RESEARCH LETTER

Heavy metal tolerance genes alter cellular thermodynamics in *Pseudomonas putida* and river *Pseudomonas* spp. and influence amebal predation

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Abstract

Predation rates were measured for two *Acanthamoeba castellanii* strains feeding on metal-tolerant and metal-sensitive strains of *Pseudomonas putida* and compared with cellular thermodynamic data. Predation rates by *A. castellanii* strain ATCC 30010 correlated with cell volume of the prey. To explore whether this observation could be environmentally relevant, pseudomonad species were isolated from a pristine and a metal-contaminated river and were paired based on phylogenetic and physiological relatedness. Then, cellular thermodynamics and predation rates were measured on the most similar pseudomonad pair. Under cadmium stress, the strain from contaminated river sediments, *Pseudomonas* sp. CF150, exited metabolic dormancy faster than its pair from pristine sediments, *Pseudomonas* sp. N9, but consumed available resources less efficiently (more energy was lost as heat). Predation rates by both strains of ameba were greater on *Pseudomonas* sp. CF150 than on *Pseudomonas* sp. N9 at the highest cadmium concentration.

Introduction

Chronic heavy metal contamination suppresses the functioning of ecosystems worldwide (Helgen & Moore, 1996). Many species of bacteria in these systems adapt to resist toxic effects of metals, so microbial diversity remains high (Gough & Stahl, 2011; Berg *et al.*, 2012). Yet, the stressor suppresses cell functions such as respiration and metabolism (Kandeler *et al.*, 2000; Ramsey *et al.*, 2005; Stanaway *et al.*, 2012) and the flow of energy to higher trophic levels (Pratt *et al.*, 1997; Iwasaki *et al.*, 2009). A possible explanation for these observations is that over time, genes for tolerance mechanisms become widespread and abundant (Coombs & Barkay, 2004; Martinez *et al.*, 2006; Sobecky & Coombs, 2009; Nongkhlaw *et al.*, 2012), but impose a continual energetic cost (Gibbons *et al.*, 2011). The genetic mechanisms of heavy metal resistance in bacteria are well documented (Silver & Phung, 2005; Monsieurs *et al.*, 2011; Ray *et al.*, 2013), yet there is little knowledge of how these genes influence energy flow and trophic interactions.

Microbial communities in river sediments grow on particles coated with metal oxides and oxyhydroxides. Heavy metals such as cadmium and copper often replace iron and manganese in the metal oxide coatings in contaminated rivers (Nimick & Moore, 1994). Bacteria that grow on sediments are therefore chronically exposed to toxic metals (Brown *et al.*, 1999). To tolerate chronic heavy metal stress, bacteria express genes to expel, bind, or reduce the metal (Silver & Phung, 1996; Nies, 1999, 2003). Microcalorimetry was previously used to quantify the energetic cost of heavy metal tolerance genes on growth yield and metabolism (Gibbons *et al.*, 2011). Cellular thermodynamics were compared between isogenic strains of *Pseudomonas putida* KT2440, each with different single tolerance gene deletions. The study demonstrated that under cadmium stress, metal efflux transporters allow for enhanced competition (faster exit from metabolic dormancy) at the cost of depressed growth. This study appears to provide the first evidence that tolerance genes may limit biomass availability for higher trophic levels.

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Protozoa, the primary consumers of bacteria in freshwater systems (Finlay *et al.*, 1988), differentiate among prey based on size (Kinner *et al.*, 1998; Rønn *et al.*, 2002), C/N/P ratios (Gruber *et al.*, 2009), and even extracellular polysaccharides. For example, Wildschutte *et al.* (2004) observed ameba discriminating among prey species that differed only in O-antigen compositions. Some tolerance gene proteins extend beyond the cell wall and can induce morphological and physiological changes in bacteria (Nies, 1999; Gibbons *et al.*, 2011). Because ameba are highly selective, they may discriminate between prey based on cellular effects related to the presence or absence of tolerance genes.

In the present study, amebal predation and microcalorimetric assays were used to explore mechanisms by which tolerance genes may limit energy flow in an ecosystem suppressed by heavy metal mine waste contamination (Fig. 1). Two strains of *Acanthamoeba castellanii* were studied: a culture-collected species and an environmentally derived species. With these strains, amebal predation rates on *P. putida* KT2440 and two new metal-sensitive environmental isolates were measured under varying concentrations of cadmium. This controlled approach allowed us to determine whether tolerance genes affect predation in the experimental system. Environmentally relevant *Pseudomonas* spp. were isolated from contaminated and uncontaminated river sediments. Next, the isolates were screened to find a pair of organisms, one from each river, with highly similar 16S rRNA gene sequences and strong phylogenetic similarity as determined by multigene phylogeny (Supporting Information, Fig. S1), but with differing levels of cadmium tolerance. Microcalorimetry was used to measure cellular thermodynamics under varied cadmium concentrations. Lastly, amebal predation rates were measured on these environmental pseudomonads. Our hypothesis was that bacterial cellular changes that accompany cadmium stress would influence amebal predation rates. Our next hypothesis was that under cadmium stress, metal-tolerant bacteria from contaminated sediments would grow rapidly, but produce less biomass than the bacteria from pristine sediments.

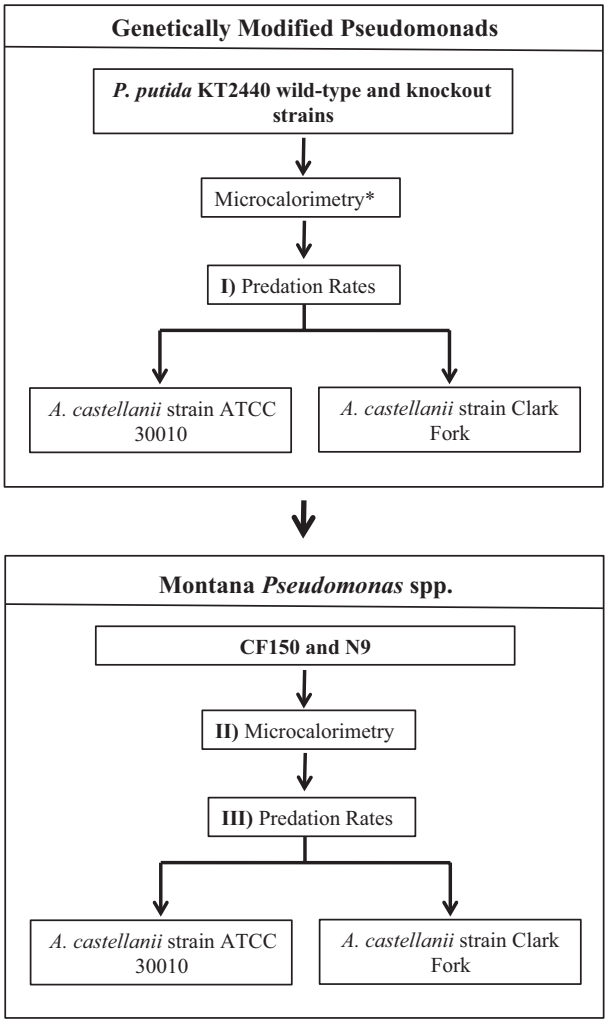

Fig. 1. Flow chart of the experimental approach and test species. For each test, organisms were exposed to varying concentrations of cadmium. Roman numerals correspond to specific Results and discussion section headings. *These data were obtained by Gibbons *et al.* (2011).

Materials and methods

Pseudomonas putida KT2440

Three metal-tolerant *P. putida* KT2440 strains were subjected to predation assays: KT2440 (wild type), the CzcCBA1 efflux transporter (Δ*czcA1*) deletion mutant (KT1), and the P-type ATPase ion pump (Δ*cadA2*) deletion mutant (KT4). Previous work determined the maximum Cd^{2+} tolerance of each organism as 380, 190, and 23 μM for the wild-type, KT1, and KT4 strains, respectively. These genes were shown to be partly responsible for cadmium resistance in these organisms (Leedjarv *et al.*, 2008) and represent the sole difference between the strains. For predation assays with *A. castellanii* strain ATCC 30010, strains were cultivated on Bacto R2A solid media (20% nutrient strength). For assays with *A. castellanii* strain Clark Fork, strains were grown on solid defined media (15.5 mM K_xPO_4, pH 7.5/0.2% peptone/0.2% glucose/2.0% agar).

Predator isolation, propagation, and preparation

Two ameba were studied. *Acanthamoeba castellanii* strain ATCC 30010 was axenically cultured in ATCC medium

354 for 14 days and then diluted to 5×10^5 cells mL^{-1} as determined by hemocytometry. A second ameba was isolated from sediments of the heavy metal-contaminated Clark Fork River at the headwaters near Silver Bow Creek in Montana. Sediment samples were added to sterile tubes and filled with river water. The tubes were gently agitated on a vortex mixer, and 20 μL aliquots were dispensed onto the middle of a diluted Bacto R2A plate (20% nutrient strength) previously streaked with *Escherichia coli* ATCC 8739 bacterial cells. To avoid conditioning the ameba to prefer metal-tolerant organisms, cadmium was not incorporated into the agar. Amebic cysts were scraped from areas of clearing, suspended in phosphate-buffered saline (PBS; pH 7.2), and re-inoculated onto plates. Over several months of repeated subculture, an *A. castellanii* isolate (identified following Jahn *et al.*, 1979, and named '*A. castellanii* strain Clark Fork') outcompeted other protozoa and was the only species present. To harvest the ameba, cysts were identified via microscopy, collected from the plates, suspended in PBS, centrifuged five times at a Relative Centrifugal Force of 100, and diluted to 5×10^5 cells mL^{-1}.

Predation measurements

Bacterial cells in late log phase growth were diluted to 4×10^7 cells mL^{-1}. Assay plates were composed of agar (Difco 281230) containing 0, 1, or 10 μM $CdCl_2$. Plates amended with 100 μM $CdCl_2$ were prepared, but not used in this study because bacteria grew nonuniformly. Whatman 1001–6508 filter pads (10-mm diameter) were placed in the center of the agar plates. Using sterile 10 μL inoculation loops, bacterial strains were streaked outward from the filter pad (adapted from Wildschutte *et al.*, 2004; The environmental bacteria were alternately streaked for a total of eight streaks per plate. The *P. putida* strains were streaked in the same manner. Then, the filter pad was inoculated with 20 μL of the 5×10^5 cells mL^{-1} *A. castellanii* suspension. Clearing was measured from the center of the filter pad to the end of the cleared zone every 2 days at 20 °C until a streak line was consumed (6–8 days). Bacterial cell density on control assay plates was determined by collecting three core samples (1.1 mm diameter) from streak lines during stationary phase, suspending them in 9-mL PBS tubes, vortexing them for 10 s, and counting cells in a Petroff–Hausser chamber (Hausser Scientific, Horsham, PA). Bacterial length and width were measured on at least 20 cells using ImageJ software (Abramoff *et al.*, 2004). The mean cell volume was calculated as described by Montesinos *et al.* (1983). The amebal consumption rate was determined by dividing the total cell number consumed at each time interval by the elapsed time. Statistical analysis between groups was performed using one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test. Pearson's correlation tests assessed relationships between cell size and feeding rates.

Isolation of bacterial strains

Pseudomonads were isolated from the pristine Middle Fork of the Flathead River, near the Nyack floodplain in Montana. Sediment (2 g) was added to filter-sterilized subsurface water (8 mL) and tetrasodium pyrophosphate (2 mL; 0.1 M) in a 15-mL centrifuge tube. Tubes were vortex-mixed for 30 s, shaken, and vortexed again for 30 s. Diluted Alcoclear CCP-II cationic dispersant (200 μL; 1 g of 50% CCP-II into 99 g filter-sterilized deionized water) was added, and the tube was sonicated for 10 min (Branson 3210R-DTH, 50/60 Hz, 117V). After sonication, samples were vortex-mixed for 1 min and allowed to settle for 15 min. Suspensions were serially diluted (1:10, 1:100, and 1:1000) in filter-sterilized subsurface water and plated on dilute R2A agar (Difco) plates (10% nutrient strength). Plates were incubated aerobically for 2–4 weeks at 10 °C in the dark (*in situ* temperature ranged from 4 °C to 12 °C seasonally). Visible bacterial colonies were selected among culturable heterotrophs for subculturing. All isolates were subsequently cultivated on dilute R2A agar (20% nutrient strength) at 10 °C.

Metal-tolerant bacteria were isolated from sediments obtained from the headwaters of the heavy metal-contaminated Clark Fork River at Silver Bow Creek in Montana. Sediment (10 g) was added to sterile tubes containing sterile PBS. The tubes were mildly sonicated for 10 min (see above), and suspensions were decanted into new sterile tubes followed by serial dilution. To select for Cd-tolerant bacteria, 100 μL $CdCl_2$ solutions with concentrations of up to 3.5 mM $CdCl_2$ were added to the surface of the plates and let dry. Aliquots (100 μL) from the sediment dilutions were spread on the $CdCl_2$-amended plates. Individual colonies were streaked on agar plates to obtain pseudomonad monocultures. All strains were identified using conventional molecular techniques (described in the Supporting Information).

Montana strain comparisons

One metal-tolerant isolate (*Pseudomonas* sp. CF150) from contaminated sediments was paired with one metal-sensitive isolate (*Pseudomonas* sp. N9) from pristine sediments. Based on 16S rRNA gene, both bacterial strains were 100.0% identical at 795 base pairs to *Pseudomonas reactans*. To further determine phylogenetic relatedness among these two strains, a multigene phylogeny was

generated using 8 functional genes: *gatA*: aspartyl/glutamyl-tRNA aminotransferase subunit A; *ubiD*: aromatic acid decarboxylase; *trpD*: anthranilate phosphoribosyltransferase; *mqo1*: malate quinone oxidoreductase; *cafA*: ribonuclease G; *rpl1*: 50S ribosomal subunit protein L9; *trmD*: tRNA (guanine-N(1)-)-methyltransferase; and *rpsR*: 30S ribosomal subunit protein S18. The multigene phylogeny supported the high degree of relatedness between the Montana *Pseudomonas* spp. (Fig. S1; for more detail on the multigene phylogenetic analysis, see the Supplemental Information). To compare phenotypic traits between the strains, each strain was inoculated in test kits (API 50 CH, bioMérieux). The strains were 96% similar on physiological tests. The only difference was that *Pseudomonas* sp. CF150 metabolized L-arabitol and erythritol, whereas *Pseudomonas* sp. N9 did not. The similarity between the strains as measured by additional phenotype tests (API 20 NE, bioMérieux) and enzyme assays (API ZYM, bioMérieux) was 86% and 75%, respectively. In lysogeny broth, *Pseudomonas* sp. CF150 grew up to 1.75 mM cadmium, and *Pseudomonas* sp. N9 grew up to 1.5 mM cadmium. Each isolate was also surveyed for the presence of heavy metal tolerance genes. The genomes of both organisms were sequenced using an Illumina sequencing platform, and the resulting genome scaffolds were analyzed and annotated as described in the Supporting Information. Two heavy metal tolerance genes were detected in *Pseudomonas* sp. CF150, and 5 were detected in *Pseudomonas* sp. N9. Efflux genes were characterized in each strain (Supporting Information). CzcA family efflux protein (a RND protein) was present in both strains; this gene enables organisms to tolerate high levels of metal stress (Leedjarv *et al.*, 2008). Cell sizes were determined for all isolates under differing cadmium levels (Table 1).

Microcalorimetry measurements

Calorimetry measures differences in metabolic output caused by variations in metal stress and individual resistance mechanisms. Thermogram peaks begin as the organism responds to the addition of a limiting resource (glucose). Heat output increases until all the resource is consumed and then drops rapidly. The thermograms allow the calculation of peak height (the maximum metabolic heat flow rate during growth), peak time (time to reach peak output, a combination of both lag phase and metabolic rate), metabolic rate (rate of heat output during log growth phase), and total heat produced by each strain (an efficiency measure, as more heat output results in less biomass).

Strains grown from stock on Bacto R2A plates (0.2% nutrient strength) for 48 h (25 °C) were transferred into a modified M1 minimal growth medium composed of 0.1 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $MgSO_4{\cdot}7\ H_2O$, 0.25 g L^{-1} KNO_3, 0.02 g L^{-1} anhydrous $CaCl_2$, and 0.6% (wt/vol) D-glucose. For growth in the calorimeter, M1 medium was amended with $CdCl_2$ to make 0, 0.01, 0.1, and 1 mM cadmium concentrations. Calorimetry assays were run in a VP-ITC instrument (Microcal VP-ITC user's manuals, 2002; MicroCal LLC, Northampton, MA) and analyzed using VP-Viewer2000 software. Each experiment started with the same amount of glucose (101 ± 5 nmol) so that there would be a fixed amount of carbon and energy ($\Delta G_{gluc} = -2870$ kJ mol^{-1}). Initial and final glucose concentrations were measured with fluorometric glucose assay kits (K606-100; BioVision, Mountain View, CA) according to the manufacturer's instructions. The metabolic rate was obtained by calculating the rate of the heat output. Detailed methods are described in Gibbons *et al.* (2011).

Table 1. Cell sizes: dimensions of the Montana *Pseudomonas* spp. and *P. putida* KT2440

	Montana *Pseudomonas* spp.		*P. putida* KT2440		
Dimension	CF150	N9	Wild-type	KT1	KT4
0 Cd (μM)					
Length (μm)	1.61 ± 0.08	1.60 ± 0.06	0.97 ± 0.05	0.97 ± 0.06	1.18 ± 0.05
Width (μm)	0.62 ± 0.03	0.53 ± 0.02	0.74 ± 0.04	0.71 ± 0.04	0.63 ± 0.02
Volume (μm^3)	0.43	0.31	0.31	0.29	0.31
1 Cd (μM)					
Length (μm)	1.64 ± 0.09	1.51 ± 0.07	0.95 ± 0.04	0.85 ± 0.06	1.15 ± 0.06
Width (μm)	0.62 ± 0.03	0.58 ± 0.03	0.57 ± 0.02	0.48 ± 0.03	0.62 ± 0.02
Volume (μm^3)	0.43	0.35	0.19	0.12	0.29
10 Cd (μM)					
Length (μm)	1.61 ± 0.06	1.49 ± 0.07	0.95 ± 0.09	0.93 ± 0.07	1.22 ± 0.05
Width (μm)	0.67 ± 0.03	0.51 ± 0.02	0.58 ± 0.03	0.42 ± 0.02	0.62 ± 0.02
Volume (μm^3)	0.48	0.26	0.20	0.11	0.31

n = 20–28 for length and width measurements. Numbers are given as mean ± SE.

Results and discussion

Amebal predation of *P. putida* KT2440

In the absence of cadmium, *A. castellanii* strain ATCC 30010 consumed the wild-type *P. putida* KT2440 and *P. putida* strain KT1 (*czcA1* gene deletion) at a faster rate than *P. putida* strain KT4 (*cadA2* gene deletion; $P < 0.001$; Fig. 2a; Table 2). As the metal concentration increased to 1 and 10 μM cadmium, the wild type was consumed faster than both KT1 and KT4. This feeding behavior may be explained by the fact that ameba have been observed to consume smaller bacteria faster than larger bacteria (Dillon & Parry, 2009). Also, nanoflagellates, which can feed similarly to ameba (Xinyao *et al.*, 2006), were shown to preferentially graze smaller *P. putida* cells faster than larger cells (Fu *et al.*, 2003). The volumes of the wild type and KT1 were smaller than KT4 at 1 and 10 μM cadmium, and they were consumed faster than KT4. The feeding rate on KT1 positively correlated with its volume (Pearson's $r = 0.217$; $P = 0.10$), whereas the feeding rate on KT4 negatively correlated with its volume (Pearson's $r = -0.301$; $P < 0.001$).

Similar to the *A. castellanii* strain ATCC 30010, *A. castellanii* strain Clark Fork consumed more wild-type and KT1 cells than KT4 cells at 0 μM cadmium ($P < 0.001$; Fig. 2b). These results correspond to the trends observed with the ATCC *A. castellanii* in the absence of cadmium. However, the relationship between prey volume and feeding rate did not exist under cadmium exposure with *A. castellanii* strain Clark Fork. At 1 μM Cd, there was no difference in bacterial consumption rate between the two ameba strains, but at 10 μM cadmium, *A. castellanii* strain Clark Fork consumed more KT4 cells than the wild type. In these experiments, the only difference in the prey organisms is the presence and/or absence of one of two metal tolerance genes (*czcA* or *cadA2*). In the wild-type *P. putida*, the *cadA2* gene is constitutively expressed and serves as a housekeeping metal resistance mechanism. When *cadA2* is deleted, *P. putida* strain KT4 (the *cadA2* knockout mutant) becomes 16 times more sensitive to cadmium exposure than the wild type. Strains that lack the *cadA* gene accumulate intracellular cadmium upon exposure (Leedjarv *et al.*, 2008), which may deter predators. However, our data suggest that in the case of *A. castellanii* strain Clark Fork that was isolated from contaminated sediments, the predator was not deterred by an increase in its prey's intracellular cadmium levels.

Thermodynamic measures of environmental pseudomonads

At a moderate cadmium concentration (0.10 mM), the peak height (maximum metabolic heat flow rate) was

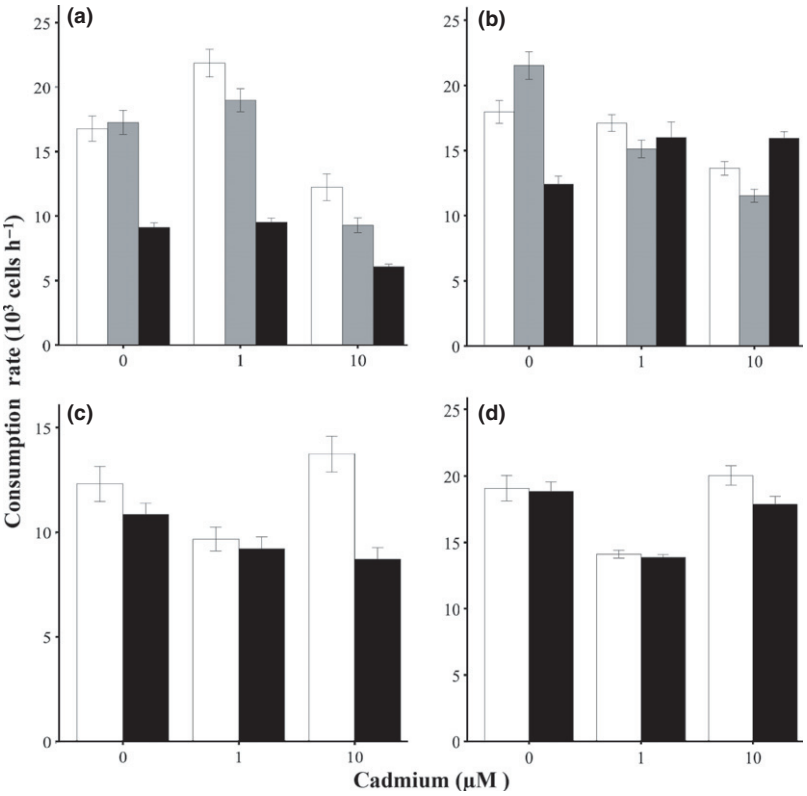

Fig. 2. (a) ATCC *Acanthamoeba castellanii* (b) *A. castellanii* strain Clark Fork consumption rates of *Pseudomonas putida* KT2440. White bars represent KT2440, gray bars represent KT1, and black bars represent KT4. (c) ATCC *A. castellanii* and (d) *A. castellanii* strain Clark Fork consumption rates of the Montana *Pseudomonas* spp. CF150 (white bars) and N9 (black bars). Error bars represent standard error.

Table 2. Mean consumption rates (cells h^{-1}) of Montana *Pseudomonas* spp. and *P. putida* strain KT2440

A. castellanii strain	Cd (μM)	Montana *Pseudomonas* spp.				*P. putida* strain KT2440					
		CF 150	N9	*F*	P	KT2440	KT1	KT4	*F*	*P*
ATCC	0	$1.2 \times 10^4 \pm 1 \times 10^3$	$1.1 \times 10^4 \pm 1 \times 10^3$	2.23	0.140	$1.7 \times 10^4 \pm 1 \times 10^3_{\alpha}$	$1.7 \times 10^4 \pm 1 \times 10^3_{\alpha}$	$9.1 \times 10^3 \pm 4 \times 10^2_{\beta}$	42.86	< 0.001
	1	$9.7 \times 10^3 \pm 6 \times 10^2$	$9.2 \times 10^3 \pm 6 \times 10^2$	0.33	0.569	$2.2 \times 10^4 \pm 1 \times 10^3_{\alpha}$	$1.9 \times 10^4 \pm 1 \times 10^3_{\beta}$	$9.5 \times 10^3 \pm 3 \times 10^2_{\chi}$	91.34	< 0.001
	10	$1.3 \times 10^4 \pm 1 \times 10^3$	$8.7 \times 10^3 \pm 6 \times 10^2$	24.5	< 0.001	$1.2 \times 10^4 \pm 1 \times 10^3_{\alpha}$	$9.3 \times 10^3 \pm 1 \times 10^2_{\beta}$	$6.1 \times 10^3 \pm 2 \times 10^2_{\chi}$	34.13	< 0.001
Clark Fork	0	$1.9 \times 10^4 \pm 1 \times 10^3$	$1.9 \times 10^4 \pm 1 \times 10^3$	0.04	0.851	$1.8 \times 10^4 \pm 1 \times 10^3_{\alpha}$	$2.2 \times 10^4 \pm 1 \times 10^3_{\beta}$	$1.2 \times 10^4 \pm 1 \times 10^3_{\chi}$	31.24	< 0.001
	1	$1.4 \times 10^4 \pm 1 \times 10^2$	$1.4 \times 10^4 \pm 1 \times 10^3$	0.45	0.513	$1.7 \times 10^4 \pm 1 \times 10^3$	$1.5 \times 10^4 \pm 1 \times 10^3$	$1.6 \times 10^4 \pm 1 \times 10^3$	1.297	0.280
	10	$2.0 \times 10^4 \pm 1 \times 10^3$	$1.8 \times 10^4 \pm 1 \times 10^3$	5.19	0.039	$1.4 \times 10^4 \pm 1 \times 10^3_{\alpha}$	$1.2 \times 10^4 \pm 1 \times 10^3_{\beta}$	$1.6 \times 10^4 \pm 1 \times 10^3_{\chi}$	17.43	< 0.001

Numbers are given as mean ± SE. n = 8–80 for consumption rate measures. A one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test was performed to test the probability of a significant difference in measured values between strains and obtain F and P values. Subscripted symbols α, β, and χ indicate statistical groupings from the *post hoc* test.

higher in *Pseudomonas* sp. CF150 than in *Pseudomonas* sp. N9 (F = 19.24; P = 0.012; Fig. 3a; Table 3). The peak heights of both strains dropped at elevated cadmium concentrations (1.0 mM). At 1.0 mM cadmium, the performance (peak time) of *Pseudomonas* sp. N9 halved relative to peak times of both strains at each cadmium concentration (F = 51.4; P < 0.001; Fig. 3b). Exposure to high cadmium concentrations reduced the performance of *Pseudomonas* sp. N9. Because both strains tolerate at least 1.5 mM cadmium, a divergence in performance at only the highest cadmium level was expected. These results correspond to our previous finding with *P. putida* KT2440, in which the strains that were more susceptible to Cd toxicity, KT1 and KT4 (Leedjarv *et al.*, 2008), performed poorly when exposed to cadmium relative to the tolerant wild-type strain (Gibbons *et al.*, 2011). The performance between the environmental strains remained unchanged from 0.00 mM to 0.10 mM cadmium. This is different from the previous study in which the wild-type strain was less competitive in the absence of cadmium.

A faster transition from metabolic dormancy to maximum resource consumption could offer an advantage over competitors in highly contaminated sites. However, such a response would reduce biomass production and potential energy flow to higher trophic levels. In contrast, the organisms without enhanced metal resistance produced more biomass, but are unable to process available glucose as soon as their metal-tolerant counterpart strains. These trends are supported by the energetics of growth of both the *P. putida* KT2440 wild type and mutants (KT1 and KT4) (Gibbons *et al.*, 2011) and the Montana isolates. *Pseudomonas* sp. CF150 produced more biomass than *Pseudomonas* sp. N9 at 0.00 and 0.01 mM cadmium (269% and 140%, respectively; Fig. 3c). However, at 1.0 mM cadmium, biomass production by *Pseudomonas* sp. CF150 decreased (while the overall speed of glucose consumption by the *Pseudomonas* sp. CF150 strain increased relative to *Pseudomonas* sp. N9), and *Pseudomonas* sp. N9 yielded 288% more biomass (F = 11.42; P = 0.004). The KT1 strain also yielded more biomass (400% relative to the wild type) at 1.0 mM cadmium, but took longer to consume the available glucose than the wild type.

Amebal predation of Montana pseudomonads

Acanthamoeba castellanii strain ATCC 30010 consumed *Pseudomonas* spp. CF150 and N9 at the same rate at 0 and 1 μM cadmium (Fig. 2c). When the cadmium level increased to 10 μM, *Pseudomonas* sp. CF150 was consumed at a rate of 49% more cells per hour than *Pseudomonas* sp. N9. Furthermore, *Pseudomonas* sp. CF150 was larger than *Pseudomonas* sp. N9, so the predator

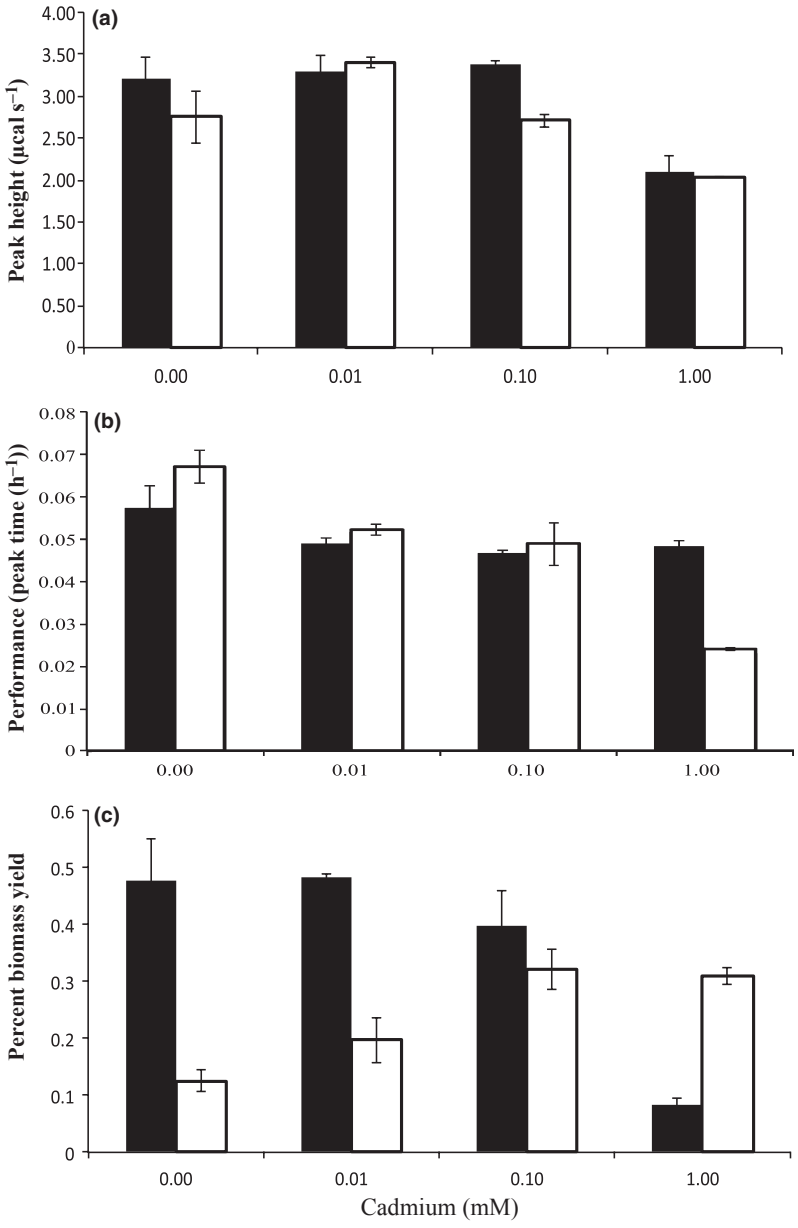

Fig. 3. Thermodynamic measures. (a) Peak height (b) performance and (c) percent biomass yield of the *Pseudomonas* spp. strains CF150 (black bar) and N9 (white bar) in the presence of different cadmium concentrations. Error bars represent the standard error.

consumed more volume per cell. Similar to the ATCC ameba strain, the consumption rate of these *Pseudomonas* strains by *A. castellanii* strain Clark Fork was the same at 0 and 1 μM (Fig. 2d). Further, as with the other ameba strain, more *Pseudomonas* sp. CF150 cells were consumed than *Pseudomonas* sp. N9 at 10 μM, which may be related to the greater biomass yield of *Pseudomonas* sp. CF150 at 0.01 mM cadmium (Fig. 3c).

Conclusion

This work reveals a possible factor for altered energy flow between trophic levels in contaminated ecosystems. The genes enabling an organism to survive in contaminated systems alter cellular metabolism, biomass yield, and predation rates. Under metal stress, metal-tolerant bacterial strains can exit metabolic dormancy faster than susceptible strains, but at the cost of a decreased growth yield. This corroborates work by von Stockar *et al.* (2006), which outlines theoretical and empirical support for the idea that an inverse relationship exists between an organism's growth rate and its biomass yield. In freshwater systems, many cells remain inactive, and the flux of nutrients is constant (Lennon & Jones, 2011). The ability to quickly ramp up metabolic activity during a pulse of nutrients and also withstand a stressor by employing tolerance genes would

Table 3. Principal calorimetric parameters obtained for *Pseudomonas* spp. CF150 and N9 in the presence of $CdCl_2$ at 25 °C

		Montana *Pseudomonas* spp.			
Cadmium (mmol)	Thermokinetic parameters	CF150	N9	*F*	*P*
0.00	Q_T (mcal)*	42.5 ± 1.5	43.6 ± 0.4	0.44	0.543
	P_{max} (μcal s^{-1})†	3.20 ± 0.26	2.75 ± 0.31	1.28	0.319
	t_{max} (h^{-1})‡	0.057 ± 0.005	0.067 ± 0.004	2.50	0.189
	Yield§	0.48 ± 0.07	0.13 ± 0.02	21.55	0.010
	Q_T/C-mol of Biomass (10^{-11})¶	3.04 ± 0.54	11.84 ± 2.03	17.52	0.013
0.01	Q_T (mcal)*	49.8 ± 0.4	51.1 ± 0.9	2.16	0.216
	P_{max} (μcal s^{-1})†	3.30 ± 0.19	3.40 ± 0.06	0.37	0.576
	t_{max} (h^{-1})‡	0.049 ± 0.001	0.052 ± 0.001	3.01	0.158
	Yield§	0.48 ± 0.01	0.20 ± 0.04	88.35	0.001
	Q_T/C-mol of boimass (10^{-11})¶	3.34 ± 0.03	8.84 ± 2.34	14.56	0.019
0.10	Q_T (mcal)*	50.9 ± 0.6	47.3 ± 1.3	6.33	0.066
	P_{max} (μcal s^{-1})†	3.37 ± 0.06	2.71 ± 0.08	19.24	0.012
	t_{max} (h^{-1})‡	0.047 ± 0.001	0.049 ± 0.005	0.03	0.873
	Yield§	0.40 ± 0.06	0.32 ± 0.03	1.10	0.353
	Q_T/C-mol of boimass (10^{-11})¶	4.36 ± 0.65	4.88 ± 0.59	0.36	0.582
1.00	Q_T (mcal)*	46.7 ± 0.2	41.4 ± 0.2	362.65	0.001
	P_{max} (μcal s^{-1})†	2.10 ± 0.18	2.04 ± 0.00	0.51	0.527
	t_{max} (h^{-1})‡	0.048 ± 0.001	0.024 ± 0.000	519.29	0.001
	Yield§	0.08 ± 0.01	0.31 ± 0.02	127.65	0.001
	Q_T/C-mol of boimass (10^{-11})¶	19.07 ± 2.67	4.33 ± 0.19	18.20	0.024

*Total heat released by the microbial growth reaction as measured by the VP-ITC (mean ± SE, $n = 3$).

†Maximum heat flow rate of the microbial growth reaction (mean ± SE, $n = 3$).

‡Time to reach peak of power/time curve (mean ± SE, $n = 3$).

§Carbon moles of biomass divided by the carbon moles of glucose consumed (mean ± SE, $n = 3$).

¶Total heat released per carbon mole of biomass (μcal/C-mol × 10^{-11}) (mean ± SE, $n = 3$). A one-way analysis of variance (ANOVA) was performed to test the probability of a significant difference in measured values between strains and obtain *F* and *P* values.

confer a strong selective advantage. Our results, coupled with the fact that stressed organisms can acquire an abundance of tolerance genes (Hemme *et al.*, 2010; Zhang *et al.*, 2013), may provide a starting point toward understanding the observed biomass and respiration suppression in contaminated systems (Kandeler *et al.*, 2000; Ramsey *et al.*, 2005; Gough *et al.*, 2008; Stanaway *et al.*, 2012).

Much ecotoxicology research seeks to determine lethal doses of pollutants to single species. These tests ensure that contaminant concentrations in the environment will be kept lower than what is required to kill a test species. This approach misses the important factor of how sublethal concentrations of pollution affect interactions between species, energy flow, and ecosystem health. Further investigation into the abundance and energetic costs of tolerance genes as they relate to energy flow and predator–prey interactions will improve our understanding of ecosystem responses to chronic stress.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Identification of bacterial strains.
Table S1. Table of NCBI prokaryotic genome annotation pipeline outputs for isolates *Pseudomonas* sp. CF150 and *Pseudomonas* sp. N9.
Fig. S1. Neighbor joining tree of multi-gene phylogenetic analysis among the metal tolerance and metal sensitive microbial isolates and known pseudomonad species.