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Phylogeny Poorly Predicts the Utility of a Challenging Horizontally Transferred Gene in *Methylobacterium* Strains

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Horizontal gene transfer plays a crucial role in microbial evolution. While much is known about the mechanisms that determine whether physical DNA can be transferred into a new host, the factors determining the utility of the transferred genes are less clear. We have explored this issue using dichloromethane consumption in *Methylobacterium* strains. *Methylobacterium extorquens* DM4 expresses a dichloromethane dehalogenase (DcmA) that has been acquired through horizontal gene transfer and allows the strain to grow on dichloromethane as the sole carbon and energy source. We transferred the *dcmA* gene into six *Methylobacterium* strains that include both close and distant evolutionary relatives. The transconjugants varied in their ability to grow on dichloromethane, but their fitness on dichloromethane did not correlate with the phylogeny of the parental strains or with any single tested physiological factor. This work highlights an important limiting factor in horizontal gene transfer, namely, the capacity of the recipient strain to accommodate the stress and metabolic disruption resulting from the acquisition of a new enzyme or pathway. Understanding these limitations may help to rationalize historical examples of horizontal transfer and aid deliberate genetic transfers in biotechnology for metabolic engineering.

The recent accumulation of genome sequences from diverse bacterial clades has demonstrated the crucial role of horizontal gene transfer (HGT) in bacterial evolution (1, 2). It has become clear that genes and operons have consistently moved between distant bacterial strains, with important implications for bacterial evolution, physiology, and ecology (3). In appreciating the significant impact of HGT, it is important to also consider the factors that limit transfer (4). If a gene is beneficial when acquired by one strain, why do we not observe that gene transferring into other, closely related strains?

One possible explanation for the rarity of successful horizontal transfers is that recipients that would benefit from the transfer have little opportunity to acquire the corresponding DNA (5), and that factors such as ecological differentiation and barriers to genetic exchange may prevent a strain from encountering a potential donor (4, 6). Additionally, transfer events may be rare even in the presence of a donor (7), since the likelihood of stably integrating and expressing newly acquired DNA is predicted to decrease with increasing genetic distance and will limit the frequency and breadth of transfer (8–10). These factors suggest that ecology and phylogeny should largely determine transfer frequencies, based on how likely a strain is to encounter a donor (ecology) and to acquire, stably integrate, and express the transferred DNA (phylogeny) (11).

Another significant, yet poorly investigated, barrier to HGT depends on how efficiently a recipient can use its new ability. A newly acquired gene or pathway may place novel stresses on the host, either by disrupting existing metabolic and regulatory networks (12, 13) or by producing new toxic metabolites (13, 14). The fitness cost of such stresses is determined by the host physiology. A beneficial ability with costly side effects will preferentially spread to those recipients best able to accommodate its associated stresses, leading to a gene distribution shaped by physiology rather than by phylogeny.

We used dichloromethane catabolism in strains of *Methylobacterium* to explore factors that limit the functional incorporation of a horizontally transferred gene. Dichloromethane (DCM) is an industrial solvent that has reached significant concentrations in the environment only in the last 50 years. Among several strains isolated for their ability to grow on DCM as the sole carbon and energy source (15, 16), a strain of *Methylobacterium extorquens* known as DM4 has been investigated in the most detail. Through HGT, this strain has acquired a gene, *dcmA*, encoding a cytoplasmic glutathione *S*-transferase that converts DCM to formaldehyde, with the concomitant release of two molecules of hydrochloric acid (17, 18). In *M. extorquens* DM4, the *dcmA* gene lies within a 126-kb genomic island that shows clear evidence of horizontal transfer, including a lower GC content (19). Within this genomic island, the *dcm* cluster contains three other genes, including the transcriptional regulator *dcmR* and two proteins of unknown function *dcmB* and *dcmC* and is flanked on both sides by IS*1354* elements. This four-gene *dcm* islet is conserved within most DCM-degrading strains (18). However, a strain of *M. extorquens* DM4 with a deletion of the genomic island, known as DM4-2cr (20), requires only the *dcmA* gene to recover growth on DCM (21). While the other genes in the genomic island may influence growth on DCM, they are not essential.

Growth with DCM is very challenging for the cell (Fig. 1). First,

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FIG 1 Growth on DCM presents several challenges to the host. One molecule of DCM is converted to one molecule of formaldehyde and two molecules of HCl. Stress-inducing compounds are indicated in red. In addition to the stresses resulting from HCl and formaldehyde, the glutathione conjugate intermediate is highly mutagenic.

the strain must accommodate the protons and chloride produced intracellularly as a by-product of DCM dehalogenation (22–24). Additionally, the *S*-chloromethylglutathione intermediate formed during the dehalogenation reaction is highly reactive and mutagenic (25–27). Finally, the cell must quickly channel the formaldehyde product into its native one-carbon metabolic pathways to minimize any resulting toxicity (28). Given these challenges, it is not surprising that deliberate transfer of the *dcmA* gene to two other strains of *M. extorquens*, AM1 and CM4, was sufficient to enable growth on DCM only in strain CM4 (21). However, it remains unclear why strain AM1 was unable to grow on DCM, or how prevalent is the ability among methylotrophic bacteria to grow on DCM using *dcmA*. We have addressed these questions by transferring *dcmA* into a broad range of *Methylobacterium* strains, quantifying their success at using their new catabolic potential, and investigating the factors that influence this success.

MATERIALS AND METHODS

Media and chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. *Escherichia coli* was grown in LB at 37°C with various antibiotic concentrations. *Methylobacterium* strains were grown at 30°C in liquid culture in M-PIPES (29) supplemented with 3.5 mM succinate or 5 mM DCM as noted. Antibiotics were added to a final concentration of 12.5 μg/ml for tetracycline, 10 μg/ml for streptomycin, or 50 μg/ml for kanamycin. Unless otherwise noted, DCM cultures were grown in 10 ml of medium in gas-tight 50-ml screw-top flasks sealed with Teflon tape and Mininert valves (Supelco, Bellefonte, PA). Valves were surface sterilized with ethanol and dried in a laminar flow hood before use. A freshly prepared 100 mM stock of DCM in water was used to inoculate the flasks.

Plasmid construction. Plasmids and strains used in this study are listed in Tables 1 and 2. Plasmid pJM10 was constructed by cloning the AatII-SacI fragment containing *kanR2* from pCM184 (30) into pME8220 (21) digested with AatII-SacI to remove the 5′ end of *tetA*. Plasmid pJM40 was constructed by amplifying superecliptic *pHluorin* (31) and using Gibson assembly to clone it into pHC08 (32) as a translational fusion to *mCherry*, yielding pJM25. The entire *mCherry-pHluorin* expression cassette was amplified from pJM25 by PCR to add BamHI and SacI restriction sites. The digested PCR fragment was then cloned into pME8220 and pCM62 to yield pJM40 and pJM41, respectively. Plasmid pJM53 was constructed by amplifying 500-bp regions upstream and downstream of the hypoxanthine phosphoribosyl transferase (*hpt*) from the genome of *M. extorquens* DM4. Using Gibson assembly, these fragments were combined with a Venus expression cassette in pPS04 (P. Swanson and C. J. Marx, unpublished results), a kanamycin resistance derivative of pCM433 (33).

Plasmid matings. Plasmids were transferred to recipient *Methylobacterium* strains using triparental matings as described previously (34). The *dcmA* gene was deleted from *M. extorquens* DM4 using a derivative of pCM433, which resulted in a mutant unable to grow on DCM (F. Bringel and S. Vuilleumier, unpublished results). *M. extorquens* DM4 Δ*dcmA-Venus* (strain CM4250) was constructed from *M. extorquens* DM4 Δ*dcmA* using pJM53 and following established protocols (33). Integration of Venus into the *hpt* locus of *M. extorquens* DM4 Δ*dcmA* was selectively neutral during growth on DCM.

Growth rate measurements. *Methylobacterium* strains were grown to saturation in 48-well plates under each of the conditions being tested. Cultures were then diluted 64× into 640 μl of fresh medium. Over 48 h, optical densities at 600 nm (OD_{600}) were measured every 30 to 45 min using an automated system (29). Growth rates were calculated using CurveFitter (29).

Competitive fitness assays. Competitive fitness for growth on DCM was measured by competing each strain against *M. extorquens* DM4 Δ*dcmA-Venus*(pJM10) generally following a previous protocol (32). In brief, each strain was grown to saturation in M-PIPES–succinate–kanamycin and then diluted 100× into M-PIPES–DCM. The exception was *M. extorquens* AM1 Δ*cel-mCherry*(pJM10), which cannot grow on DCM alone. This strain was grown in M-PIPES–DCM–tetracycline, where the tetracycline is dissolved in 80% ethanol and therefore provides 14 mM ethanol for growth. After 3 days, the cultures were diluted and mixed in fresh M-PIPES–DCM. *M. extorquens* DM4 Δ*dcmA-Venus*(pJM10) was added to each flask at an OD of 0.001. The test strain was then added at an OD of 0.005. A 450-μl portion of each culture was removed, mixed with 50 μl of dimethyl sulfoxide (DMSO), and frozen at −80°C. The mixed cultures were then grown for 3 days.

At the end of the growth phase, the population ratios of the samples, before and after growth on DCM, were determined using flow cytometry. Postgrowth samples were diluted into fresh M-PIPES to a final OD of ~0.015. Fluorescence was measured on an LSRII flow cytometer (BD, Franklin Lakes, NJ). Venus was excited at 488 nm and measured at 530 nm with a 30-nm bandpass filter. When available, mCherry was excited at 561 nm and measured at 620/40 nm. The competitive fitness was calculated as $\log[(R_1{*}N)/R_0]/\log[(1 - R_1){*}N/(1 - R_0)]$, where R_0 and R_1 represent the population fraction of the test strain before and after mixed growth and N represents the fold increase in the population density. When the test strain was labeled with mCherry, the population fraction was calculated as the ratio of mCherry-positive cells to Venus-positive cells. When the test strain was unlabeled, the population fraction was the ratio of Venus-negative cells to Venus-positive cells.

Chloride measurements. Chloride released by dehalogenation was measured using the method of Jörg and Bertau (35), comparing the absorbance against that obtained with M-PIPES without added carbon.

Total protein measurements. Total protein was measured by growing the desired culture in 10 ml of M-PIPES–DCM for 3 days. The cultures were concentrated by centrifugation and resuspended in 1 ml of PBS plus 1 mM EDTA. Cells were lysed using a FastPrep-24 (MP Bio, Santa Ana, CA) and lysing matrix B (MP Bio). Lysates were centrifuged, and total protein in the supernatant was quantified using the Bradford quick-start assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard.

Dehalogenase activity measurements in whole cells. Strains were grown at 30°C in 50 ml of M-PIPES in 300-ml screw-top Erlenmeyer flasks fitted with Mininert stoppers (Supelco), using 5 mM DCM with or without the addition of 3.5 mM succinate as the carbon and energy source. Cultures were harvested in exponential phase after measurement of their ODs and centrifuged for 15 min at 8,000 rpm. The final pH of the cultures was measured in the spent medium supernatant. The cell pellets were resuspended in 2 ml of M-PIPES, and the protein concentration in cell

TABLE 1 Plasmids used in this study

Plasmid	Description	Reference
pCM433	*oriT*^{RP4} *cat bla tetA sacB*	33
pJM10	*dcmA kanR2*	This work
pJM40	P_{Tac}-*mCherry-pHluorin dcmA tetA*	This work
pJM41	P_{Tac}-*mCherry-pHluorin tetA*	This work
pJM53	pPS04 *hpt*::*Venus*	This work
pPS04	*kanR2* replaces *cat*, *bla*, and *tetA* of pCM433	P. Swanson and C. J. Marx, unpublished data

TABLE 2 Strains used in this study

Designation	Strain	Genotype	Reference
AM1	CM3120	*M. extorquens* AM1 Δ*cel katA*::*mCherry*	L. Chubiz and C. J. Marx, unpublished data
PA1	CM3839	*M. extorquens* PA1 Δ*cel hpt*::*mCherry*	D. Nayak and C. J. Marx, unpublished data
DM4 Δ*dcmA*	CM4250	*M. extorquens* DM4 Δ*dcmA hpt*::*Venus*	This work
DM4-2cr	DM4-2cr	*M. extorquens* DM4 with a deletion spanning *dcmABC*	20
CM4	CM4	*M. extorquens* CM4	45
BJ001	BJ001	*M. extorquens* BJ001	41
M. nodulans	ORS 2060	*M. nodulans* ORS 2060	42
M. radiotolerans	JCM 2831	*M. radiotolerans* JCM 2831	43

suspensions was determined by a commercial bicinchoninic acid protein assay kit (Sigma) adapted to microplate format (0 to 10 μg protein, using BSA as a reference). Chloride concentration in the final spent medium was determined as described above, adapting the assay to microplate format. DCM dehalogenase activity was determined by the Nash method as described previously (36), with minor modifications. Briefly, concentrated cell suspensions of the different strains (200 to 500 μl in M-PIPES) were incubated at 30°C in a total volume of 1 ml M-PIPES containing 7.5 mM potassium sulfite, 2 mM reduced glutathione, and 20 mM DCM added last to the mixture from a 100 mM stock in M-PIPES. Aliquots (60 μl) were taken at different times, added to 540 μl Nash reagent (4 ml 30% [wt/vol] ammonium acetate with 0.4% acetylacetone, 1 ml 1% [wt/vol] iodine in acetone, 10 ml H_2O), and incubated for 30 min at 65°C, and absorbance was measured at 412 nm. Activity was expressed as μmol formaldehyde produced per minute per mg protein, using an ε_{412} value of 7,812 M^{-1} cm^{-1} for the produced dimethyldihydropyridine derivative.

Internal pH measurements. Strains were grown to saturation in M-PIPES–succinate–tetracycline, diluted 100× in M-PIPES–DCM, and grown for 3 days. Strain AM1 Δ*cel*(pJM40) was the exception, as it cannot grow on DCM alone. Consequently, this strain was grown in M-PIPES–DCM–tetracycline, where the tetracycline was dissolved in 80% ethanol and provided 14 mM ethanol. A negative control, *M. extorquens* DM4 Δ*dcmA*(pJM41), was inoculated into M-PIPES–succinate–tetracycline and grown to saturation overnight.

Cultures were diluted to 2 ml of fresh M-PIPES at an OD of 0.015. Fluorescence was measured on an LSRII flow cytometer (BD). pHluorin was excited at 488 nm and measured at 530/30 nm. mCherry was excited at 561 nm and measured at 620/40 nm. Cultures were gated on forward and side scatter to isolate cells and then gated to remove events with low mCherry (pH-independent) fluorescence.

For each sample, a time zero measurement was made by collecting 50,000 cells. A 100-μl portion of a 100 mM DCM stock was added to the tube and briefly vortexed, and the tube was put back on the flow cytometer for continuous sampling. Approximately 20 s elapsed between the addition of DCM and stable measurements of the population fluorescence.

To calibrate the pH biosensor, cultures were diluted into a solution containing 20 mM buffer, 50 mM NaCl, 3 mM KCl, 10 μM valinomycin, and 10 μM nigericin. Buffers used for calibration were MES at pH 5.1, 5.3, 5.5, 5.7, and 5.9 and PIPES at pH 6.1, 6.3, 6.5, 6.7, 6.9, 7.1, and 7.3. Three technical replicates were performed for each combination of strain and pH. Four calibrations were performed for each experiment, using *M. extorquens* DM4-2cr(pJM40) (20), *M. extorquens* DM4 Δ*dcmA*(pJM41), *M. radiotolerans*(pJM40), and *M. nodulans*(pJM40).

Internal pH values were calculated by comparison to the calibration curve. The ratio of green (pH-dependent) to red (pH-independent) fluorescence was calculated for each cell. For the calibration and time zero samples, the population geometric mean was calculated for this ratio. For the time course samples, the ratio and timestamp of each cell were exported as comma-separated values and imported into Matlab. Further processing in Matlab using a custom script removed outliers, divided the cells into 5-s bins, calculated the geometric mean of the fluorescence ratio for each bin, and converted that mean fluorescence into a measured pH by comparison to the appropriate calibration curve.

Phylogenetic analyses. Phylogenetic trees using 16S rRNA gene sequences were constructed in the Ribosomal Database Project (37). Phylogenetic trees based on 400 conserved proteins were constructed with PhyloPhlAn (38).

RESULTS

To determine the prevalence of the capacity to grow on DCM, we constitutively expressed *dcmA* from its native promoter on a broad-host-range plasmid (21). The plasmid was transferred by conjugation into seven *Methylobacterium* strains, including five that belong to the same species, *M. extorquens* (Fig. 2; also, see Fig. S1 in the supplemental material). As recipients, we tested the previously studied strains *M. extorquens* AM1, CM4, and DM4 (21) as well as the recently sequenced strains *M. extorquens* PA1 (39, 40), *M. extorquens* BJ001 (formerly *M. populi*) (40, 41), *M. nodulans* (42), and *M. radiotolerans* (43). To aid in accurate growth rate and fitness measurements, some recipients were modified by genomic insertions of fluorescent markers and gene disruption to prevent cell clumping, as previously described for strain *M. extorquens* AM1. The strains AM1 Δ*cel-mCherry* (CM3120, referred to here as AM1) and PA1 Δ*cel-mCherry* (CM3839, referred to here as PA1) lack the cellulose biosynthetic cluster (30) and express a red fluorescent protein (32), and DM4 Δ*dcmA-Venus* (CM4250, referred to here as DM4 Δ*dcmA*) lacks the chromosomal *dcmA* gene and expresses a yellow fluorescent protein.

Six of the strains, with the notable exception of AM1, were able to use pJM10 to grow on DCM as the sole carbon and energy source (Fig. 3). Four of the strains grew planktonically and showed a linear relationship between the final optical density and the amount of chloride released, indicating that chloride generation is linked to cell growth to the same extent across these strains. The remaining three strains, *M. extorquens* CM4(pJM10), *M. extorquens* BJ001(pJM10), and *M. radiotolerans*(pJM10), grew as clumpy particulates, rendering measurements of optical density impractical. When we grew these strains on DCM, lysed the cells, and measured total protein, we observed a linear relationship between total protein and chloride released (see Fig. S2 in the supplemental material).

We next asked whether these differences in yield would translate into differences in competitive fitness. Most strains that grew poorly on DCM in pure culture were also less fit in competition (Fig. 4). However, *M. radiotolerans*(pJM10) and *M. extorquens* BJ001(pJM10) were more and less fit, respectively, than their individual yields would predict (see Fig. S3A in the supplemental material). The phylogenetic distance between a test strain and *M. extorquens* DM4 did not predict the competitive fitness during growth on DCM (see Fig. S3B in the supplemental material).

Given the observed variation in competitive fitness on DCM,

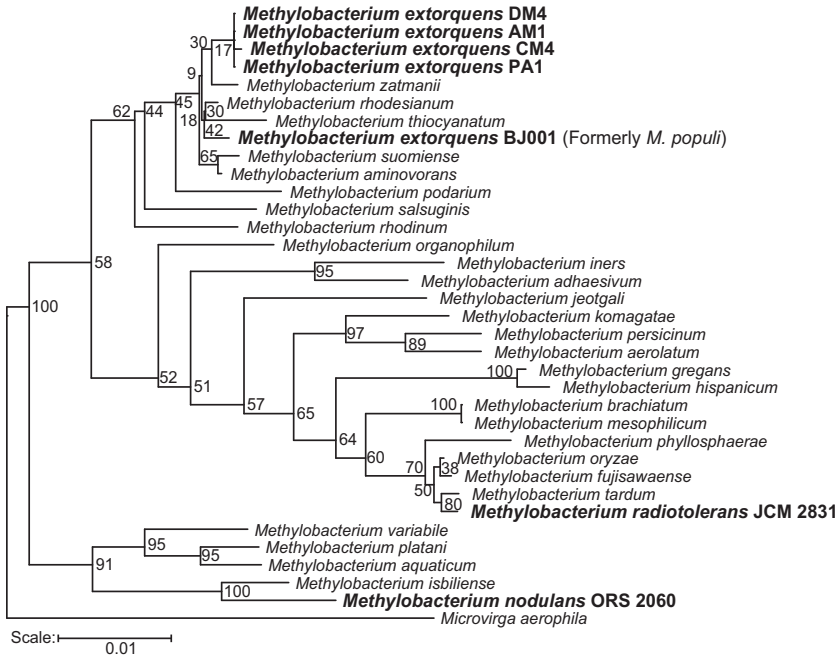

FIG 2 The DCM degradation pathway can be transferred to diverse *Methylobacterium* species. Strains used in this work are in bold. The phylogenetic tree was built using the Ribosomal Database Project (37), based on 16S rRNA genes.

we sought to identify physiological traits that differed between the transconjugants and correlated with fitness. We first tested whether the strains expressed functional DcmA, as this enzyme is essential for growth on DCM. All transconjugants expressed active DcmA (see Fig. S4 in the supplemental material), in agreement with previous results (21). However, AM1(pJM10) showed significantly lower dehalogenase activity than the other transconjugants. Next we compared the ability of the transconjugants to respond to the predicted stresses of hydrochloric acid and formaldehyde production. None of the transconjugant strains was able to grow on 2 mM formaldehyde as the sole carbon and energy source (data not shown). For comparison, despite being unable to grow on DCM, AM1 can grow on 0.5 mM formaldehyde (data not shown). We next used an automated system to measure growth rates under various medium conditions (29). Somewhat unexpectedly, DM4 Δ*dcmA*(pJM10) was the most sensitive to growth in high external chloride or low external pH (Fig. 5). Altogether, the measured growth rates at high external chloride or low pH did not correlate to competitive fitness on DCM (see Fig. S5 in the supplemental material).

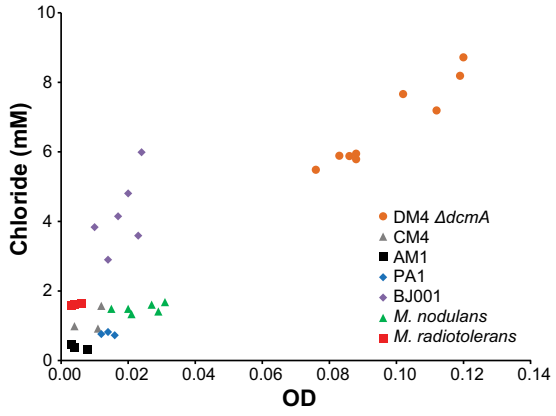

FIG 3 Transconjugant *Methylobacterium* strains vary in their ability to grow on DCM as the sole carbon and energy source. All strains contain the pJM10 plasmid expressing DcmA. Cultures were grown in sealed flasks with M-PIPES medium containing 5 mM DCM. After 3 days' growth, the optical density and supernatant chloride concentrations were measured using a spectrophotometer. Each point represents a biological replicate. The experiment was repeated at least three times for each strain, with results similar to those shown.

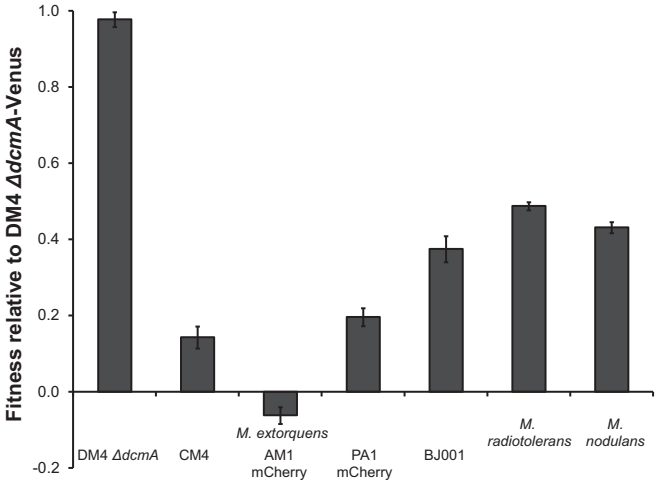

FIG 4 Competitive fitness of transconjugant *Methylobacterium* strains containing pJM10. Fitness is measured relative to DM4 Δ*dcmA-Venus*. Cultures were grown in pure culture using DCM as the sole carbon and energy source. The strains were then individually competed against DM4 Δ*dcmA-Venus*. Error bars show one standard deviation, calculated from three biological replicates. Each competition was repeated at least three times, with results similar to those shown here.

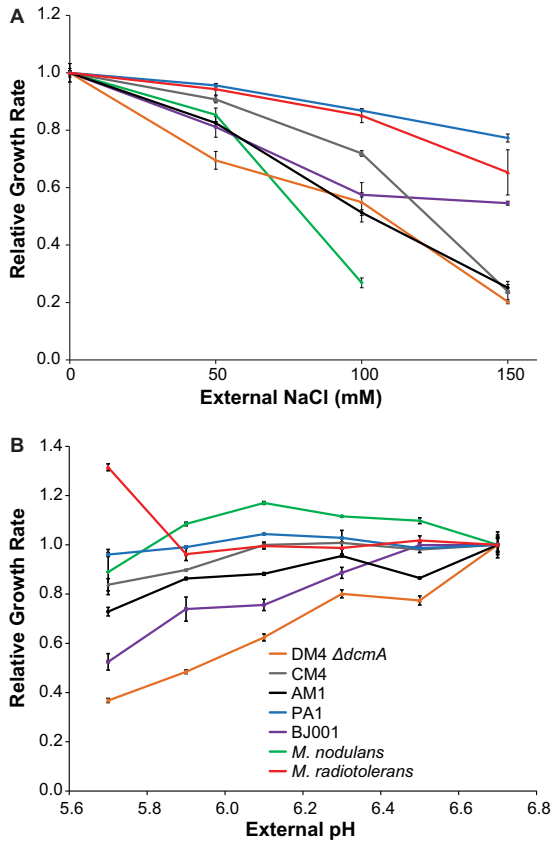

FIG 5 Transconjugant *Methylobacterium* strains containing pJM10 vary in their sensitivity to high external concentrations of NaCl (A) and to low external pH (B). Cultures were grown in M-PIPES containing succinate as the carbon and energy source. Growth rates were measured in 48-well plates using an automated system. *M. nodulans* grows as a biofilm at 150 mM NaCl, making growth rates difficult to measure. Error bars show one standard deviation, calculated from three biological replicates. The experiment was repeated three times, with similar results.

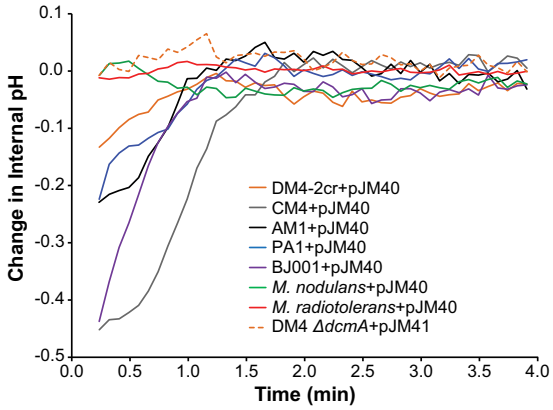

FIG 6 Internal pH decreases transiently upon addition of DCM. pJM40 expresses both the pH biosensor and DcmA. pJM41 contains only the biosensor and serves as a negative control. Internal pH upon addition of 5 mM DCM was measured in cell suspensions of *dcmA*-containing transconjugants using a pH-sensitive GFP translationally fused to a pH-insensitive mCherry (see Materials and Methods; also, see Fig. S5 in the supplemental material) (dead time before reliable fluorescence measurements, approximately 20 s).

Adding hydrochloric acid to the growth medium does not exactly reflect the stress associated with intracellular production of hydrochloric acid upon DCM dehalogenation. We therefore sought to measure how the transconjugant strains responded to the dehalogenation of DCM. We used a fluorescent biosensor to measure internal pH on a rapid, approximately 5-s time scale (44). When this biosensor was coexpressed with DcmA, we could measure the change in internal pH upon addition of DCM (Fig. 6; also, see Fig. S6 in the supplemental material). We found only a weak correlation between smaller transient decreases in intracellular pH and increased fitness of the strain on DCM (Fig. S7 in the supplemental material).

DISCUSSION

DCM is a very challenging substrate, as its catabolism imposes multiple types of stress on the host cell: protons and chloride ions need to be extruded from the cell interior; the reactive intermediate of DCM dehalogenation, *S*-chloromethylglutathione, is mutagenic; and formaldehyde, the product of DCM dehalogenation, must be efficiently transformed to minimize its toxic effects. The combination of these stresses may explain why transferring *dcmA* into naive *Methylobacterium* strains allowed only poor growth on DCM. In contrast to previous work, however, we found that the ability to use DcmA to grow on DCM was widespread and that only *M. extorquens* AM1 remained unable to grow on DCM after *dcmA* transfer. Most evidently, phylogeny was a poor predictor of a strain's ability to exploit the dehalogenase DcmA and grow on DCM under the investigated conditions. *M. extorquens* strains AM1 and CM4 have 16S rRNA gene sequences identical to those of the natural isolate *M. extorquens* DM4 but are the least successful at growing on DCM. Meanwhile, the organisms most distantly related to *M. extorquens* DM4, *M. nodulans* and *M. radiotolerans*, are among the most successful.

In this study, we were able to set aside many of the factors predicted to limit productive HGT in nature. We know that the necessary *dcmA* gene was introduced into the new host, can stably replicate, and is functionally expressed. Despite these facts, all of the transconjugants are much less fit that the original donor, *M. extorquens* DM4, and some strains show little or no growth. Other factors clearly limit the growth of these transconjugants on DCM, and we used a series of physiological assays in an attempt to identify these factors.

We expected that transconjugant growth on DCM would be limited by one or more of the following: DcmA expression, tolerance to intracellular production of HCl, efficient use of formaldehyde, and the mutagenic effects of the glutathione-conjugant intermediate. None of the assays that we used precisely replicates the overall stress involved in growing on DCM. That a single physiological parameter was not predictive of fitness on DCM may simply reflect the limitations of these assays. However, we hypothesize that this inconsistency stems from the multiple stresses imposed by growth on DCM, combined with differing abilities of the recipients to cope with these stresses. For example, some strains may be more sensitive to intracellular chloride production, while others are limited by the intracellular production of protons. In such a scenario, no single physiological parameter could predict fitness on DCM.

All of the strains showed a linear relationship between chloride produced and the final optical density or total protein of the culture. We conclude that dehalogenation is productive in each of

these strains; if DCM is dehalogenated to formaldehyde, the cells use that formaldehyde to grow. Consequently, we do not expect that inefficient metabolism of formaldehyde limits growth in the transconjugants.

The natural isolate, *M. extorquens* DM4, grows slowly in media with low external pH but maintains its internal pH when dechlorinating DCM. We hypothesize that DM4 has adapted to growth on DCM by becoming more permeable to protons, a trait that would be beneficial when the dechlorination of DCM produces intracellular protons but detrimental when the extracellular pH is low. Similarly, the poor growth of DM4 on medium containing high concentrations of chloride may reflect a selection for chloride excretion mechanisms that result in detrimental leaky import under these laboratory conditions.

Two other *M. extorquens* isolates, AM1 and CM4, struggle to maintain their internal pH upon addition of DCM and are also the least fit when growing on DCM. This result with CM4 is surprising, as it naturally has the ability to catabolize chloromethane, which also involves release of a proton and a chloride ion (45). While these results suggest that strains AM1 and CM4 grow poorly on DCM at least partly because of their inability to tolerate the protons released during dechlorination, we also note that *M. extorquens* BJ001(pJM10) has a relatively high fitness on DCM yet also has a transient decrease in pH upon addition of DCM. While maintaining a stable internal pH during dechlorination is clearly an important factor in determining fitness, it therefore cannot be the only one.

Conclusions. We have transferred the challenging, one-gene pathway for dichloromethane catabolism from *M. extorquens* DM4 into a range of naive recipient strains. All but one of the transconjugants could use the new pathway to grow on dichloromethane, but no strain could use this new ability as effectively as the donor. Among the strains tested, those with a negligible evolutionary distance from the donor were less successful at exploiting their new ability than the more distant relatives. Moreover, physiological measurements suggested that the type of stress limiting growth on DCM varied between transconjugants. These results demonstrate the necessity for evolutionary refinement following the horizontal acquisition of new genes (46). In the future, experimental laboratory evolution of HGT recipients may help to unravel the factors limiting the utility of a newly acquired gene as well as the biochemical mechanisms available to overcome those limitations.

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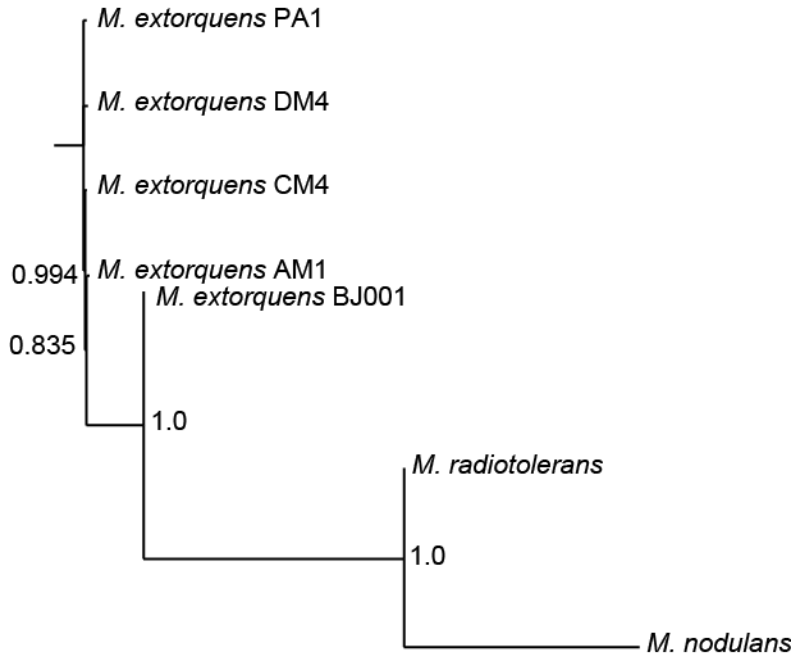
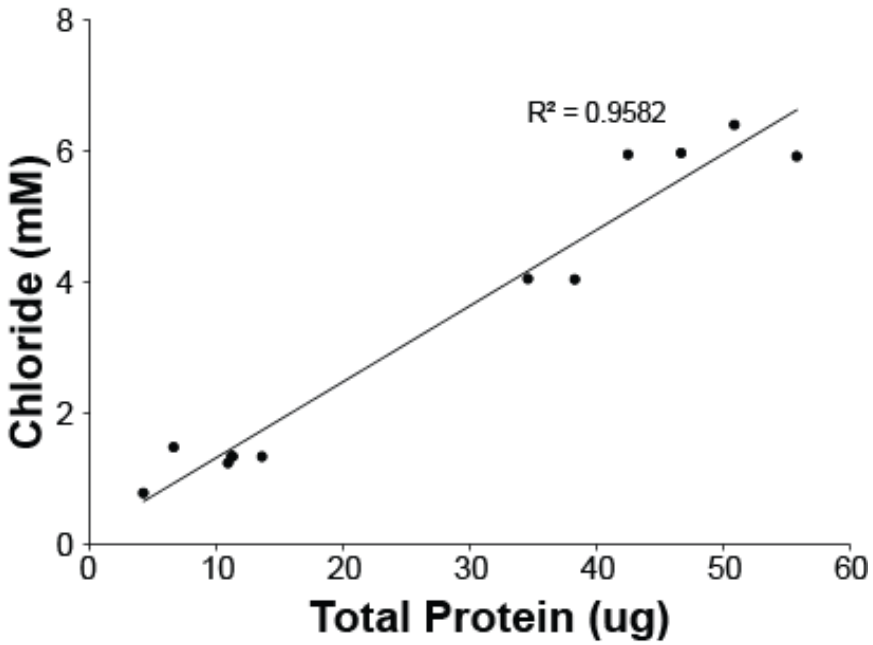
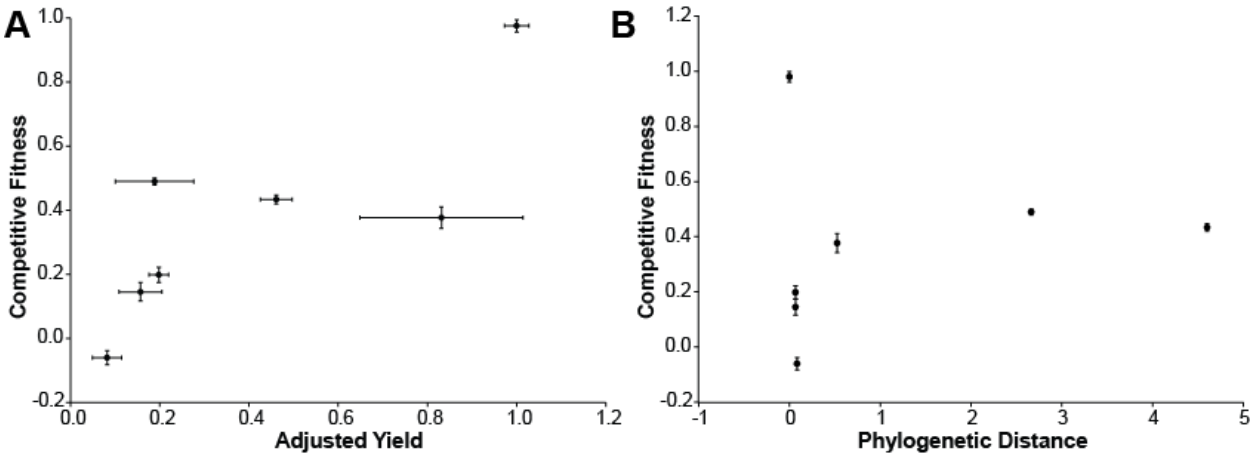


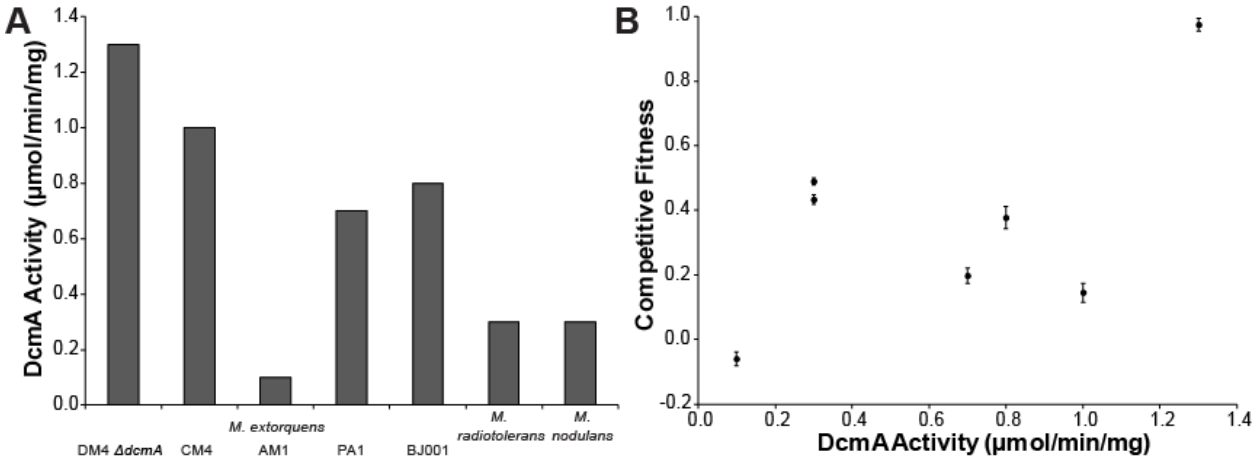
SI Figure 1: A phylogenetic tree based on 400 conserved proteins is consistent with the 16S rRNA tree. The phylogenetic tree of the seven *Methylobacterium strains* was built using PhyloPhlAn (38). The conserved proteins were selected based on ubiquity among the entire tree of life. Protein homologs were identified using a threshold of 90% sequence similarity. The most informative residues were extracted from each protein, concatenated, and used as the input for the phylogenetic tree.



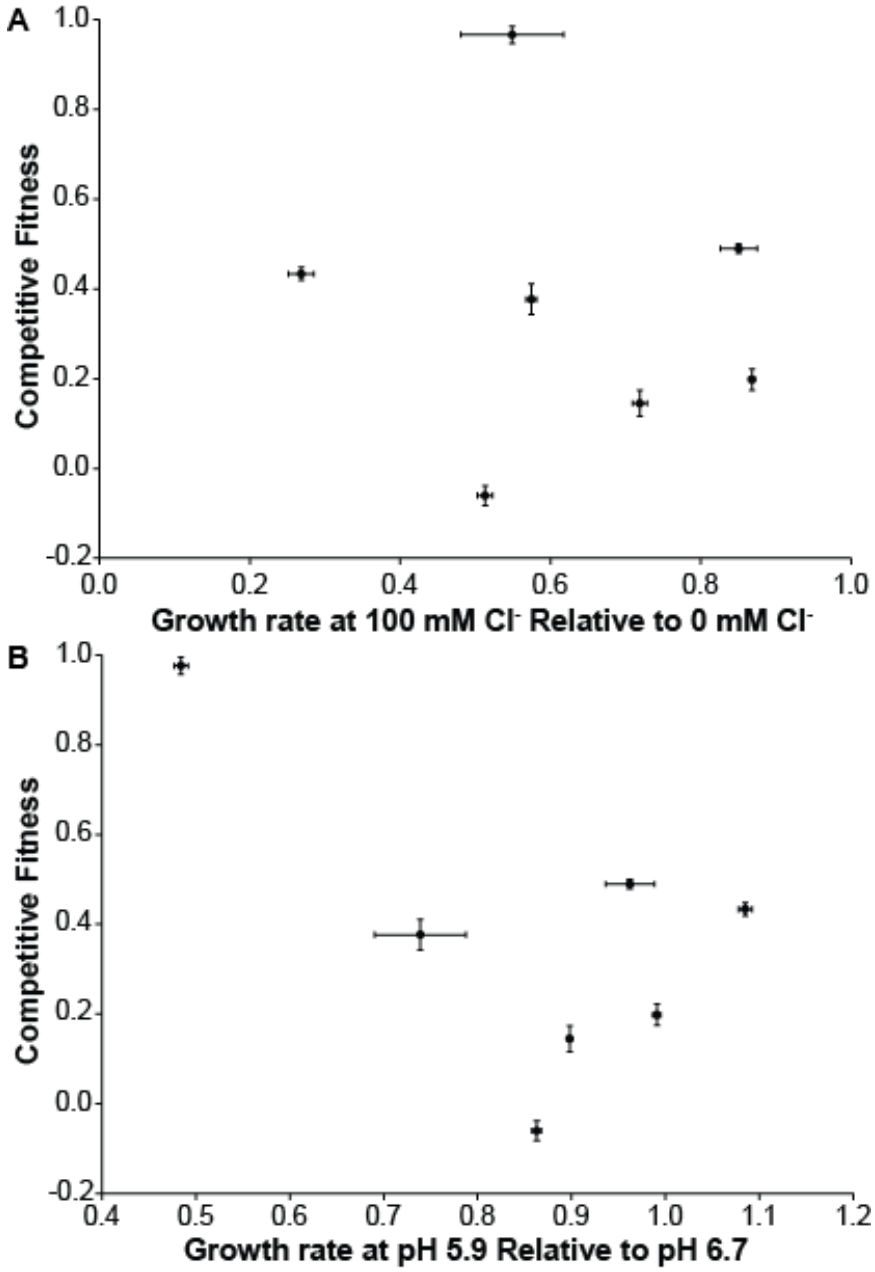
SI Figure 2: Non-planktonic strains grown on DCM show a linear relationship between total protein produced and chloride released. DM4 Δ*dcmA* +pJM10 (planktonic control), CM4+pJM10, BJ001+pJM10, and *M. radiotolerans*+pJM10 were grown in sealed flasks containing 10 mL of M-PIPES + 5 mM DCM as the sole carbon and energy source. After three days' growth, chloride concentrations were measured in the supernatant. Cells were then pelleted by centrifugation, lysed using a bead beater, and the total protein in the supernatant was measured with a Bradford assay.



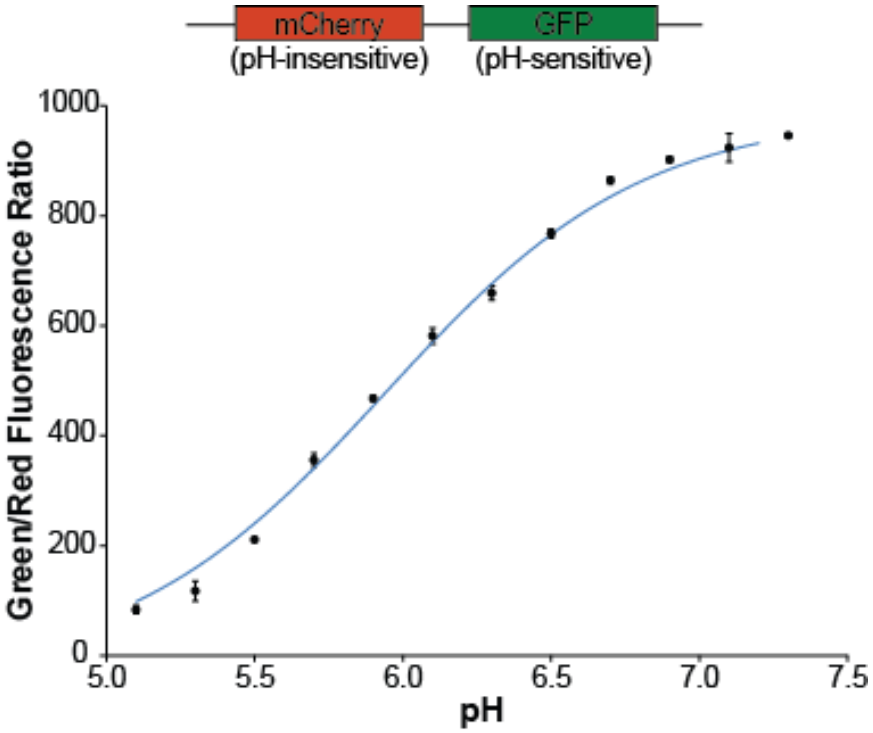
SI Figure 3: (A) Transconjugant yield on DCM in pure culture is generally predictive of competitive fitness. The outliers are BJ001, which shows lower fitness than would be predicted based on yield, and *M. radiotolerans*, which shows higher fitness than predicted. (B) Phylogenetic distance is not an effective predictor of competitive fitness. Error bars show one standard deviation, calculated from three biological replicates.



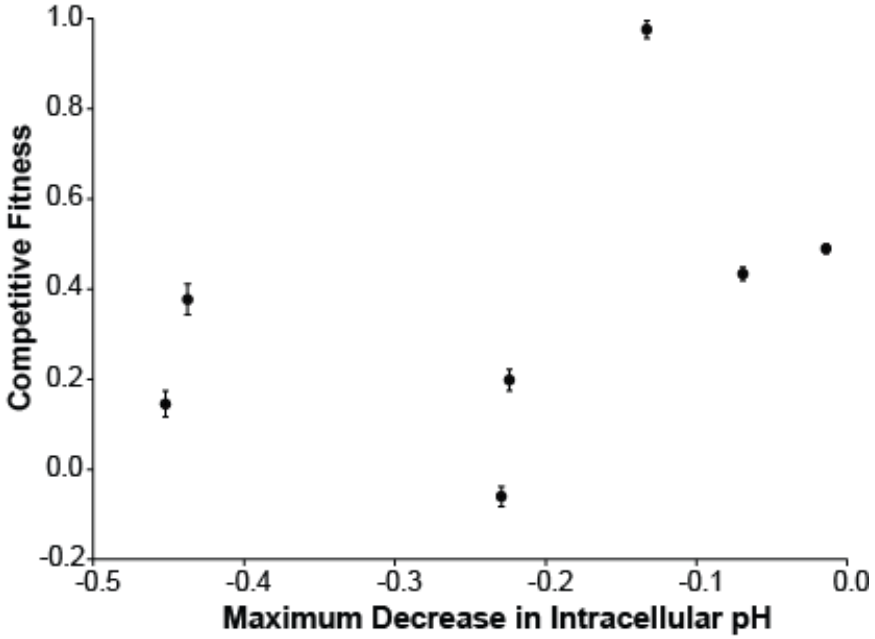
SI Figure 4: (A) All pJM10 transconjugants show DcmA activity in cell suspensions of cultures grown with 3.5 mM succinate and 5 mM DCM. (B) *In vitro* DcmA activity does not predict *in vivo* competitive fitness. The outliers, AM1 and DM4 Δ*dcmA*, have the lowest and highest specific activities, respectively. However, strains with intermediate fitness cannot be resolved based on DcmA activity.



SI Figure 5: Neither growth rate in media containing high external chloride concentrations nor growth rate in media buffered to low pH are predictive of growth with DCM. (A) The ratio of growth rates when grown with 100 mM NaCl compared to unmodified M-PIPES+succinate does not predict the competitive fitness on DCM. (B) The ratio of growth rates when grown in M-PIPES+succinate at pH 5.9 compared to pH 6.7 is similarly ineffective at predicting competitive fitness on DCM.



SI Figure 6: Calibration curve for the intracellular pH biosensor. A pH-insensitive red fluorescent protein is translationally fused to a pH-sensitive green fluorescent protein. The ratio of green to red fluorescence is indicative of the intracellular pH. For calibration, cells expressing the biosensor construct were incubated in buffer of the indicated pH containing valinomycin and nigericin prior to measurement by flow cytometry. Error bars show one standard deviation calculated from three replicates.



SI Figure 7: The maximum decrease in intracellular pH on addition of 5 mM is not predictive of competitive fitness on DCM. From each of the curves in Figure 4, the lowest intracellular pH measurement was taken as representative of the strain's ability to maintain its intracellular pH under a DCM challenge.