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Use of Microcalorimetry To Determine the Costs and Benefits to *Pseudomonas putida* Strain KT2440 of Harboring Cadmium Efflux Genes▽

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A novel microcalorimetric approach was used to analyze the responses of a metal-tolerant soil bacterium (*Pseudomonas putida* strain KT2440) to metal resistance gene deletions in cadmium-amended media. As hypothesized, under cadmium stress, the wild-type strain benefited from the resistance genes by entering the exponential growth phase earlier than two knockout strains. In the absence of cadmium, strain KT1, carrying a deletion in the main component (*czcA1*) of a Cd/Zn chemiosmotic efflux transporter (CzcCBA1), grew more efficiently than the wild type and released ~700 kJ (per mole of biomass carbon) less heat than the wild-type strain, showing the energetic cost of maintaining CzcCBA1 in the absence of cadmium. A second mutant strain (KT4) carrying a different gene deletion, Δ*cadA2*, which encodes the main Cd/Pb efflux transporter (a P-type ATPase), did not survive beyond moderate cadmium concentrations and exhibited a decreased growth yield in the absence of cadmium. Therefore, CadA2 plays an essential role in cadmium resistance and perhaps serves an additional function. The results of this study provide direct evidence that heavy metal cation efflux mechanisms facilitate shorter lag phases in the presence of metals and that the maintenance and expression of tolerance genes carry quantifiable energetic costs and benefits.

Human activities have contaminated ecosystems around the world with persistent heavy metals (30). Metal-associated stress alters microbial community structure and metabolism (14, 15, 40) and therefore affects higher trophic interactions. Metal efflux pumps exploit the cellular pool of energy (ATP and chemiosmotic gradients) to pump metal ions out of a cell. Consequently, they should encumber the host organism with the energetic cost of maintaining and expressing the genes coding for them (10, 32, 42). The overall cost of encoding these traits, while necessary for survival in a contaminated environment, should decrease fitness in environments with low concentrations of metals (43). The energetic costs of maintaining metal resistance genes in the absence of bioavailable heavy metals in bacteria are generally assumed but apparently have never been directly quantified.

The main toxic effect of cadmium on bacterial cells is to bind to and denature protein (33, 34). Cadmium-protein binding induces a cascade of other putative changes in the cell, such as the release of redox-active metals bound to proteins and the general disruption of many protein-mediated processes (20, 44). Efflux pumps are a way of directly and efficiently dealing with cadmium, by transporting it out of the cell. Other cellular responses to cadmium involve the large-scale synthesis of molecules, such as glutathione, polysaccharides, and chaperone proteins, to bind cadmium and cope with cadmium-induced protein denaturation (13, 16, 20, 33).

Most bacteria nonspecifically import cadmium into the cell along with other divalent metal ions (31). The result can be seen in the evolution of multiple cadmium resistance mechanisms in bacteria (44). Modeling of the complete genome of *Pseudomonas putida* KT2440, a model soil bacterium (2, 25, 28), showed that there are four primary operons putatively responsible for cadmium tolerance (6). Two of these operons encode CBA transporters from the RND (resistance-nodulation-cell division) protein family (CzcCBA1 and CzcCBA2), and two encode P-type ATPases (CadA1 and CadA2). Prior studies have shown that CzcCBA2 confers weak resistance to zinc and that CadA1 provides no measurable heavy metal resistance in KT2440 under laboratory conditions, but CadA1 does confer low-level zinc resistance when heterologously expressed in *Escherichia coli* (23). CzcCBA1 and CadA2 were shown previously to be solely responsible for cadmium resistance in *P. putida* KT2440 (23). Leedjärv et al. (23) discovered that CzcCBA1 is constitutively transcribed at low levels and that its transcription increases by up to 3 orders of magnitude in the presence of several heavy metals, including cadmium. CadA2 is constitutively expressed at a relatively high level, but its expression is also augmented by up to 10-fold in the presence of multiple heavy metals, including cadmium (23, 27).

In order to assess the metabolic cost of metal resistance, isothermal microcalorimetry was used to compare energetic parameters between wild-type *P. putida* strain KT2440 and the two knockout strains carrying a deletion in either the *czcA1* or the *cadA2* gene (developed and described previously by Leed-

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järv et al. [23]) in cadmium-free and cadmium-amended media. Several prior investigations demonstrated that metabolic heat output is directly comparable to microbial growth curves and can indicate the growth phase of a bacterial culture (7, 8, 49). The growth yield (biomass/substrate consumed), metabolic rate (rate of heat output during log growth phase), peak time (time to reach peak heat output, a combination of both lag phase and metabolic rate), and total heat produced by three strains in media containing varied cadmium concentrations were calculated. The tested hypothesis was that in the absence of cadmium, strains with deletions in a single metal tolerance gene would exhibit shorter lag phases, higher growth yields, increased metabolic rates, and fewer calories burned per cell due to the benefit of not carrying tolerance genes in an uncontaminated environment. Conversely, for the wild-type strain, similar trends were hypothesized (higher growth yields, shorter lag phases, increased metabolic rates, and fewer calories burned per cell) when grown in metal-containing media, which would demonstrate the benefits of carrying tolerance genes in a contaminated environment. The data show that the benefit of harboring heavy metal resistance genes in the presence of cadmium is an accelerated ability to respond to and process resources (i.e., glucose), while the cost is manifested mainly as a decreased growth yield.

MATERIALS AND METHODS

Bacterial strains and media. Three heavy metal-resistant *P. putida* strains were used in these experiments: KT2440 (wild type), the CzcCBA1 efflux transporter mutant KT1 (Δ*czcA1*), and the P-type ATPase ion pump mutant KT4 (Δ*cadA2*) (23). Strains grown from stock on 0.2% Bacto R2A plates for 48 h (25°C) were transferred into a modified M1 minimal growth medium composed of 0.1 g/liter KH_2PO_4, 0.1 g/liter $MgSO_4 \cdot 7H_2O$, 0.25 g/liter KNO_3, 0.02 g/liter 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. A 0.22-μm-filter-sterilized 800 mM cadmium chloride stock solution was used for all experiments.

Cell and medium preparation for calorimetry. Colonies were transferred from 0.2% Bacto R2A plates into 30 ml of modified M1 medium in sterile 50-ml conical tubes. Culture tubes were incubated for 24 h at 25°C on an orbital shaker (100 rpm) and then centrifuged at 3,000 × *g* for 15 min. The pellets were washed twice in glucose-free M1 medium and resuspended in 5 ml of glucose-free M1 medium (with 0, 0.01, 0.1, or 1 mM Cd). A Petroff-Hausser counting chamber was used to determine the titer of suspended cells. Solutions were adjusted to a final cell titer of $6.0 \times 10^6 \pm 2.0 \times 10^6$ cells ml^{-1} with glucose-free M1 medium (with 0, 0.01, 0.1, or 1 mM Cd). A working glucose solution was prepared for injection into the calorimeter cell by adding 56 μl of a 10% (wt/vol) glucose stock solution to 9.944 ml of M1 medium (with 0, 0.01, 0.1, or 1 mM Cd) and loaded into the automatic injection syringe on the calorimeter.

Isothermal titration calorimetry. All calorimetry assays were run in a VP-ITC instrument (MicroCal LLC, Northampton, MA) and analyzed by using VP-Viewer2000 software. The temperature in the instrument was set at 25°C, the reference power (μcal s^{-1}) was set to 11.6, the initial injection delay after equilibration was set at 240 s, the feedback mode/gain was on high, and the ITC equilibrium options were set to "fast equil. & auto." Injection syringe parameters were as follows: the volume was set to 50 μl, the duration was 62.4 s with a spacing of 86,400 s (24 h), and the number of injections was set to 4. The sample cell was filled with 1.416 ml of M1 medium (without glucose) with either 0, 0.01, 0.1, or 1.0 mM cadmium chloride, while nanopure water was used in the reference cell. The final cell volume was 1.57 ml, containing 101 ± 5 nmol glucose and $6.0 \times 10^6 \pm 2.0 \times 10^6$ bacterial cells.

Each experiment started with the same amount of glucose (101 ± 5 nmol) so that there would be a fixed amount of carbon and energy ($\Delta G_{gluc} = -2{,}870$ kJ/mol): the more energy consumed by efflux (i.e., metal tolerance), the less energy available for anabolic processes. 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 lower detection limit for the glucose assay was 0.2 nmol.

Calorimeter conditions. Since the injection port design in the VP-ITC unit prohibited the injection of multiple solutions during an experiment, glucose injection was delayed, and cells were manually added by using a micropipette, replacing the syringe in time for the medium and cells to equilibrate before glucose injection. The removal of the syringe after equilibration led to a reproducible endothermic peak of 5,000 ± 400 μcal, which was factored into the final heat output calculation.

Since the calorimeter chamber could not be aerated, glucose limitation was used to prevent anaerobic growth. The initial glucose concentration was 101 ± 5 nmol, and the initial amount of dissolved oxygen in the medium was approximately 700 nM (measured by a Fibox 3 Minisensor oxygen meter [PreSens, Regensburg, Germany]). Due to the nature of the VP-ITC instrument, it was not possible to directly measure the increase in biomass during the course of an experiment. Only initial and final cell counts could be obtained. Regardless, the change in biomass was so small (a single order of magnitude) that traditional growth curves would have been inaccurate at that scale. In any case, this was not an issue, since others have shown previously that growth curves for bacteria are strongly correlated to the heat output (7, 8, 49). The metabolic rate was obtained by calculating the rate of the heat output, which is closely associated with the metabolic rate, as demonstrated previously by others (7, 11, 19).

Cell size and biomass. Cell size was determined by growing each strain with different concentrations of cadmium in 30-ml volumes (in a 50-ml conical tube) of the same medium used in the calorimeter on an orbital shaker (100 rpm) at 25°C. Incubation times were determined based on the peak times from the calorimeter data (described below). Control experiments were performed to ensure that the cell size did not differ between cells grown in tubes and those grown in the calorimeter at the same cadmium concentration. Cells were washed and then photographed with a digital camera. The length and width of 20 cells were measured by using Image J software (2008). The volume was calculated by assuming a cylinder with hemispherical caps (29). Conversion factors for bacterial cell volume to carbon moles (C-mol) were taken from the literature (3, 4, 29). The mean cell volume data were used for the yield calculations.

RESULTS

The raw thermograms for all three *P. putida* KT2440 strains subjected to four different cadmium treatments (0, 0.01, 0.1, and 1 mM Cd) are shown in Fig. 1. Replicate thermograms from each growth condition were averaged for the wild type ($n = 3$), KT1 ($n = 3$), and KT4 ($n = 2$) and showed a high degree of reproducibility (Table 1).

Growth kinetics. The peak time (combined lag phase and metabolic rate) for the wild type increased with increasing cadmium concentrations but was significantly shorter than the peak time for KT4 at 0.01 mM cadmium (Table 1) and significantly shorter than the peak times for KT1 at 0.1 mM and 1 mM cadmium (~90% and 29% shorter, respectively) (Table 1). KT1, however, had a shorter peak time (~40%) than the wild type in cadmium-free medium ($P = 0.013$). The peak time for KT1 lagged by more than 24 h between 0.01 and 0.1 mM cadmium. This did not occur in the wild type (Fig. 1 and 2A). The peak time for KT4 was not statistically different from the wild-type peak time in the absence of cadmium ($P = 0.542$). The peak time for KT4 was significantly longer than the peak time for KT1 and the wild type at 0.01 mM cadmium (Table 1). No growth or heat evolution was observed for KT4 at 0.1 or 1 mM cadmium.

To determine the effect of cadmium exposure on the metabolic rate (k), the slope of the natural log of the thermogram during the exponential growth phase was calculated (41). The metabolic rate of the wild type did not change significantly between 0 and 0.01 mM cadmium and between 0.1 and 1 mM cadmium ($P = 0.276$ and $P = 0.096$, respectively), but it decreased significantly between 0.01 and 0.1 mM cadmium ($P < 0.001$). KT1 had 50% and 200% higher metabolic rates than the wild type at 0 and 1 mM cadmium, respectively ($P = 0.007$

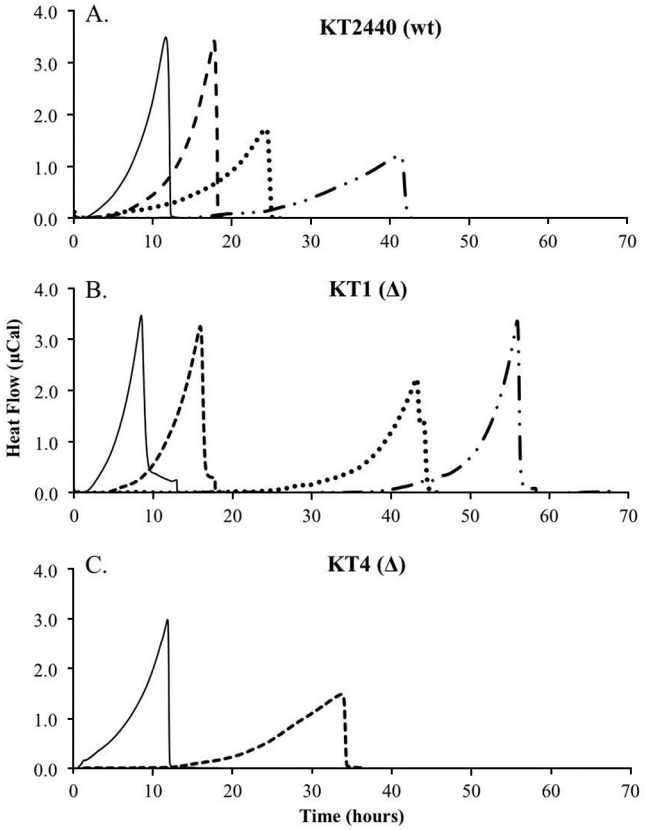

FIG. 1. Thermograms of *P. putida* KT2440 strains (KT1, *czcA1* knockout; KT4, *cadA2* knockout) growing on 100 nmol glucose in the presence of 0, 0.01, 0.1, and 1 mM $CdCl_2$. The solid line shows growth on 0 mM Cd, the dashed line shows growth on 0.01 mM Cd, the dotted line shows growth on 0.1 mM Cd, and the line with a dash followed by two dots shows growth on 1 mM Cd. wt, wild type.

and $P = 0.004$, respectively), with a metabolic rate comparable to that of the wild type at 0.01 mM cadmium (Fig. 2B). The metabolic rate for KT4 was not significantly different from the wild-type rate in the absence of cadmium, but KT4 had a metabolic rate 2 times lower than that of the other strains at 0.01 mM cadmium (Table 1). Metabolic rates correlated well with the thermogram peak heights (Pearson's $r = 0.672$; $n = 35$), which express the maximum metabolic intensity (Table 1).

Biomass yield. KT1 had significantly higher biomass yields than the wild type at cadmium concentrations of 0, 0.1, and 1 mM (80%, 80%, and 400%, respectively) (Table 1 and Fig. 2C). The wild-type biomass yield increased up to 0.01 mM cadmium and then began to decline. KT1 had an observable increase in biomass yield up to 0.1 mM cadmium and then began to drop back down at 1 mM cadmium (Table 1). The biomass yield for KT4 increased from 0 to 0.01 mM cadmium and fell to zero (no growth) at 0.1 mM and 1 mM cadmium. The cell volume (i.e., cell mass) ranged from 0.3 to 1.1 μm^3 for all strains. The cell volumes for KT2440 and KT1 rose at least 2-fold from 0 to 0.01 and 0.1 mM Cd and then dropped back to their original size at 1 mM Cd (data not shown). In KT4, the average cell volume did not change across the different metal concentrations at which growth was observed (data not shown). For each experiment a total of 101 ± 5 nmol glucose was initially present in the medium. For KT4, at 0.1 and 1 mM cadmium, none of the glucose was consumed during the course of the experiment, but in all other experiments 100% of the initial glucose was consumed (the final glucose concentration was below the detection limit of 0.2 nmol).

Energetic efficiency. The wild type grew less efficiently at cadmium concentrations of 0 and 1 mM than did KT1. The wild-type heat output varied significantly across the different metal concentrations ($P < 0.001$), with the highest growth efficiency at cadmium concentrations of 0.01 and 0.1 mM (Table 1). KT1 appeared to follow the same trend as the wild type, with significant differences in heat released at the different metal concentrations ($P = 0.047$). The heat released per carbon mole of biomass for KT4 did not vary significantly between 0 and 0.01 mM Cd but was significantly higher than that of the other two strains at both metal concentrations (Table 1).

DISCUSSION

Initially, we were concerned that there would not be enough dissolved oxygen in the calorimeter chamber for the aerobic respiration of all available glucose. The initial molar ratio of dissolved oxygen to glucose in solution was 7:1, and 6 mol of oxygen is theoretically consumed for every 1 mol of glucose in a complete combustion reaction. Even so, as oxygen concentrations drop, the likelihood of nitrate reduction increases. Prior work showed that shifts from aerobic to anaerobic metabolism, or switching between food sources, manifest themselves as separate peaks on the thermogram (polyphasic growth), but this was not observed in our experiments (1). The thermograms of all the strains under various cadmium conditions show single peaks, which suggests that all metabolic activity was due to the aerobic consumption of glucose (Fig. 1).

Efflux genes influence growth kinetics. The wild-type organism (*P. putida* KT2440) was able to consume a fixed quantity of glucose before either KT1 (Δ*czcA1*) or KT4 (Δ*cadA2*) under cadmium stress. The accelerated ability to process glucose was due to a shortened lag phase rather than a higher metabolic rate. At cadmium concentrations above 0.1 mM, the wild-type strain consumed all the available glucose before KT1 could exit from the lag phase (Fig. 1). Given that an accelerated ability to process glucose under conditions of metal stress is a benefit to the organism, the wild type outperformed the other strains (Fig. 2A). Concordantly, strain KT1 had a shorter peak time than that of the wild type in the absence of cadmium (Fig. 2A), suggesting that the presence of the *czcA1* gene impairs competitive performance under pristine or other conditions when metals are not bioavailable by prolonging the lag phase. This supports the hypothesis that heavy metal efflux transporter genes are costly to maintain in the absence of metals, even when they are transcriptionally regulated. KT4 performed relatively poorly in all experiments and was completely inhibited by cadmium concentrations above 0.01 mM (Fig. 1). CadA2, therefore, appears to be important for the growth and survival of KT2440 in both contaminated and uncontaminated environments, suggesting a significant homeostatic role in addition to mediating cadmium resistance.

The metabolic rate and peak height data did not agree with our initial hypothesis that increasing metal concentrations would depress the metabolic rates of the mutant strains and

TABLE 1. Results from calorimetric assays obtained for *Pseudomonas putida* KT2440 strains in the presence of $CdCl_2$ at 25°C[b]

Thermokinetic parameter[a]	Mean value ± SE for strain			*F*	*P*
	KT2440	KT1	KT4		
0 mM Cd					
Q_T (−μcal)	42,127 ± 3,973	39,474 ± 1,053	46,357 ± 446.0	1.3920	0.331
P_{max} (−μcal s^{-1})	2.980 ± 0.260	3.260 ± 0.160	3.120 ± 0.150	0.4730	0.648
$t_{max\ (h)}$	11.06 ± 0.700	7.800 ± 0.320	9.910 ± 1.890	3.8170	0.099
k (h^{-1}) (10^1)	3.170^{α} ± 0.240	4.680^{β} ± 0.340	2.550^{α} ± 0.020	44.811	**0.001**
Yield	0.281^{α} ± 0.044	0.499^{β} ± 0.043	0.159^{α} ± 0.001	16.524	**0.006**
Q_T/C-mol biomass (10^{-11})	3.640^{α} ± 0.360	1.910^{β} ± 0.200	6.940^{χ} ± 0.200	66.370	**0.000**
0.01 mM Cd					
Q_T (−μcal)	48,012 ± 2,787	45,091 ± 1,894	44,483 ± 1,900	0.6380	0.567
P_{max} (−μcal s^{-1})	3.520^{α} ± 0.190	3.160^{α} ± 0.060	1.490^{β} ± 0.010	56.238	**0.000**
t_{max} (h)	15.08^{α} ± 1.340	15.06^{α} ± 0.490	30.94^{β} ± 2.710	34.983	**0.001**
k (h^{-1}) (10^1)	3.210^{α} ± 0.470	3.460^{α} ± 0.460	1.770^{β} ± 0.020	11.098	**0.014**
Yield	0.676^{α} ± 0.086	0.794^{α} ± 0.033	0.217^{β} ± 0.054	18.583	**0.005**
Q_T/C-mol biomass (10^{-11})	1.750^{α} ± 0.280	1.360^{α} ± 0.100	5.120^{β} ± 1.060	17.390	**0.006**
0.1 mM Cd					
Q_T (−μcal)	43,539 ± 368.0	46,830 ± 385.0	0 ± 0	38.178	**0.003**
P_{max} (−μcal s^{-1})	1.800 ± 0.060	2.200 ± 0.020	0 ± 0	35.977	**0.004**
t_{max} (h)	24.47 ± 1.460	46.61 ± 2.610	0 ± 0	54.884	**0.002**
k (h^{-1}) (10^1)	1.520 ± 0.080	2.330 ± 0.200	0 ± 0	42.391	**0.003**
Yield	0.482 ± 0.048	0.854 ± 0.001	0 ± 0	60.127	**0.001**
Q_T/C-mol biomass (10^{-11})	2.190 ± 0.260	1.300 ± 0.010	0 ± 0	38.178	**0.003**
1 mM Cd					
Q_T (−μcal)	42,765 ± 1,293	51,698 ± 923.0	0 ± 0	31.634	**0.005**
P_{max} (−μcal s^{-1})	0.990 ± 0.180	3.230 ± 0.210	0 ± 0	66.814	**0.001**
t_{max} (h)	41.75 ± 0.450	54.00 ± 0.970	0 ± 0	130.68	**0.000**
k (h^{-1}) (10^1)	1.140 ± 0.230	2.530 ± 0.170	0 ± 0	71.821	**0.001**
Yield	0.159 ± 0.002	0.700 ± 0.072	0 ± 0	56.102	**0.002**
Q_T/C-mol biomass (10^{-11})	6.390 ± 0.170	1.790 ± 0.200	0 ± 0	31.634	**0.005**

[a] Q_T, total heat released by the microbial growth reaction as measured by the VP-ITC instrument ($n = 3$); P_{max}, maximum heat flow rate of the microbial growth reaction ($n = 3$); t_{max}, time to reach peak of power/time curve ($n = 3$); k, metabolic rate constant ($n = 3$); yield, carbon moles biomass divided by the carbon moles glucose consumed ($n = 3$); Q_T/C-mol biomass, total heat released per carbon mole biomass (μcal/C-mol × 10^{-11}) ($n = 3$).

[b] *F* and *P* values were obtained by a one-way analysis of variance (ANOVA) with a Tukey post-hoc test. Significant *P* values are in boldface type. The superscripted symbols α, β, and χ indicate statistical groupings from the post-hoc test.

accelerate the metabolic rate of our wild-type strain. However, cadmium concentrations above 0.01 mM reduced the log heat output rate of the wild type, while KT1 exhibited consistent heat output and comparatively higher metabolic rates under all conditions. The drop in the wild-type metabolic rate at 0.01 mM Cd implies a threshold above which the Cd-dependent expression of the *czcA1* gene confers enhanced resistance to cadmium, as suggested by the jump in lag phase between 0.01 and 0.1 mM cadmium for KT1 (Fig. 2A) and as reported previously (23). It appears that the CzcCBA1 transporter minimizes the metabolic rate at higher cadmium concentrations (lowering the rate of log-phase heat output), perhaps due to a lower intracellular cadmium concentration and decreased need to repair intracellular damage, while shortening the lag phase at the same time. The slight rise in the heat output rate for KT1 at 1 mM cadmium could be attributed to the need for elevated levels of macromolecular synthesis and repair of cellular damage from increasing intracellular levels of cadmium. This putative rise in levels of macromolecular synthesis could also drive the higher metabolic rates observed at elevated cadmium levels (5, 12), which would explain the increased log-phase metabolic rate (relative to that of the wild type) of strain KT1 at 1 mM cadmium. This provides a potential mechanism for observations made by prior investigations, which have shown an increase in the rate of microbial respiration in the presence of heavy metals (17, 36).

Effects of the presence and/or absence of efflux genes on biomass yield and metabolic efficiency. The biomass yield for KT1 was almost twice the wild-type biomass yield with no cadmium, and KT1 maintained the highest yields across all cadmium concentrations (Fig. 2C). The biomass yield for KT4 remained lower than those of the other two strains across all metal concentrations (Table 1). The biomass data suggest that wild-type *P. putida* prefers rapid resource processing, in a tradeoff between biomass yield and the resource consumption rate. The CBA transporter appears to be the cause of the decreased biomass yield (either directly or indirectly), which supports the idea that metal resistance genes suppress productivity by diverting energy toward efflux and gene maintenance. A curious effect observed for the wild type and KT1 was an increase in biomass yield coupled with an unchanging or slightly greater heat output. This seems counterintuitive but might be explained by the energy density of the biomass (26). Organisms with metal resistance mechanisms are known to vary protein expression and carbohydrate production under conditions of metal stress (23, 32, 46), altering the proportion of the different classes of macromolecules in the cell. Steady or increasing biomass yields coupled with higher metabolic heat

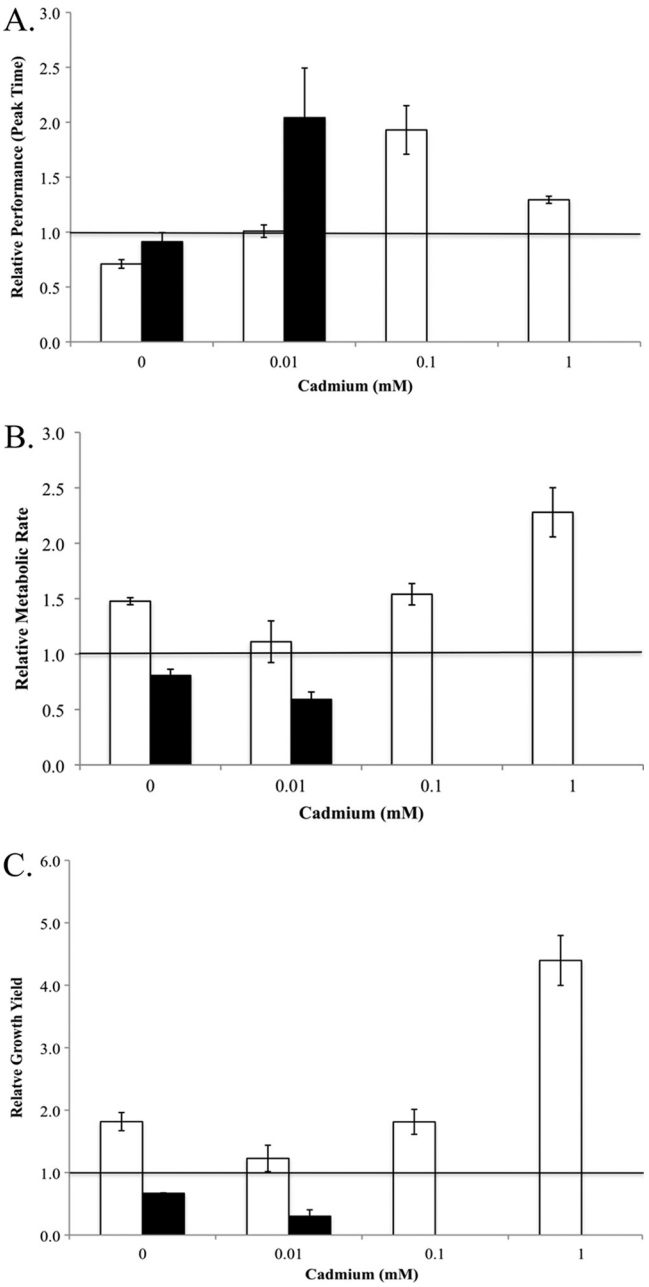

FIG. 2. Performance (A), metabolic rate (B), and growth yield (C) of *P. putida* KT2440 knockout strains relative to the wild-type strain (represented by the horizontal line at 1.0 on the *y* axis) in the presence of different cadmium concentrations. The white bars represent KT1 (*czcA1* knockout) data, and the black bars represent KT4 (*cadA2* knockout) data. Performance values >1 indicate a slower transition from stationary phase to active metabolism than the wild type, whereas performance values <1 indicate a faster transition from a stationary state to active metabolism than the wild type. The metabolic rate was measured as the slope of the log of the power-versus-time curve (μcal/s) during exponential growth. The growth yield was calculated as carbon moles biomass produced per carbon mole glucose consumed.

output were also observed previously by von Stockar and Liu (47) when they analyzed the thermokinetic profiles of microbes growing on increasingly electron-poor substrates. The enthalpy of combustion and the elemental biomass composition changed when cells were grown on these substrates (9, 22, 47). Perhaps, the wild type and KT1 manufacture a proportionally larger amount of polysaccharides than protein under conditions of metal stress (especially KT1, which lacks CzcCBA1 expression), lowering the total amount of stored energy per carbon mole of biomass and freeing up more energy to be lost as heat (45). Increased polysaccharide production and other significant shifts in cellular metabolism and gene expression in metal-stressed *P. putida* were observed previously (37, 39), but further investigations are needed to test this hypothesis.

The heat output per unit biomass may be calculated as the ratio of the catabolic processes to the anabolic processes, providing a measure of cellular inefficiency (inefficiency = ΔH_{heat}/C-mol biomass) (47, 48). The wild-type strain produced approximately 720 kJ more heat per carbon mole of biomass than did KT1 in the absence of cadmium (~50% less efficient), which most likely reflects the cost of *czcA1* gene maintenance in energy units. Also, the wild type produces 2,670 kJ more heat per carbon mole biomass than KT1 at 1 mM cadmium (equivalent to consuming almost 1 mole additional glucose per carbon mole biomass), indicating again that the wild type diverts resources to metal resistance and rapid resource consumption at the expense of efficiency and that KT1 is more energetically efficient, but slower to exit from lag phase, than the wild type under all tested conditions. KT4 produced 1,380 kJ more heat than the wild type in the absence of cadmium and three times more heat per carbon mole than both the wild type and KT1 at 0.01 mM cadmium. These data further highlight the importance of CadA2 to the cell. The wild type reached an energetic minimum at intermediate (0.01 and 0.1 mM) cadmium concentrations (Table 1), suggesting that the wild-type organism grows most efficiently in the presence of intermediate concentrations of cadmium.

Implications for future work. Efflux genes are involved in a variety of resistance processes, including toxin export, tolerance of antibiotics, and heavy metal efflux (24, 32, 35). Each of these mechanisms incurs a metabolic cost. This work demonstrates that the energetic cost for growing in a cadmium-contaminated system is a lower biomass yield. It also shows that even when efflux genes are under transcriptional regulation, the cost of maintaining them under pristine conditions is still high. Despite this cost, the results indicate that the benefit of harboring the CzcCBA1 chemiosmotic transporter in a contaminated environment is an accelerated ability to gain access to resources. This enhanced capacity to rapidly exit from metabolic dormancy would allow a bacterium to outcompete other slower (although more efficient) organisms for limited or intermittently available resources. Viewed in an ecological context, one could suggest that low-level metal stress decreases metabolic efficiency and depresses gross microbial biomass production at the bottom of the food chain. Previous studies demonstrated that metal stress mechanisms and the bioaccumulation of metals may protect bacteria from predation (18, 21, 38, 50), further limiting the movement of energy and carbon up the food chain. This organism-level cost to biomass yield and metabolic efficiency induced by the presence of a chronic or intermittent stressor (e.g., heavy metals or organic pollutants), even at subinhibitory concentrations, could affect higher trophic levels, constraining total ecosystem productivity.

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