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The Mercury-Tolerant Microbiota of the Zooplankton *Daphnia* Aids in Host Survival and Maintains Fecundity under Mercury Stress

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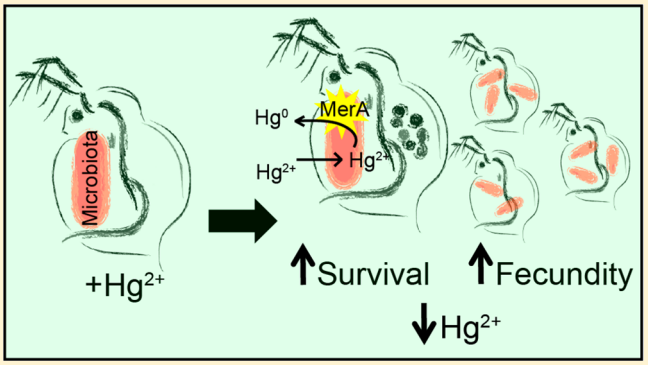
Supporting Information

ABSTRACT: Many aquatic organisms can thrive in polluted environments by having the genetic capability to withstand suboptimal conditions. However, the contributions of microbiomes under these stressful environments are poorly understood. We investigated whether a mercury-tolerant microbiota can extend its phenotype to its host by ameliorating host survival and fecundity under mercury-stress. We isolated microbiota members from various clones of *Daphnia magna*, screened for the mercury-biotransforming *merA* gene, and determined their mercury tolerance levels. We then introduced the mercury-tolerant microbiota, *Pseudomonas*-10, to axenic *D. magna* and quantified its *merA* gene expression, mercury reduction capability, and measured its impact on host survival and fecundity. The expression of the *merA* gene was up-regulated in *Pseudomonas*-10, both in isolation and in host-association with mercury exposure. *Pseudomonas*-10 is also capable of significantly reducing mercury concentration in the medium. Notably, mercury-exposed daphnids containing only *Pseudomonas*-10 exhibited higher survival and fecundity than mercury-exposed daphnids supplemented with parental microbiome. Our study showed that zooplankton, such as *Daphnia*, naturally harbor microbiome members that are eco-responsive and tolerant to mercury exposure and can aid in host survival and maintain host fecundity in a mercury-contaminated environment. This study further demonstrates that under stressful environmental conditions, the fitness of the host can depend on the genotype and the phenotype of its microbiome.



INTRODUCTION

Daphnia is a ubiquitous keystone zooplankton species found in many aquatic ecosystems, capable of growing in both pristine and polluted environments. It can withstand many environmental stressors, hence, is widely used as a model organism for numerous research,[1−7] including studies on the effects of temperature fluctuations,[8,9] food availability,[10−12] predations,[13−15] and exposure to metal pollutants (such as mercury, cadmium, copper, arsenic).[16−23] To date, mercury studies on *Daphnia* have focused mainly on the mechanisms of uptake, accumulation, and elimination in *D. magna*.[16,24−26] While host responses to stressors are evaluated primarily, the responses and contributions of their microbiomes are often overlooked. Aside from host genetics, microbiomes can also be an important component in host survival in deleterious environments. For instance, the gut microbiome of desert woodrats enhances the host's ability to ingest plants with toxic secondary compounds,[27] and the gut microbiota of a coffee berry borer can detoxify the caffeine ingested by its host, allowing the beetle to subsist on the otherwise toxic caffeine-laden berries.[28]

Mercury is a well-known metal contaminant that is bioaccumulated and biomagnified in aquatic food webs.[29−31] Many studies on mercury pollution and its neurotoxicity have been reported.[30,32−34] Three major species of mercury can be found in the environment: inorganic mercury (Hg^{2+}), organic methylmercury (MeHg including the mono- and dimethyl forms), and elemental mercury (Hg^0). Hg^0 and Hg^{2+} are commonly released into the atmosphere via anthropogenic and natural sources, while deposition of atmospheric mercury leads to mercury contamination of terrestrial and aquatic ecosystems.[31,33,35] Anaerobic microorganisms harboring *hgcAB* genes (encoding a corrinoid-dependent protein and an associated ferredoxin protein, respectively) have been shown to methylate mercury, producing the highly bioaccumulative MeHg from inorganic Hg^{2+}.[36−39] Demethylation of MeHg to Hg^{2+} can be facilitated by microorganisms containing *merB* that encodes an organomercurial lyase. Inorganic Hg^{2+} can be further converted into the less toxic, extremely volatile Hg^0 by microorganisms expressing the *merA* gene that encodes a mercury reductase.[40−43] Therefore, microorganisms harboring these mercury-biotransformation genes have the potential to impact mercury speciation, cycling, and concentration in the environment. Although mercury tolerance and detoxification in

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many bacteria species have been reported,[42−44] most of these bacteria were identified in water columns, sediments, and soil.[45−47] In addition, most studies on the biotransformation of mercury in natural ecosystems focused on bacteria found in specific geographical environmental habitats,[45−47] with only a few studies on mercury-biotransforming bacteria found in animal hosts.[48,49] The contribution of these bacteria on host mercury tolerance and survival, however, has never been investigated.

In this study, we investigated the role of microbiomes on host fitness under mercury stress. We first assessed the microbiome community structure of *D. magna* collected from a seasonally mercury-polluted site (Yolo Bypass, California).[50] We then isolated members of the microbiota, determined their mercury tolerance levels, and measured *merA* gene expression and mercury reduction of the mercury-tolerant microbiota member (*Pseudomonas*-10) in isolation and in association with the daphnid host. We found that *Daphnia* is an environmental reservoir of mercury-tolerant bacteria that could potentially biotransform mercury into less-toxic form by up-regulating the expression of *merA* gene upon exposure to elevated levels of mercury and reducing the concentration of mercury in the medium. Most importantly, we found that a daphnid microbiota member can aid in the host survival and allow the host to produce viable offspring even when exposed to mercury contamination.

MATERIALS AND METHODS

***Daphnia* Animal Collection and Husbandry.** *D. magna* CAY (California Yolo-bypass) was sampled in the inlet ponds (38°31′45.4″N, 121°36′28.9″W) that are part of the Yolo Bypass Wildlife Area (Davis, California, U.S.A). Other *D. magna* clones, DE-K35-Iinb1 (Germany) and FI-Xinb3 (Finland)[51] are part of the *Daphnia* collection in our laboratory. Daphnid husbandry was carried out as previously published.[52] Animals were routinely cultured in artificial *Daphnia* medium (ADaM)[53] at 20 °C with 14:10 light/dark photoperiod and fed daily with *Scenedesmus obliquus* (~2×10^7 cells). Experiments with *D. magna* were also carried out in the same media and conditions.

16S rRNA Fragments High-Throughput Sequencing and Data Analysis. Total genomic DNA (gDNA) (n = 3) was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). Equimolar DNA concentration was sent to the Argonne National Laboratory Sequencing Core for library preparation and sequencing of the 16S rRNA V4 region (PCR-amplified with 515F and 806R primers, Table S1), using the MiSeq Illumina sequencing platform. Additional details are provided under Supporting Information B.

D. magna sequences were demultiplexed and checked for chimeras, with low quality sequence and short sequence reads (<150 bp) removed prior to postanalysis with the Quantitative Insights Into Microbial Ecology (QIIME 1.8) pipeline.[54] Operational taxonomic units (OTUs) were clustered at 97% sequence similarity with Uclust.[55] Representative sequences from the clustered OTUs were picked for taxonomic identification based on RDP classifier 2.2.[56] Singletons, chloroplast, and mitochondria sequences were filtered out of the OTU table prior to alignment of OTU representative sequences with PyNAST.[54] Samples were rarified at a minimum of 10 sequences and a maximum depth of 12030 sequences in steps of 10. For microbiome composition analysis, assignments of bacterial taxon were performed using BLAST 2.22.2.[57] Information on NCBI data availability of the raw sequence reads are provided in Supporting Information E.

Bacteria Isolation and Identification, Growth Conditions, and *merA* Screening. *D. magna* microbiota were isolated from various *D. magna* clones: the newly collected CAY clone and clones in the laboratory collections (FI-Xinb3 and DE-K35-Iinb1) as well as sediment samples from Yolo Bypass. Individual *D. magna* were washed three times with 1 mL of sterile ADaM, homogenized in 200 μL of sterile ADaM, plated on various agar media, including LB, R2A (Teknova, Hollister, CA), 10x-diluted R2A with and without N-acetylglucosamine supplement, and MacConkey (Teknova, Hollister, CA), and incubated at room temperature (22−23 °C) for several days. Sediment samples were also plated on the same set of agar media and incubated at room temperature for several days. Agar medium contains 1.5% (w/v) granulated agar. Colonies exhibiting different phenotypes were repeatedly streaked for single colonies. Pure microbiota isolates were cultured in R2A liquid media and stored at −80 °C in autoclaved glycerol.

Genetic identification of bacterial isolates and *merA* screening were carried out by sequencing the partial PCR-amplified 16S rRNA and *merA* fragments. PCR amplification was carried out with MyTaq Red (Bioline, Taunton, MA) using the following PCR program: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, and a final extension step at 72 °C for 5 min. Universal 16S primer set 8F and 1492R[58] and *merA* primer sets MERA5 and MERA1[44] or merA-128_F and merA-993_R, were used to amplify 16S rRNA and *merA* genes, respectively. Additional details are provided in Supporting Information C. Primers used in this study are listed in Table S1. GenBank accession numbers for the partial 16S rRNA and *merA* sequences are found in the Supporting Information E. Details on 16S rRNA phylogenetic analysis are provided in Supporting Information D.

Bacteria Mercury Minimal Inhibitory Concentration (MIC) Assays. Mercury MIC assays of twenty-seven bacterial isolates were carried out at room temperature (22−23 °C) in 96-well microtiter plates containing 200 μL of R2A media with different concentrations of mercury (0.1, 0.2, 0.4, 0.6, 0.8, and 1 to 15 μM, with 1 μM increment). The bacteria MIC was determined using 1 to 15 μM first, followed by the lower mercury concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1 μM) for bacteria that have MIC < 1 μM. Each well containing the defined mercury concentrations was inoculated with 2 μL of bacterial inoculum and visible bacterial growth, defined as increase in culture turbidity, was visually checked and measured as increased in absorbance/optical density at 600 nm (OD_{600}). Cultures grown in the absence of mercury were used as positive controls, while uninoculated R2A media were used as negative controls. The MIC is the lowest mercury concentration where bacterial growth (culture turbidity) was not observed with the naked eye,[59] and the difference in OD_{600} values between measured and negative control (uninoculated R2A media) is less than 0.01.[60] The MIC assays were repeated three times, and the lowest mercury concentration where *no growth* occurred[59] after 3 days of incubation indicates the mercury MIC for that particular bacterial isolate. Mercury stock solution (1000 μg/mL mercury in 10% nitric acid) was purchased from SPEX CertiPrep (Metuchen, NJ). OD_{600} was determined daily using SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

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RNA Extraction and cDNA Synthesis. Since *Pseudomonas*-10 exhibited the highest mercury MIC (8 μM) among the *merA*-positive microbiota isolates identified in this study (Table 1), this microbiota isolate was used to study *merA* gene expression. To quantify *merA* gene expression in isolation, *Pseudomonas*-10 was grown aerobically at room temperature (22−23 °C) in LB media until the midexponential growth phase (OD_{600} = 0.3). Aliquots (10 mL) of the cultures were transferred to new culture flasks, followed by no mercury exposure (0 μM) or exposure to 2.5 μM or 5 μM of mercury for 15 min at room temperature. After treatments, 2 mL aliquots were pelleted by centrifugation (21, 200 × *g*) at room temperature for 2 min. Cell pellets were immediately resuspended in Trizol (Fisher Scientific, Hampton, NH) and stored at −80 °C.

Table 1. List of Identified *D. magna* Microbiota Isolates, Mercury MIC, and *merA* Gene Screening

16S rRNA Identification[a]	Source[b]	Mercury MIC[c] (μM)	*merA*[d]
Acidovorax-4	FI-Xinb3	11	
Curtobacterium-20	CAY	11	
***Pseudomonas*-10**	**DE-K35-Iinb1**	**8**	+
***Acinetobacter*-1**	**DE-K35-Iinb1 (ADaM)**	**6**	+
Brevundimonas-6	DE-K35-Iinb1	6	
Pseudomonas-28	CAY	6	
***Hydrogenophaga*-8**	**DE-K35-Iinb1**	**5**	+
***Variovorax*-11**	**FI-Xinb3**	**5**	+
Bacillus-19	CAY	5	
Rhodococcus-24	CAY	5	
Blastomonas-12	CAY	4	
Runella-13	CAY	4	
Bacillus-16	CAY	4	
Bacillus-5	FI-Xinb3	3	
Micrococcus-17	CAY	3	
***Pseudomonas*-23**	**CAY**	**3**	+
Shinella-26	CAY	3	
Aeromonas-27	CAY	3	
Arthrobacter-2	DE-K35-Iinb1	2	
Aeromonas-3	FI-Xinb3	2	
Citricoccus-14	Sediment, Yolo Bypass	2	
Flavihumibacter-21	CAY	2	
Curtobacterium-15	CAY	1	
Flavihumibacter-25	CAY	1	
Exiguobacterium-7	DE-K35-Iinb1	0.8	
Flavihumibacter-22	CAY	0.8	
Lysinibacillus-18	Sediment, Yolo Bypass	0.6	

[a]Numbers after the genus names indicated the laboratory collection numbers, so as to distinguish between isolates from the same genera. [b]Bacteria were isolated either from *D. magna* crushed bodies (CAY, DE-K35-Iinb1, or Fl-Xinb3 clones), Yolo Bypass sediment (where the *D. magna* CAY clones were collected), or the ADaM culture medium. [c]Lowest mercury concentration with no bacterial growth after 3 days of incubation at room temperature (22−23 °C). [d]Presence of *merA* gene determined by PCR and sequencing, indicated with + and bold lettering.

For measuring *merA* expression of daphnid-associated *Pseudomonas*-10, *D. magna* CAY harboring *Pseudomonas*-10 exposed to 50 nM mercury for 4 h on day 5 (Experiment 1, n = 55; Experiment 2, n = 50) or unexposed (Experiment 1, n = 50; Experiment 2, n = 53) were harvested, washed once with sterile ADaM, homogenized in Trizol, and stored at −80 °C. The mercury LD_{50} for *D. magna* is 51.5 nM (Figure S3); hence, the mercury concentration of 50 nM was chosen for the mercury stress experiments in *D. magna* CAY. Gnotobiotic *D. magna* were generated as described for survival assays.[52]

RNA was extracted with Direct-zol RNA Miniprep Plus (Zymo research, Irvine, CA) according to the manufacturer's instructions. Eluted total RNA was further treated with DNase I (Promega, Madison, WI) for 30 min at 37 °C, followed by inactivation at 65 °C for 10 min and purification using Direct-zol RNA Miniprep Plus. RNA concentrations were estimated using either a NanoDrop spectrophotometer or a Qubit fluorometer (Fisher Scientific, Hampton, NH). cDNA was synthesized using SuperScript III and random hexamers (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. For no-host RNA samples, 200 ng of RNA was used as a template for cDNA synthesis. For daphnid-associated RNA samples, depletion of daphnid host RNA was first carried out using a Dynabeads mRNA purification Kit (Fisher Scientific, Hampton, NH), where the Dynabeads Oligo $(dT)_{25}$ binds to host RNA that contains a poly-A tail. Unbound bacterial RNA was collected and purified using Direct-zol RNA Miniprep Plus. cDNA synthesis was then carried out using 2−3 μg of RNA as templates. Reactions without reverse transcriptase were used as controls (NRT).

Quantitative Real-Time PCR (qRT-PCR) and Semi-quantitative PCR (Semi-qPCR). qRT-PCR reactions (10 μL) were set up using a PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Each reaction contains 2 μL of cDNA, 0.4 μM of each specific primer (merA-Pse10_F and merA-Pse10_R or glnA_F and glnA_R; Table S1), and 1X SYBR green master mix. qRT-PCR was performed on an Applied Biosystems 7500 real-time PCR system with the following thermal cycling steps: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificities of the *merA* and *glnA* primers were tested with regular PCR and DNA gel electrophoresis, as well as in qRT-PCR with a Dissociation Stage (melt curve analysis). No amplification of no template control (NTC) and no reverse transcriptase (NRT) reactions served as negative controls. Serially diluted *Pseudomonas*-10 gDNA samples, extracted with a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), were used as positive controls and to generate calibration curves. The C_T values of the samples were within the linear dynamic range of the calibration curves for *glnA* and *merA*, with R^2 values of >0.99. All samples were normalized to the expression of the glutamine synthetase (*glnA*) housekeeping gene,[61] and relative gene expression (fold change) is calculated using the $2^{-\Delta\Delta C_T}$ method.[62] Data were log-transformed for statistical analysis. The assay was carried out three times, each with three technical replicates.

Semi-qPCR was carried out, similar to a protocol published previously,[63] to measure *merA* expression in daphnid-associated *Pseudomonas*-10. The reactions (10 μL) were set up using a MyTaq Red master mix, 3 μL of cDNA and 1 μM of each specific primer (merA-Pse10_F and merA-Pse10_R or glnA_F and glnA_R) with the following PCR program: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 20 s, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. NRT and NTC were used as negative controls. Amplified products (3 μL) were analyzed with 2% (w/v) agarose gel electrophoresis, and the gel images

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were captured using the ChemiDoc System (BioRad, Hercules, California). Fluorescence was determined with Image Lab v5 software (Bio-Rad) and calculated by subtracting background fluorescence of the corresponding NRT reactions for both *merA* and *glnA*, followed by normalization to the corresponding *glnA* samples. The assay was carried out twice, each with three technical replicates.

***D. magna* Mercury Lethal Dose 50 (LD_{50}), Survival and Fecundity Assays.** Mercury LD_{50} of *D. magna* was determined in nonaxenic daphnids cultured in 80 mL of ADaM medium containing 10, 50, 75, and 100 nM mercury. Daphnids cultured in ADaM without mercury (0 μM mercury) served as controls. The mercury LD_{50} experiments were carried out in 10% nitric acid-washed experimental jars (with 0.22 μM filter caps) containing 4 daphnids per experimental jar (n = 4 for each treatment), and these were fed with nonaxenic *S. obliquus* (~2×10^7 cells per jar). LD_{50} was determined using a 4-parameter logistic regression (Figure S3). The LD_{50} of *D. magna* CAY after 2 days of mercury exposure is 51.5 nM, and as such, 50 nM of mercury was used as the applied concentration in the experiments that involved mercury exposure in *D. magna* CAY. The LD_{50} assays were carried out with four replicates.

Survival assays were carried out twice in 10% nitric acid-washed experimental jars (with 0.22 μM filter caps) containing 80 mL of sterile ADaM with one daphnid per jar which were fed with axenic *S. obliquus* (~8×10^7 cells/jar). Bacteria-free eggs were generated with antibiotic-treatment[52] using the third egg clutches of reproductively synchronized F2 adults and were separated into 3 groups: Bacteria-free (Bac-Free), bacteria supplemented with parental microbiome (Bac-Suppl), and *Pseudomonas*-10-infected (Pse-Inf). *Pseudomonas*-10 was used as it exhibits the highest MIC (8 μM) among the *merA*-positive microbiota isolates identified in this study (Table 1). Mercury stress (50 nM) was introduced on day 5. Survival was monitored daily for a period of 18 days. Fecundity assays were carried out twice, set up similarly as the survival assays, in 80 mL of sterile ADaM with one daphnid per jar which were fed with axenic *S. obliquus* (~8×10^7 cells/jar). Live hatchlings were counted and removed from the experimental jars daily for a period of 18 days. Mercury stress (50 nM) was added on day 5. At the end of the experiments, the sterility of the daphnids in the Bac-Free group and the presence of bacteria in the Bac-Suppl and Pse-Inf groups were verified by PCR using a 327F and 936R primer set (Table S1), targeting the bacterial 16S rRNA gene.[52] In addition, crushed daphnids from each experimental group were also plated on LB or R2A agar media (Sigma, St. Louis, MO) to verify that there was no bacterial growth from the Bac-Free group, mixed bacterial growth from Bac-Suppl group (indicated by the growth of bacteria exhibiting various morphotypes), and pure bacterial growth from Pse-Inf group. The identity of *Pseudomonas*-10 from the Pse-Inf groups was further confirmed via sequencing of the 16S rRNA using 8F and 1492R.[58]

Mercury Biotransformation by *Pseudomonas*-10. Total mercury (Hg^{2+} and Hg^0, MeHg, and other Hg species) concentrations in the ADaM medium of *Pseudomonas*-10 only (Pse), bacteria-free daphnids (Bac-Free), and daphnids infected with *Pseudomonas*-10 (Pse-Inf) were determined by cold vapor atomic absorption spectrometry using a DMA-80 (Milestone, Inc.), calibrated with aqueous standards and accuracy checked against standard reference material BCR-414 (EPA Standard Method 7473).[64] Experimental jars were set up similar to the survival assays, except that each experimental jar contained 5 daphnids. The experimental jars containing ADaM medium (80 mL) only served as controls. The experimental jars of the Pse group contained ADaM medium (80 mL) and *Pseudomonas*-10 (200 μL of ADaM-washed bacterial culture suspension diluted to OD = ~ 0.6). Mercury (50 nM) was added to the experimental jars on day 5. Samples were collected and filtered through a 0.22 μm filter on day 5, before (No Hg) and after (D5) the addition of mercury, and on day 8 (D8). Mercury samples were stored at 4 °C until analysis. Total mercury in the samples was measured directly with cold vapor atomic absorption spectrometry without any pretreatment. Data were obtained from at least 3 replicates for each condition: ADaM medium (n = 3), Pse (n = 3), Bac-Free (n = 4), Pse-Inf (n = 4).

Statistical Analysis. Data were presented as mean values with standard errors, except for box plots where medians (horizontal lines within the boxes) were shown with 25% to 75% quartiles, and maximum and minimum values were shown as whiskers. For experiments measuring the differences in gene expression and mercury reduction between treatments, data were log-transformed (when necessary) to fit the assumption of normal distribution and were tested for homogeneity of variance prior to performing one-way ANOVA with Tukey's HSD posthoc test or Student's t test using JMP 14. Statistical analysis for differences in survival rates was conducted using the Mantel Cox log-rank test in GraphPad Prism 5.01, with Bonferroni corrected p-value for multiple comparisons. The Kruskal–Wallis test and the Wilcoxon each pair test were used to compare differences in fecundity using JMP 14.0.

RESULTS AND DISCUSSION

Microbiome Composition of *D. magna* CAY. To investigate whether *D. magna* CAY (collected from California Yolo Bypass) harbors mercury-tolerant and *merA*-positive bacteria, we first determined the composition of the *D. magna* CAY microbiome via 16S rRNA amplicon sequencing. A total of 1295 OTUs were identified from the samples (n = 3). After removing singletons and mitochondrial and chloroplast sequences, 416 OTUs remained and were assigned into 73 genera, 32 families, 10 orders, 9 classes, and 1 phylum (Table S2). Only bacteria with ≥1% average relative abundance (from 3 samples) were included in the comparison shown in Figure 1. The top three most abundant microbiota in *D. magna* CAY are bacteria from the genus *Limnohabitans*

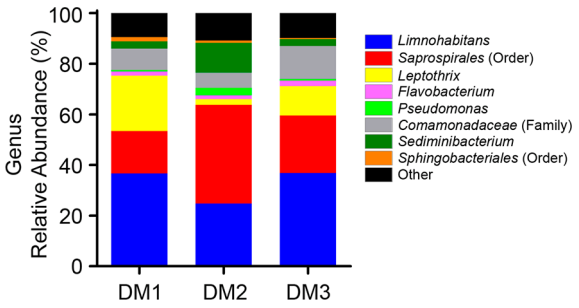

Figure 1. Relative abundance of major taxonomic groups of microbiota from *D. magna* CAY. Relative abundance of microbiota members that are ≥1% (average abundance of all 3 samples). Genera with less than 1% are grouped as "Other". Some of the microbiota were identified only at the family and order levels using BLAST. *D. magna* samples (n = 3): DM1, DM2, and DM3.

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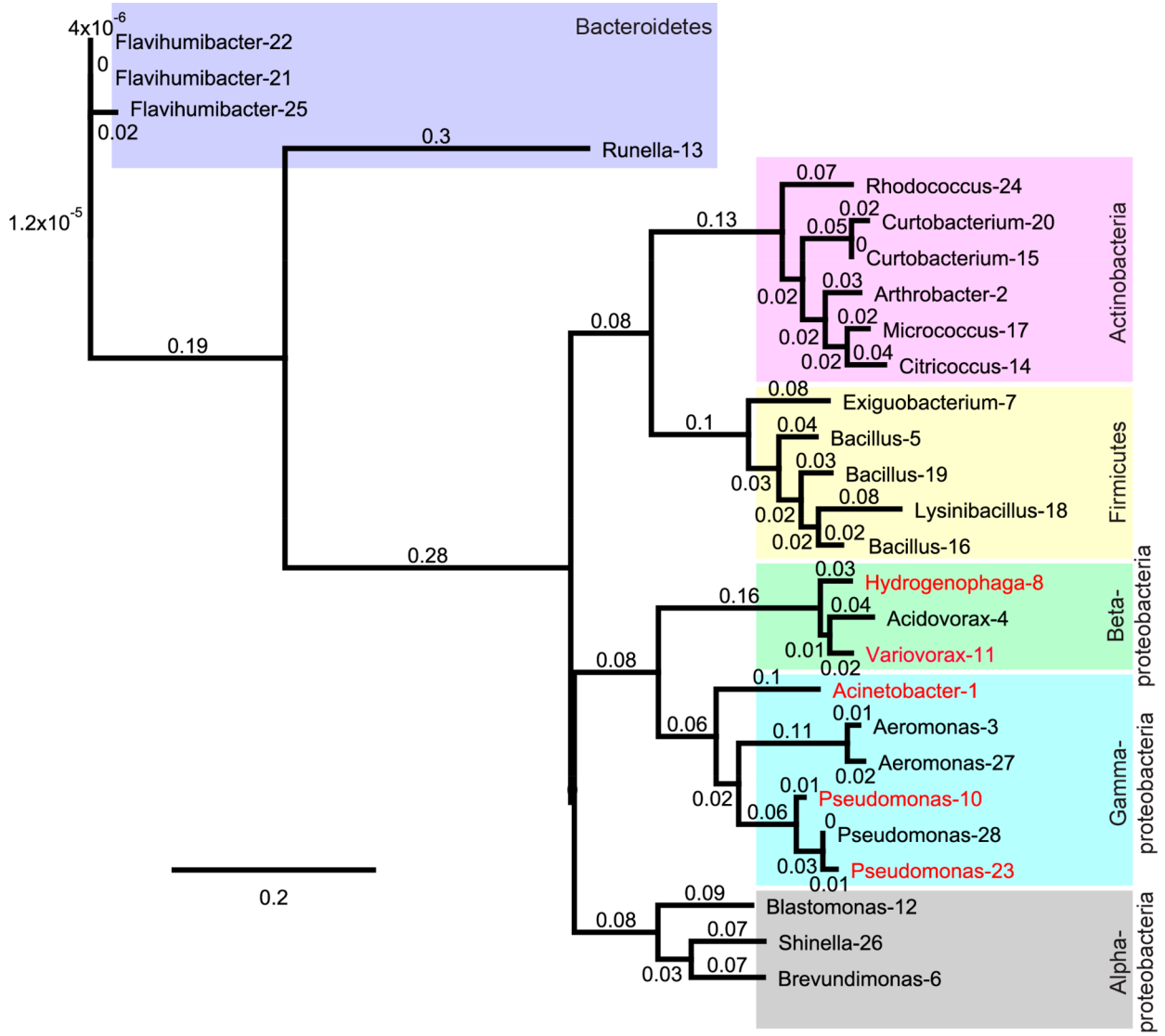

Figure 2. 16S rRNA phylogenetic tree of isolated *D. magna* microbiota. 16S rRNA sequences of the 27 isolates were first aligned using MUSCLE, followed by tree-construction using maximum likelihood method (PhyML), with Jukes-Cantor substitution model and 250 bootstrap replicates. *merA*-positive microbiota isolates identified in this study (Table 1) are in red. Numbers after the bacteria name represent laboratory collection ID number. Bootstrap values and scale bar are indicated as substitution per site.

(32.8%, average abundance), the order Saprospirales (26.2%), and the genus *Leptothrix* (11.9%). Other identified microbiota members that are ≥1% (average abundance) include bacteria from the family Comamonadaceae (9.2%), the genera *Sediminibacterium* (5.8%), *Flavobacterium* (1.8%), and *Pseudomonas* (1.3%), and the order Sphingobacteriales (1%). Since *Pseudomonas* has been shown to be pathogenic to *D. magna*,[65−67] it is interesting that *Pseudomonas* was found to be >1% in the microbiota of *D. magna* CAY. The presence of *Pseudomonas* as part of the *Daphnia* microbiome has also been reported in different *Daphnia* species,[51,68,69] suggesting that these *Pseudomonas* microbiome members may be non-pathogenic, as opposed to the isolates (*P. aeruginosa* PAO1 and strain DD1) used in the reported studies.

Notably, the microbial composition and structure of *D. magna* CAY reported here is similar to the microbiomes of other *D. magna* coming from different geographical locations,[51,68,70] such as the dominance of *Limnohabitans* and the Saprospirales group for instance, suggesting active selection of microbiome by the daphnid host. *Limnohabitans* have been reported to increase fecundity in the *Daphnia* host[71] and are abundant in the host's filter apparatus.[72] Indeed, host genetics have been shown to play a role in the structuring and maintaining of different microbiota community abundances in *Daphnia*[51] and in *Drosophila*.[73−75] Collectively, our data indicates that the newly collected *D. magna* CAY clones harbor microbiota similar to other published *D. magna* clones and reinforces the idea that host factors influence the microbial composition of the *Daphnia* microbiome.

Mercury Minimal Inhibitory Concentration (MIC) and *merA* Screening of Microbiota Isolates. Many bacteria are capable of mercury detoxification, commonly through the expression of *mer* operons.[42−44] One of the central players of this widespread mercury-detoxification system is MerA, a mercuric reductase that is capable of reducing a more reactive, cationic form of mercury (Hg^{2+}) to a relative inert, volatile monatomic mercury vapor (Hg^{0}), which can diffuse through the cell membrane.[40−42] Cross-referencing the 73 assigned

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genera found in *D. magna* CAY with NCBI databases, 41 genera were found to contain species that putatively harbor *merA* genes (Table S2). We therefore hypothesize that *Daphnia* could harbor *merA*-positive bacteria capable of detoxifying mercury through biotransformation. To test this hypothesis, we isolated pure cultures of the microbiota members from the newly collected *D. magna* CAY and two published *D. magna* clones (DE-K35-Iinb1 and FI-Xinb3) in the laboratory collection as well as their environments (ADaM culture media and the sediment from Yolo Bypass sampling site). We then determined the mercury MICs of individual isolated bacteria and screened them for the presence of the *merA* gene. We isolated 27 bacteria from different *D. magna* clones and their environments (Table 1) and determined their identities and phylogeny using partial 16S rRNA sequences. The well-resolved phylogenetic tree showed the phylum/class groupings of *D. magna* CAY microbiota isolates (Figure 2). Several of the bacterial isolates (*Acidovorax*, *Acinetobacter*, *Blastomonas*, *Exiguobacterium*, *Hydrogenophaga*, and *Pseudomonas*) have homologous 16s rRNA sequences identified in the *D. magna* CAY microbiome (Figure 1 and Table S2). In addition, some of these isolated bacteria (*Acidovorax*, *Acinetobacter*, *Aeromonas*, *Hydrogenophaga*, and *Pseudomonas*) were also identified as microbiome members of other *Daphnia* species.[68,69,76] We were not able to isolate and culture the dominant microbiota member, *Limnohabitans*, despite numerous attempts using several different media and methods of isolation.

Table 1 also shows the level of mercury tolerance (reported as MIC) of the isolated *D. magna* microbiota. Highest mercury MIC was observed with *Acidovorax*-4 (11 μM) and *Curtobacterium*-20 (11 μM), followed by *Pseudomonas*-10 (8 μM). Other microbiota isolates exhibited low to medium MICs ranging from 0.6 to 6 μM. To date, most bacteria tested for mercury tolerance are either free-living bacteria or bacteria found in the sediments.[45,77−79] Host-associated bacteria that are mercury-tolerant have been reported only in a few hosts, including fish gut bacteria grown in media containing 12.5 μM of mercury,[48] bacteria isolated from nodules of leguminous plants, and marine sponges exhibiting MICs of 30 μM and >100 μM of mercury, respectively.[49,80] Hence, our study and that of others indicate that host-associated microbiomes are also potential sources of mercury-detoxifying bacteria.

To determine if mercury-tolerant bacteria contain *merA* genes, we screened all 27 bacteria isolates for the presence of *merA* using published primer sets (Table S1).[44,77,81] Initial screening identified partial *merA* genes in *Acinetobacter*-1 and *Hydrogenophaga*-8 using primer set MERA5 and MERA1;[44] other primer sets did not yield amplified *merA* fragments. Using the partial *merA* sequences from *Hydrogenophaga*-8 and *Acinetobacter*-1, we designed a primer set, merA-128_F and merA-993_R (Table S1), targeting the conserved sequence regions (Figure S1), and further identified three more bacteria isolates containing *merA*. In all, *merA* fragments were amplified from five microbiota isolates: three γ-proteobacteria isolates (*Pseudomonas*-10, *Pseudomonas*-23, and *Acinetobacter*-1) and two β-proteobacteria isolates (*Variovorax*-11 and *Hydrogenophaga*-8). Homology sequence searches using BLAST showed high sequence similarity to known *merA* sequences (Table S3 and Figure S1), indicating that *Pseudomonas*-10, *Pseudomonas*-23, *Acinetobacter*-1, *Variovorax*-11, and *Hydrogenophaga*-8 harbor *merA* genes. It is noteworthy that all these five *merA*-positive isolates also exhibited moderate to high mercury MICs, ranging from 3 to 8 μM (Table 1), hinting that these bacteria isolates likely contain functional *merA* genes. The ability of the primer set merA-128_F and merA-993_R in amplifying *merA* fragments from both β-proteobacteria and γ-proteobacteria, indicates that it can be used for the *merA* screening of environmental samples.

Although many other isolated microbiota exhibited high mercury tolerance or belong to genera that putatively contain *merA*-positive species, we were unable to amplify their *merA* gene, including *Acidovorax*-4 and *Curtobacterium*-20. In addition, while *Pseudomonas*-10 and -23 harbor the *merA* gene, we were unable to detect the presence of *merA* in the congeneric *Pseudomonas*-28. It is possible that these bacterial isolates contain nucleotide polymorphisms in the binding regions of the designed *merA* primer sets or that these particular species/strains do not contain *merA* but exhibit mercury tolerance by other yet-to-be identified mechanisms. We are continuing our effort in using new *merA* primer sets to screen our remaining microbiota isolates as well as screen for the presence of *merB*, which will be part of another study.

MerA exists as a homodimer protein, and three major conserved regions/residues critical for MerA activity have been identified.[42] At the N-terminus, a stretch of residues containing two cysteines forms a redox-active site, while the C-terminus short cysteine pair aids in Hg^{2+} binding to the N-terminal redox-active site of the corresponding monomer. In addition, two tyrosine residues are also involved in Hg^{2+} binding.[42] While most of the conserved active regions/residues are beyond the identified *merA* sequences of the microbiota isolates, the first Hg^{2+}-binding tyrosine is found in *Pseudomonas*-10, *Hydrogenophaga*-8, and *Acinetobacter*-1 (Figure S1). The well-resolved MerA tree also showed divergence of MerA between *Pseudomonas* isolates and the other three microbiota isolates (Figure S1). This is not surprising since *merA* found in *Pseudomonas* has been suggested to be more distantly related to the *merA* of other Gram-negative bacteria.[82] While the partial MerA sequences between *Pseudomonas*-10 and -23 determined in this study are identical, they exhibited distinct mercury tolerance levels. Possible sequence variations may exist in their C- and N-terminus active sites, which may explain the tolerance differences between these two *Pseudomonas* isolates. Together, our data showed that *Daphnia* microbiomes contain mercury-tolerant bacteria that harbor *merA* genes, which imply their potential to biotransform mercury from the toxic Hg^{2+} to less toxic Hg^0 species.

Expression of Bacterial *merA* Gene in Isolation and in Association with *D. magna* after Mercury Exposure. To test the functionality of the *merA* gene, we exposed exponentially growing *Pseudomonas*-10 to 2.5 and 5 μM of mercury for 15 min and compared *merA* expressions between mercury-exposed and unexposed cultures (0 μM). *Pseudomonas*-10 was chosen as it exhibits the highest mercury MIC (8 μM) among the *merA*-positive microbiota isolates identified in this study (Table 1). Using qRT-PCR, relative expression of *merA* was found to be more than 300-fold higher in cells exposed to mercury compared to unexposed cells (one-way ANOVA, $F = 130.79$, $p = 0.00001$ Figure 3). We also visualized the qRT-PCR amplified products with agarose gel electrophoresis and quantified the relative fluorescence (Figure S2). As expected, higher fluorescence was detected in samples exposed to mercury compared to the unexposed samples. Expression of *merA* is not significantly different between cells exposed to 2.5 and 5 μM mercury (Tukey's HSD Posthoc test,

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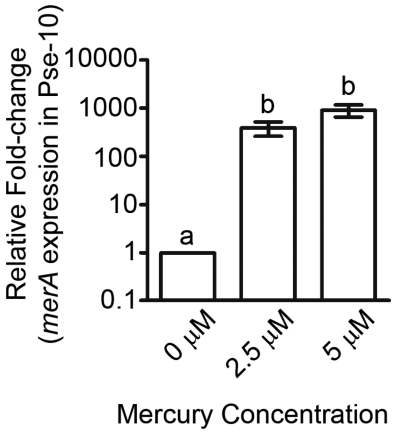

Figure 3. *merA* expression in the microbiota isolate *Pseudomonas*-10. Relative fold-change of *merA* expression in *Pseudomonas*-10 (Pse-10) bacterial cultures exposed to 0, 2.5, and 5 μM of mercury in LB media, determined from 3 independent experiments with 3 technical replicates. Data were log-transformed and were analyzed using one-way ANOVA with Tukey's HSD Posthoc test. Column with different letters are significantly different ($p < 0.05$).

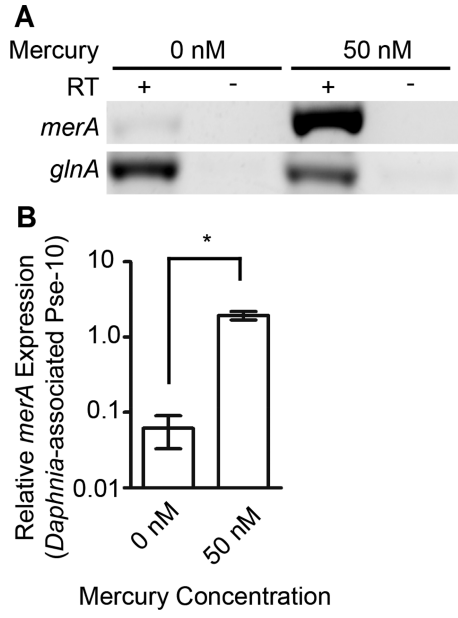

Figure 4. Expression of *merA* in the daphnid-associated *Pseudomonas*-10. (A) Agarose gel electrophoresis of amplified *merA* and *glnA* (housekeeping gene for normalization). (B) Relative *merA* expression in host-associated *Pseudomonas*-10 exposed to 50 nM mercury, determined from 2 independent experiments with 3 technical replicates. *, $p = 0.0001$ (Student's t test). RT, reverse transcriptase.

$p > 0.05$, Figure 3 and Figure S2). Similarly, increases in *merA* gene expressions have also been reported in other *Pseudomonas* strains, where over 10-fold and 30-fold increases were observed with 2 and 5 μM mercury exposures (respectively) in *Pseudomonas stutzeri* OX,[83] and more than a 2-fold increase was observed with 50 μM of mercury exposure in the *Pseudomonas* strain ATH-43.[78] Our data show that the mercury-tolerant and *merA*-positive *Pseudomonas*-10 isolate is capable of upregulating *merA* gene expression upon mercury exposure.

We further investigated if similar upregulation of *merA* also occurs in *Pseudomonas*-10 when in association with the *D. magna* host upon exposure to mercury. We first determined the lethal dose 50 (LD_{50}) of mercury in *D. magna* CAY. To do this, we exposed 5 day old nonaxenic daphnids to various concentrations of mercury for 2 days and monitored their survival. Using a 4-parameter logistic regression, we determined that the LD_{50} of mercury is 51.5 nM (Figure S3) and as such, 50 nM was chosen as the applied mercury concentration for the mercury stress experiments in *D. magna*. We infected bacteria-free *D. magna* CAY with *Pseudomonas*-10, allowed bacteria-host association to establish for 5 days, exposed the *Pseudomonas*-10-infected daphnids to 50 nM of mercury for 4 h, harvested total RNA, and determined *merA* gene expression. Several attempts using qRT-PCR to determine *merA* expression in host-associated *Pseudomonas*-10 did not yield reliable results, likely due to low abundance of bacterial cDNA and interference from the daphnid host cDNA, despite attempts to deplete host RNA before cDNA synthesis. As such, we carried out semiquantitative RT-PCR (semi-qPCR) by analyzing the amplified *merA* and *glnA* (housekeeping gene for normalization) on agarose gel and determined band intensities of the amplified products (Figure 4). Upon exposure to 50 nM of mercury, *merA* expression in daphnid-associated *Pseudomonas*-10 was upregulated approximately 31-fold, when compared to daphnid-associated *Pseudomonas*-10 without mercury treatment. This data indicates that *Pseudomonas*-10 is capable of upregulating *merA* expression in response to mercury stress even when in association with the daphnid host. The lower expression level of *merA* in daphnid-associated *Pseudomonas*-10 (31-fold), when compared to *Pseudomonas*-10 in isolation (>300-fold), is likely due to the lower mercury concentration used in host-associated *Pseudomonas*-10 exposure.

***Pseudomonas*-10 Aids in Host Survival and Maintains Host Fecundity under Mercury Stress.** Since host-associated *Pseudomonas*-10 exhibited increased *merA* expression upon mercury exposure, we investigated whether *Pseudomonas*-10 can contribute to host survival and fecundity under mercury stress. We infected *D. magna* CAY with *Pseudomonas*-10 (Pse-Inf), allowed the daphnids to grow for 5 days before exposing the daphnids to 50 nM mercury, and compared their survival rates to bacteria-free (Bac-Free) and parental-microbiome supplemented *D. magna* CAY (Bac-Suppl) over a period of 18 days (Figure 5A). We positively verified the absence of bacteria in the Bac-Free group, the presence of *Pseudomonas* in the Pse-Inf group, and the presence of various bacterial morphotypes in the Bac-Suppl group at the end of the experiment.

A significant difference in survival rates was observed between daphnid groups (Mantel-Cox log-rank test, $\chi^2 = 26.29$, $p = 0.003$). In the absence of mercury stress, Bac-Suppl daphnids (94%, $n = 29/31$) exhibited similar survival rates with Pse-Inf daphnids (87%, $n = 26/30$, $\chi^2 = 0.68$, $p = 0.41$) and significantly higher survival rates than Bac-Free daphnids (73%, $n = 22/30$, $\chi^2 = 4.16$, $p = 0.04$) at the end of the assay at day 18. This suggests that *Pseudomonas*-10 is a mutualistic symbiont and contributes to *D. magna* survival. A similar report also showed that a *Pseudomonas* strain isolated from a *D. magna* host is beneficial to the development of *D. magna* resting eggs at warm conditions.[84]

Under mercury stress, lower survival rates were observed for the three mercury-treated groups when compared to their counterparts at the end of the assay (day 18): Bac-Suppl + Hg (43%, $n = 13/30$), Pse-Inf + Hg (70%, $n = 21/30$), and Bac-

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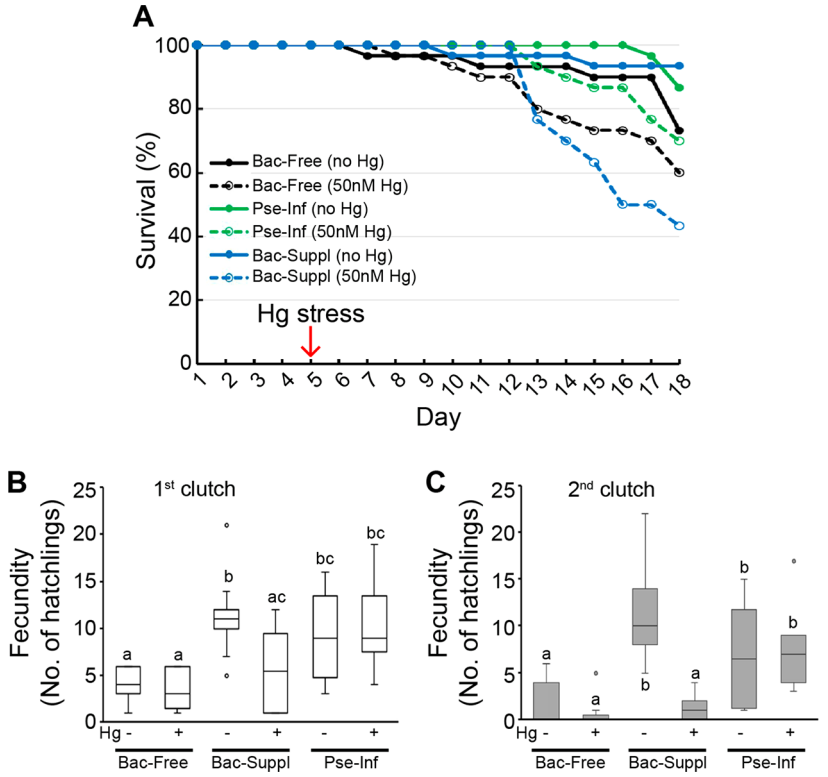

Figure 5. Survival and fecundity of gnotobiotic *D. magna* CAY harboring different microbiota under mercury stress. (A) Survival of bacteria-free daphnids (Bac-Free), bacteria-supplemented daphnids harboring parental microbiota (Bac-Suppl), and *Pseudomonas*-10 infected daphnids (Pse-Inf) with (50 nM Hg) and without (no Hg) mercury exposure. Mercury stress (Hg stress) was introduced at day 5, indicated with a red arrow. Bac-Free (no Hg), n = 30; Bac-Free (50 nM Hg), n = 30; Bac-Suppl (no Hg), n = 31; Bac-Suppl (50 nM Hg), n = 30; Pse-Inf (no Hg), n = 30; Pse-Inf (50 nM Hg), n = 30. Survival assays were repeated twice (Figure S4), but only one representative experiment is shown here. (B) Fecundity of Bac-Free, Bac-Suppl, and Pse-Inf daphnids with (50 nM Hg) and without (no Hg) mercury stress. Boxes show the 25% to 75% quartiles, medians are shown as horizontal lines (within the box), and maximum and minimum values are shown as whiskers. Columns with the same letter are not significantly different, analyzed using Kruskal–Wallis test with Wilcoxon each pair test for pairwise comparisons. Bac-Free (no Hg), n = 8; Bac-Free (50 nM Hg), n = 9; Bac-Suppl (no Hg), n = 15; Bac-Suppl (50 nM Hg), n = 10; Pse-Inf (no Hg), n = 9; Pse-Inf (50 nM Hg), n = 9. Fecundity assays were repeated twice (Figure S5).

Free + Hg (60%, n = 18/30). Daphnids from the Bac-Suppl + Hg were most severely affected under mercury stress (χ^2 = 17.22, $p < 0.0001$) while the survival rates of Pse-Inf + Hg daphnids were statistically similar to those of Pse-Inf daphnids (χ^2 = 2.18, p = 0.14). Survival rates of Bac-Free daphnids were also statistically similar under mercury and without mercury stress (χ^2 = 1.61, p = 0.21). Notably, under mercury stress, daphnids harboring *Pseudomonas*-10 (Pse-Inf + Hg) exhibited significantly higher survival rates than Bac-Suppl + Hg (χ^2 = 6.19, p = 0.01). These results suggest that the mercury-tolerant *Pseudomonas*-10 is capable of augmenting host survival under mercury stress, likely by biotransforming mercury into a less toxic form, in addition to the daphnid's ability to eliminate mercury through excretion, egestion, molting, and neonate production.[25] Intriguingly, under mercury stress, daphnids supplemented with parental microbiome (Bac-Suppl + Hg) exhibited the lowest survival (43% at day 18; n = 13/30). Similar results were obtained in a repeated experiment (Figure S4). Possibly, the microbiota composition of the Bac-Suppl daphnids is low in *merA*-positive bacteria, hence the low survival under mercury exposure, as compared to the higher abundance of *merA*-positive *Pseudomonas*-10 in Pse-Inf daphnids. It is also likely that under mercury stress, harboring various kinds of microbiota can have a detrimental effect on the health of the daphnids. Indeed, Bac-Suppl + Hg daphnids also exhibited significantly lower survival rates than BacFree + Hg daphnids (χ^2 = 14.49, p = 0.0001), implying that harboring many bacteria is beneficial under normal conditions but can be a burden under stressful environmental conditions. This is also observed on grasses with and without fungal endophytes, where the endophyte-free plants significantly fared better than endophytic-laden plants in terms of root/shoot ratio under extreme limiting-nutrient conditions.[85]

To further investigate the contributions of *Pseudomonas*-10 in host fitness, we carried out fecundity assays and compared the number of live hatchlings from the first and second clutches of all daphnid groups with and without mercury stress (Figure 5B,C and Figure S5). The six groups significantly vary in the number of first and second clutch hatchlings (Kruskal–Wallis test, χ^2 = 26.97 and 37.56, respectively, $p < 0.0001$), suggesting differences between treatments. Under no mercury stress, Bac-Suppl daphnids have a significantly higher number of first and second clutch hatchlings than the Bac-Free daphnids (Wilcoxon Each Pair test, p = 0.0004 and p = 0.0003, respectively) but did not differ with Pse-Inf daphnids (p = 0.31 and p = 0.15, respectively). This data confirms that *Pseudomonas*-10 is a beneficial symbiont and positively contributes to daphnid host fitness under normal environmental conditions. Intriguingly, untreated Bac-Suppl and Pse-inf daphnids produced a similar number of first and second clutch hatchlings as the Pse-Inf + Hg treated daphnids ($p >$ 0.05), suggesting that the fecundity of the daphnids harboring

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mercury-tolerant *Pseudomonas*-10 is maintained under mercury stress and is comparable to that of unstressed daphnids. Under mercury stress, hatchling production of Bac-Suppl + Hg treated daphnids was significantly lower than that of Bac-Suppl daphnids ($p = 0.007$, Figure 5B) and this decrease in fecundity is even more pronounced in the second clutch ($p < 0.0001$, Figure 5C). Moreover, Bac-Suppl + Hg have a similar number of hatchlings as Bac-Free and Bac-Free + Hg daphnids ($p > 0.05$). This indicates that prolonged exposure to mercury results in lower fecundity of daphnids harboring several microbiota associates. Interestingly, Bac-Suppl + Hg treated daphnids have a significantly lower number of second clutch hatchlings than the Pse-Inf + Hg daphnids ($p = 0.0011$). This further confirms that mercury-tolerant *Pseudomonas*-10 can maintain the fecundity of the daphnid host under mercury exposure, likely by up-regulating its *merA* expression and reducing the toxicity of mercury, thereby allowing the host to withstand and survive and maintain clonal reproduction in a mercury-contaminated environment.

***Pseudomonas*-10 is Capable of Biotransforming Mercury.** Since the *merA*-positive and mercury-tolerant *Pseudomonas*-10 can up-regulate *merA* expression, both in isolation and in host-association, and is capable of aiding host survival and fecundity under mercury stress, we wanted to investigate its ability to biotransform and reduce mercury (Figure 6). In a separate experiment, we measured the reduction or loss of mercury by *Pseudomonas*-10 in isolation (Pse) and in association with the daphnids (Pse-Inf) and compared that with that of the Bac-Free daphnids to tease apart the contribution of each respective partner to mercury reduction. We did not include Bac-Suppl in this study because it harbors several microbiota of unknown mercury biotransformation potentials. As expected, before the addition of mercury on day 5 (No Hg), negligible background concentrations of mercury were detected. Upon addition of 50 nM mercury on day 5 (D5), we detected initial mercury concentrations ranging from 11.6 to 15.6 nM in all groups (ADaM control, Pse, Bac-Free, and Pse-Inf). The significant mercury loss after initial mercury addition could be attributed to the attachment of mercury to the walls of the processing vessels. Nonetheless, the concentration of mercury in the ADaM only control group between D5 and D8 showed no significant differences (Tukey's HSD, $p > 0.05$), as in the D5 samples of all treatments (Tukey's HSD, $p > 0.05$), indicating that the loss of mercury due to attachment to processing vessels is consistent among samples. Intriguingly, after 3 days (day 8), a drastic 4.7-fold decrease in mercury concentrations (from 15.5 nM to 3.3 nM) was detected in the Pse group, which contained only ADaM medium and *Pseudomonas*-10 (Tukey's HSD, $p < 0.0001$). Mercury loss in the ADaM only control group between D5 and D8 is not significant, indicating that the reduction of mercury from solution in Pse group was due to the presence of *Pseudomonas*-10. This strongly suggests that *Pseudomonas*-10 biotransformed and reduced mercury in the solution, most likely with the use of its MerA enzyme, which is known to be the common mechanism for the reduction and biotransformation of Hg^{2+} to elemental Hg^0.[42] We also observed a significant reduction of mercury in the Bac-Free group after 3 days (12.9 to 6.3 nM, 2.0-fold), suggesting that the daphnids are capable of mercury uptake from their environment. This is not surprising as the assimilation of mercury by *Daphnia* has been reported.[25] A similar reduction of mercury was also detected in the Pse-Inf group after 3 days (15.6 to 5.8 nM, 2.7-fold), but mercury reduction was not significantly different from that of Bac-Free group. Hence, we could not conclusively show that *Pseudomonas*-10 significantly contributes to mercury reduction when it is in association with the daphnid host, despite its mercury biotransformation capability in isolation.

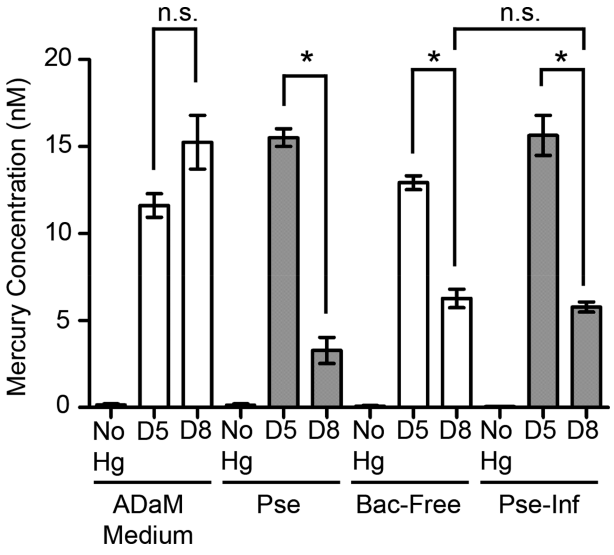

Figure 6. Mercury biotransformation by *Pseudomonas*-10. Mercury concentrations (nM of Hg^{2+}) measured in the ADaM medium control, with *Pseudomonas*-10 only (Pse), bacterial-free daphnids (Bac-Free), and daphnids infected with *Pseudomonas*-10 (Pse-Inf) on day 5, before (No Hg) and after (D5) addition of mercury, and on day 8 (D8). Experimental jars containing ADaM medium only served as controls. * indicates significant difference ($p < 0.0001$), analyzed using one-way ANOVA with Tukey's HSD Posthoc test, while n.s. indicates no significant difference ($p > 0.05$).

Host genetics indeed play a major role on metal stress tolerance via metallothionein genes, which act as metal-responsive systems upon metal exposure. *D. magna* contain three putative genes encoding the metal-binding metallothionein proteins in their genome,[86,87] and these proteins are known to play an important role in metal detoxification and homeostasis in many organisms, likely through binding and sequestration of various metals, including mercury.[88,89] While the expression of these metallothionein genes may enable the *Daphnia* host to tolerate mercury stress, we showed in this study, that the *merA*-positive and mercury-tolerant microbiota *Pseudomonas*-10 can also reduce mercury in isolation and contributes to host survival and fecundity under mercury stress, through upregulation of *merA* and thus likely reducing the mercury stress experienced by the host. To definitively show the contribution of *Pseudomonas*-10 in biotransforming mercury inside the host and to further investigate its mechanistic role during host association, daphnids containing knockouts of the host metallothionein genes and a *Pseudomonas*-10 strain harboring a *merA*-knockout need to be generated, and this will be part of another study.

Our study highlights one of the mechanisms on how an aquatic organism like *Daphnia* can potentially withstand and survive environmental stresses. Just like many hosts, *Daphnia* harbor several groups of bacteria (with different relative abundances) in their microbiome consortium, most of them with unknown functions; some microbial members maybe

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mutualistic, commensals, or parasitic depending on existing environmental conditions, or the microbiome consortium may be operating as a group of ecological guilds, with various microbial members having specific contributions to the functioning of the ecosystem (i.e., the host). Under normal conditions, the microbial consortium collectively increases *D. magna* survival and fitness as reported here and in another study.[51] But under stressful environmental conditions, the collective beneficial contribution of the microbial consortium to the host can break down (as shown in this study) and the fitness of the host may depend on the genetic and phenotypic traits of a specific microbiome member/s that can positively respond to the given environmental condition. *Pseudomonas* and other *merA*-containing bacteria only constitute a minor percentage of the microbiome consortium in *D. magna*. In this study, we did not investigate if prolonged exposure to mercury can induce changes in microbiota composition or if it can lead to the enrichment of *merA*-positive bacteria in *Daphnia*. It would be interesting to investigate the flexibility of the symbiosis under stressful environmental conditions.

In conclusion, this study is one of the few that shows the extension of the microbiome's genetic and phenotypic traits to the fitness traits of the host, resulting in a holobiont phenotype that can withstand stressful environmental conditions. The microbiome of *Daphnia* should therefore be taken into consideration specifically in ecotoxicological research where *Daphnia* is commonly used as a testing animal.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b05305.

Genbank accession numbers, list of primers, assigned taxon of *D. magna* CAY microbiota, *merA* Blastn matches, *merA* protein alignment and tree, *merA* expression in *Pseudomonas*-10, *Daphnia* mercury LD_{50} assay, replicates of survival and fecundity (PDF)

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Notes

The authors declare no competing financial interest.

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