccessful (note the RNA smearing in the *M. prunae* U15 samples compared with *M. sedula*; Fig. 3A). Furthermore, from Fig. 3A, substantial degradation of ribosomal RNA has occurred in the *M. prunae* U15 sample, suggesting that translation was also impacted. In the samples for U60, neither archaea showed any evidence of RNA degradation. The integrity of genomic DNA obtained from the U shock cultures (U15 and U60) of both species was unaffected by U shock. No specific RNase could be identified as responsible for RNA degradation in *M. prunae* at U15, but the possible role of toxin–antitoxin systems, perhaps involving one or more VapBCs, is intriguing. The current paradigm is that these toxins are activated as ribonucleases when they disassociate from

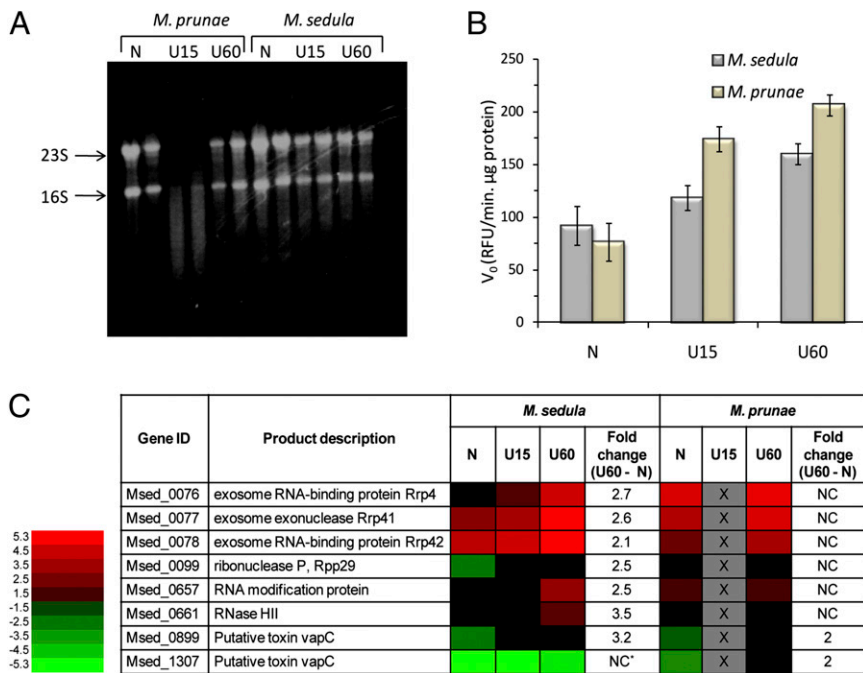


Fig. 3. Ribonuclease activity in uranium conditions compared with normal growth. (A) RNA from the three conditions: normal growth (N), cultures harvested 15 min after U shock (U15), and cultures harvested 60 min after U shock (U60). Biological repeats are shown for each condition. 23S and 16S rRNA bands have been indicated. (B) Ribonuclease activity in cell extracts for *M. sedula* and *M. prunae* from the three conditions (N, U15, and U60). (C) Heat plot showing response of selected ribonucleases in *M. sedula* and *M. prunae* to U(VI) shock, comparing N, U(15) (*M. sedula* only), and U60. RNA from *M. prunae* U15 cultures could not be reverse transcribed to cDNA and hence data from that condition is not available (X). NC, no change; fold change between U60 and N conditions <2.

the antitoxin, an event that would not be detected by the methods used here (25).

***M. sedula*, and Not *M. prunae*, Directly Oxidizes U₃O₈ to U(VI) Under Chemolithoautotrophic Conditions.** The U(VI) shock experiments revealed that, after short time exposure to the heavy metal, *M. prunae* minimizes its metabolism through degradation of cellular RNA. However, the results in Fig. 1A show that *M. prunae* is also less susceptible to solid U₃O₈ and that U(VI) levels increased in the *M. sedula* culture; note that this result was obtained for growth under heterotrophic conditions. Given that *Metallosphaera* species are capable of Fe(II) oxidation to Fe(III) for bioenergetic benefit, the possibility that U(IV) or U(V) could serve as an energy source under chemolithoautotrophic conditions was examined. In fact, oxidation of U(IV) or U(V) to U(VI) could exacerbate U toxicity. Heterotrophically grown cells were harvested and yeast extract (YE) was removed by centrifugation. Chemolithoautotrophic growth was initiated in the presence of U₃O₈ by inoculating the cells at a high initial cell density, 1.5 × 10⁸ cells/mL. There was a drop in cell density until 48 h, after which the cell density leveled off at 10⁸ cells/mL. *M. sedula*, but not *M. prunae*, was able to completely solubilize all of the spent uranium added to the culture. The yellow color in the culture was associated with the formation of a precipitate (Fig. 4B). X-ray diffraction (XRD) analysis confirmed this material to be ammonium uranyl phosphate trihydrate [NH₄UO₂PO₄ (H₂O)₃]. SEM and XRD confirmed that there was no detectable U₃O₈ in *M. sedula* cultures after incubation, whereas it was detected for abiotic control as well as *M. prunae*. This finding suggested that *M. sedula* was directly oxidizing the spent uranium to soluble U(VI), a result confirmed by inductively coupled plasma (ICP)-MS analysis (Fig. 4C). The transcriptomes of the two archaea were compared at 2 d following exposure to the spent uranium to determine if specific terminal oxidases in each archaeon were responsive to U₃O₈ (Fig. 4A, Fig. S1B, and Table S7). Although neither culture increased in cell density, the *M. sedula* and *M. prunae* transcriptomes suggested significant levels of metabolic activity and implicated the five terminal oxidase clusters associated with the *Metallosphaera* membranes, albeit in different ways and to different extents (Fig. 4A). *soxABCDD'L* (Msed_0285–Msed_0290), a proton pump encoded in a single operon in *Sulfolobus acidocaldarius* (26), was induced during growth on sulfur/tetrathionate, compared with organic carbon or iron (18, 27). Here, this operon

was highly transcribed in *M. sedula* compared with *M. prunae*. *sox-EFGHIM* (Msed_0319–Msed_0324) was first identified in *S. acidocaldarius* through the presence of *soxM*, a gene fusion of subunits I and III of cytochrome *c* oxidase (28). It was proposed that *soxF* (Rieske 2Fe-2S protein), *soxG* (cytochrome *b*), *soxH* (*coxII*), and *soxI* (hypothetical protein) belong to an operon, but *soxE* (sulfocyanin) and *soxM* (fusion of *coxI* and *coxIII*) were transcribed separately (29). The *soxM* complex for *M. sedula* was noted previously to be highly transcribed under heterotrophic compared with chemolithoautotrophic growth (27). Our results here indicate that the corresponding operon Msed_0319–Msed_0322 (*soxF*–*soxI*) was up-regulated in *M. prunae* compared with *M. sedula*, whereas Msed_0323–Msed_0324 (*soxM*, *soxE*) was down-regulated. Whether the frameshifting mutation in *soxF* in *M. prunae*, which encodes a protein responsible for transfer of electrons to SoxM (terminal electron acceptor) via SoxE, was a factor is yet to be determined. *foxAA'BCDEFGHIJ* (Msed_0468–Msed_0485) has been shown to be involved in iron (Fe²⁺) oxidation in *Sulfolobus metallicus* and *M. sedula* (18, 19). *chsA* (Msed_0504), a monoheme cytochrome *b*_{558/566}, was 3.5-fold up-regulated in *M. sedula* compared with *M. prunae*; this gene encodes a surface-exposed S-layer protein with N-glycosylated and O-glycosylated sugar residues (30). It has been shown that this gene was highly transcribed during chemolithoautotrophic growth (iron pyrite and elemental sulfur) compared with heterotrophic growth (19, 27). Rusticyanin (Msed_1206), a blue copper protein involved in iron oxidation, was up-regulated in *M. sedula* compared with *M. prunae*. Also, DoxDA (Msed_0363–Msed_0364), proteins implicated in thiosulfate and sulfur oxidation (19), were up-regulated in *M. sedula* compared with *M. prunae*.

Components of the terminal oxidase clusters discussed above that were up-regulated in *M. sedula* could all play roles in the direct oxidation of U₃O₈ by *M. sedula*. However, previous transcriptional response analysis of *M. sedula* growth on acid insoluble (pyrite) and soluble iron indicated that metal oxidation involves *foxA/foxA'* (cytochrome *c* oxidase, subunit I) in the *fox* cluster (Msed_0468–Msed_0485) (19). Exposure of both species to spent uranium under chemolithoautotrophic conditions led to significant down-regulation of *foxA* and *foxA'* (Msed_0484 and Msed_0485) transcripts for *M. prunae* compared with *M. sedula*. The frameshifting mutation in Msed_0485 (*foxA'*) could have had a polar effect on Msed_0484–Msed_0485 transcription, thereby impairing *M. prunae*'s ability to oxidize spent uranium. In

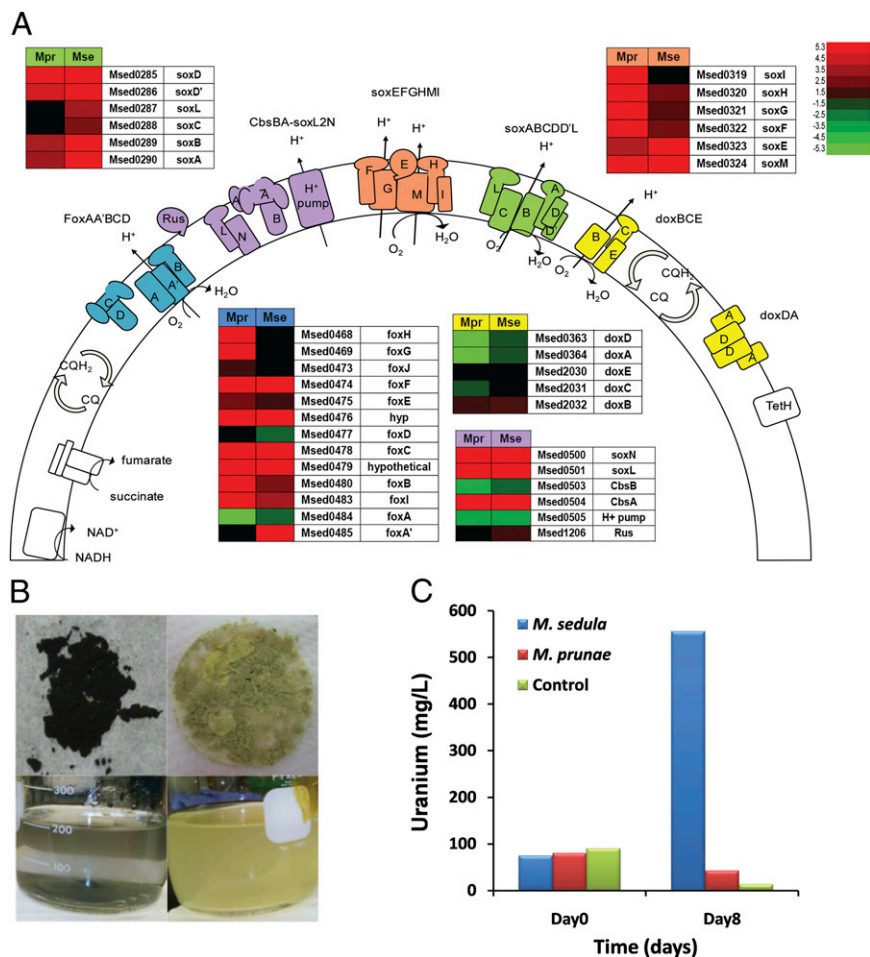


Fig. 4. Incubation of *M. sedula* (Mse) and *M. prunae* (Mpr) with U_3O_8 under chemolithoautotrophic conditions. (A) Heat plots based on mixed-effects model ANOVA analysis of transcriptional response of ORFs encoding five membrane-associated terminal oxidase complexes to chemolithoautotrophic conditions [0.1% (wt/vol) U_3O_8 with CO_2 supplementation in the headspace]. Table S7 shows the fold changes for the heat plots represented in this figure. (B) *M. sedula* and *M. prunae* and abiotic control incubated with 0.125% (wt/vol) U_3O_8 , and headspace supplemented with CO_2 . (Upper Left) U_3O_8 before inoculation. (Upper Right) Filter cake collected from *M. sedula* cultures after 7 d postinoculation. (Lower Left) Abiotic control after 7 d. (Lower Right) Yellow coloration in *M. sedula* cultures after 3 d. (C) Uranyl ion analysis of supernatant from B using ICP-MS analysis. Samples taken from *M. sedula*, *M. prunae*, and abiotic control (control) comparing day 0 and day 8.

fact, *M. prunae* was far less proficient in oxidizing Fe(II) compared with *M. sedula* (Fig. S4). Thus, it is possible that *M. sedula* is attempting to oxidize U_3O_8 to U(VI) with an iron-oxidizing complex. Not only does the generation of U(VI) by *M. sedula* likely exacerbate uranium toxicity, but the U(VI)/U(IV) redox couple (+0.26 V) (12) is bioenergetically poor compared with the Fe(III)/Fe(II) couple (+0.77 V) (14). Taken together, *M. sedula* growth on spent uranium was ultimately very limited, despite transcriptomal evidence of a high level of metabolic activity. For *M. prunae*, however, minimal amounts of U_3O_8 oxidation to U(VI) seem to be yet another mechanism for avoiding the potentially toxic effect of the solubilized heavy metal.

Discussion

Resistance to Uranium Toxicity in *M. prunae* Has Passive and Active Elements. The results presented here suggest that the *M. prunae* genome is a mutated version of the *M. sedula* genome that enables *M. prunae* to adapt to survival in an environment with high levels of uranium. In contrast to *M. sedula*, *M. prunae* exhibited few, if any, previously known signs of stress response when subjected to U(VI) shock, suggesting that it has developed mechanisms, active and passive, to resist the deleterious effect of this heavy metal. To this point, as shown in Fig. 3, *M. prunae* appears to minimize its cellular and metabolic activity by shutting down transcription and translation when suddenly confronted with toxic levels of uranium. Though specific ribonucleases have not been identified as a key factor in this process, the possible stress response role of toxin-antitoxin loci (23) needs to be considered. *M. prunae* also employs a passive mechanism to resist uranium, given that, unlike *M. sedula*, it showed minimal capacity to directly oxidize U_3O_8 to soluble U(VI), even when this was the only bioenergetic substrate available. Transcriptomic

analysis also hinted at other mechanisms, including the possible role of a siderophore that sequesters uranium because genes encoding an operon (Msed_0805–Msed_0807), which is homologous to iron complex transport system in other microorganisms (31), was highly induced during U(VI) shock in *M. prunae* and not *M. sedula*.

Microbial Extremophily—An Intrinsic or Adaptive Physiological Characteristic? In a sense, the resistance of *M. prunae* to uranium can be viewed as an expanded extremophily, an adaptation in which heavy metal resistance is superimposed on other unique physiological characteristics (thermophily and acidophily) that are germane to this genus. It is interesting that the *M. prunae* and *M. sedula* genomes are nearly identical and that no identifiable ORFs (or pieces thereof) appear in one of these archaeon's genome and not the other. The spontaneous mutations in *M. sedula* that have apparently conferred U resistance and limited the capacity to generate oxidized forms of the metal are consistent with the needs dictated by *M. prunae*'s environmental niche in a U deposit. Whether already extremophilic microorganisms, in general, are intrinsically better suited for additional adaptations to harsh environments remains to be seen. However, the results described here indicate that heavy metal extremophily is not, in all cases, necessarily a core genomic feature of thermoacidophiles.

A larger issue that arises is how to bridge microbial taxonomy between the pregenomics and postgenomics eras. What constitutes a new genus, species, or strain of a microorganism now compared with then? The close relationship between the genomes of *M. sedula* and *M. prunae* suggests that one is likely the progenitor of the other; however, their vastly different growth physiology in the presence of U, in the absence of genome sequence information, would have made this determination difficult. Going forward, one

wonders how much of our previous understanding of microbial biology, extremophily, or otherwise is based on confusion between genotype and phenotype.

Materials and Methods

Microbial Physiology. For all experiments, *M. sedula* and *M. prunae* cultures were grown aerobically in DSMZ88 media at pH 2.0 and 70 °C with a shaking rate of 90 rpm. Detailed description of DSMZ88 media composition is mentioned in *SI Materials and Methods*. Genomic DNA for genome sequencing of *M. prunae* and for quality check of U shock cultures for both species was prepared as described previously (32). For the experiments reported in Fig. 1A, 0.125 % (wt/vol) spent (nonradioactive) U₃O₈ and 0.2 % (wt/vol) YE were added to the medium. The U₃O₈ was 99.5% pure (analysis by Department of Nuclear Engineering, North Carolina State University); this form of U₃O₈ has been reported to have a mean oxidation state of +5.3, based on XANES spectra (33). Cell densities were determined by epifluorescence microscopy using acridine orange stain. For chemolithoautotrophic growth on 0.125 % (wt/vol) U₃O₈, *M. sedula* and *M. prunae* inocula were obtained by resuspending a heterotrophically grown cell pellet formed by centrifugation at 6,080 × g for 15 min in DSMZ88 media without YE and with CO₂ supplementation in the headspace. For ICP-MS (U analysis), 3-mL samples were collected at the initial time point and after 7 d. The 3-mL samples were split into two tubes as technical replicates and spun at 6,000 × g for 15 min to remove precipitates. The supernatant was collected and sent to North Carolina State University's Department of Soil Science for ICP-MS analysis. Cultures were grown under chemolithoautotrophic conditions (discussed previously) for SEM and XRD analysis of the precipitated material from *M. sedula*, *M. prunae*, and abiotic control. The material was analyzed at the Analytical Instrumentation Facility at North Carolina State University.

Transcriptional Response Experiments. The whole-genome transcriptional response experiments were designed to compare the interspecies response when challenged with solid 0.1 % (wt/vol) U₃O₈ [lower U₃O₈ % (wt/vol) was used for

the transcriptomic experiments to exclusively analyze the bioenergetics elements and not stress response] and also the response of individual species to U(VI) shock. For U(VI) shock, heterotrophic cultures [0.2 % (wt/vol) YE] were challenged in early exponential growth ($t = 13$ h, cell density $\sim 1 \times 10^8$ cells/mL) with 1 mM uranyl acetate. Cultures were harvested 15 min (U15), 60 min (U60), and 180 min (U180) after uranyl acetate addition. The reference culture was grown under the same conditions, but was not challenged with uranyl acetate. For U₃O₈ oxidation, cultures adapted from heterotrophic [0.2 % (wt/vol) YE] to chemolithoautotrophic growth [0.1 % (wt/vol) U₃O₈ with CO₂ supplementation in the headspace], and was accomplished as described above. Cultures were harvested 48 h after inoculation. For both experiments, cells were harvested by rapid chilling and then passage through a 3- μ m filter to remove precipitates followed by centrifugation at 6,080 × g for 15 min. Cell pellets were washed with Tris-EDTA buffer to remove residual media.

RNA extraction and purification was done using TRIzol reagent (Invitrogen). RNA from biological repeats for each condition was pooled together and reverse-transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was labeled with either Cy3 or Cy5 dye (GE Healthcare) and hybridized onto the *M. sedula* array, which was constructed as described previously (18, 19, 21). The same array was used for hybridizing both species, because all of the ORFs in *M. prunae* could be mapped back to *M. sedula*. The microarray slides were scanned using a Genetix scanner. Average intensity data for all spots on the array were normalized using an ANOVA mixed-effects model and analyzed using JMP Genomics (SAS Institute). The criterion for significant differential response was set at a fold change ≥ 2 . The Bonferroni corrections for the U shock and U oxidation experiments were 5.1 and 4.9, respectively. For description of U(VI), RNase assay, and 2D gel analysis, see *SI Materials and Methods*.

Figs. S1–S4 and Tables S1–S5 will be linked to the online version of the paper.

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- Merroun ML (2007) Interactions between metals and bacteria: Fundamental and applied research. *Biometals* 16:331–339.
- Barkleit A, Moll H, Bernhard G (2008) Interaction of uranium(VI) with lipopolysaccharide. *Dalton Trans* (21):2879–2886.
- Barkleit A, Moll H, Bernhard G (2009) Complexation of uranium(VI) with peptidoglycan. *Dalton Trans* (27):5379–5385.
- Appukuttan D, Rao AS, Apte SK (2006) Engineering of *Deinococcus radiodurans* R1 for bioprecipitation of uranium from dilute nuclear waste. *Appl Environ Microbiol* 72:7873–7878.
- Basnakova G, Stephens ER, Thaller MC, Rossolini GM, Macaskie LE (1998) The use of *Escherichia coli* bearing a *phoN* gene for the removal of uranium and nickel from aqueous flows. *Appl Microbiol Biotechnol* 50:266–272.
- Hu P, Brodie EL, Suzuki Y, McAdams HH, Andersen GL (2005) Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. *J Bacteriol* 187:8437–8449.
- Martinez RJ, et al. (2007) Aerobic uranium (VI) bioprecipitation by metal-resistant bacteria isolated from radionuclide- and metal-contaminated subsurface soils. *Environ Microbiol* 9:3122–3133.
- Renninger N, Knopp R, Nitsche H, Clark DS, Keasling JD (2004) Uranyl precipitation by *Pseudomonas aeruginosa* via controlled polyphosphate metabolism. *Appl Environ Microbiol* 70:7404–7412.
- Cologgi DL, Lampa-Pastirk S, Speers AM, Kelly SD, Reguera G (2011) Extracellular reduction of uranium via *Geobacter* conductive pili as a protective cellular mechanism. *Proc Natl Acad Sci USA* 108:15248–15252.
- Guay R, Silver M (1977) Ferrous iron oxidation and uranium extraction by *Thiobacillus ferrooxidans*. *Biotechnol Bioeng* 16:727–740.
- Qiu G, et al. (2011) Column bioleaching of uranium embedded in granite porphyry by a mesophilic acidophilic consortium. *Bioresour Technol* 102:4697–4702.
- Beller HR (2005) Anaerobic, nitrate-dependent oxidation of U(IV) oxide minerals by the chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Appl Environ Microbiol* 71:2170–2174.
- Beller HR, et al. (2009) Identification of c-type cytochromes involved in anaerobic, bacterial U(IV) oxidation. *Biodegradation* 20:45–53.
- DiSpirito AA, Tuovinen OH (1982) Uranous ion oxidation and carbon dioxide fixation by *Thiobacillus ferrooxidans*. *Arch Microbiol* 133:28–32.
- Fuchs T, Huber H, Teiner K, Burggraf S, Stetter KO (1995) *Metallosphaera prunae*, sp. nov., a novel metal-mobilizing, thermoacidophilic archaeum, isolated from a uranium mine in Germany. *Syst Appl Microbiol* 18:560–566.
- Huber G, Spinnier C, Gambacorta A, Stetter KO (1989) *Metallosphaera sedula* gen. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermoacidophilic archaeobacteria. *Syst Appl Microbiol* 12:38–47.
- Liu LJ, You XY, Guo X, Liu SJ, Jiang CY (2011) *Metallosphaera cuprina* sp. nov., an acidothermophilic, metal-mobilizing archaeon. *Int J Syst Evol Microbiol* 61:2395–2400.
- Auernik KS, Kelly RM (2008) Identification of components of electron transport chains in the extremely thermoacidophilic crenarchaeon *Metallosphaera sedula* through iron and sulfur compound oxidation transcriptomes. *Appl Environ Microbiol* 74:7723–7732.
- Auernik KS, Kelly RM (2010) Impact of molecular hydrogen on chalcopyrite bioleaching by the extremely thermoacidophilic archaeon *Metallosphaera sedula*. *Appl Environ Microbiol* 76:2668–2672.
- Liu LJ, et al. (2011) Complete genome sequence of *Metallosphaera cuprina*, a metal sulfide-oxidizing archaeon from a hot spring. *J Bacteriol* 193:3387–3388.
- Auernik KS, Maezato Y, Blum PH, Kelly RM (2008) The genome sequence of the metal-mobilizing, extremely thermoacidophilic archaeon *Metallosphaera sedula* provides insights into bioleaching-associated metabolism. *Appl Environ Microbiol* 74:682–692.
- Cooper CR, Daugherty AJ, Tachdjian S, Blum PH, Kelly RM (2009) Role of vapBC toxin-antitoxin loci in the thermal stress response of *Sulfolobus solfataricus*. *Biochem Soc Trans* 37:123–126.
- Maezato Y, et al. (2011) VapC6, a ribonucleolytic toxin regulates thermophilicity in the crenarchaeote *Sulfolobus solfataricus*. *RNA* 17:1381–1392.
- Villafane A, et al. (2011) CopR of *Sulfolobus solfataricus* represents a novel class of archaeal-specific copper-responsive activators of transcription. *Microbiology* 157:2808–2817.
- Gerdes K, Christensen SK, Løbner-Olesen A (2005) Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol* 3:371–382.
- Gleissner M, Kaiser U, Antonopoulos E, Schäfer G (1997) The archaeal SoxABCD complex is a proton pump in *Sulfolobus acidocaldarius*. *J Biol Chem* 272:8417–8426.
- Kappler U, Sly LI, McEwan AG (2005) Respiratory gene clusters of *Metallosphaera sedula*—differential expression and transcriptional organization. *Microbiology* 151:35–43.
- Bönisch H, Schmidt CL, Schäfer G, Ladenstein R (2002) The structure of the soluble domain of an archaeal Rieske iron-sulfur protein at 1.1 Å resolution. *J Mol Biol* 319:791–805.
- Castresana J, Lübbers M, Saraste M (1995) New archaeobacterial genes coding for redox proteins: Implications for the evolution of aerobic metabolism. *J Mol Biol* 250:202–210.
- Hettmann T, et al. (1998) Cytochrome b558/566 from the archaeon *Sulfolobus acidocaldarius*. A novel highly glycosylated, membrane-bound b-type hemoprotein. *J Biol Chem* 273:12032–12040.
- Rohrbach MR, Braun V, Köster W (1995) Ferrichrome transport in *Escherichia coli* K-12: Altered substrate specificity of mutated periplasmic FhuD and interaction of FhuD with the integral membrane protein FhuB. *J Bacteriol* 177:7186–7193.
- Geslin C, et al. (2003) PAV1, the first virus-like particle isolated from a hyperthermophilic euryarchaeote, "Pyrococcus abyssi". *J Bacteriol* 185:3888–3894.
- Salbu B, et al. (2005) Oxidation states of uranium in depleted uranium particles from Kuwait. *J Environ Radioact* 78:125–135.