IDENTIFICATION OF DIVERSE CARBON UTILIZATION PATHWAYS IN *SHEWANELLA ONEIDENSIS* MR-1 VIA EXPRESSION PROFILING

MICHAEL E. DRISCOLL[1] mdriscol@bu.edu
MARGIE F. ROMINE[2] margie.romine@pnl.gov
FRANK S. JUHN[3] frnkjn@bu.edu
MARGRETHE H. SERRES[4] mserres@mbl.edu
LEE ANNE MCCUE[2] leeann.mccue@pnl.gov
ALEX S. BELIAEV[2] alex.beliaev@pnl.gov
JAMES K. FREDRICKSON[2] jim.fredrickson@pnl.gov
TIMOTHY S. GARDNER[1,3] tgardner@bu.edu

[1] *Bioinformatics Program, Boston University, St. Boston, MA 02215, USA*
[2] *Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA*
[3] *Department of Biomedical Engineering, Boston University, Boston, MA 02215, USA*
[4] *Josephine Bay Paul Center , Marine Biological Laboratory, Woods Hole, MA, USA*

To identify pathways of carbon utilization in the metal-reducing marine bacterium *Shewanella oneidensis* MR-1, we assayed the expression of cells grown with various carbon sources using a high-density oligonucleotide Affymetrix microarray. Our expression profiles reveal genes and regulatory mechanisms which govern the sensing, import, and utilization of the nucleoside inosine, the chitin monomer N-acetylglucosamine, and a casein-derived mixture of amino acids. Our analysis suggests a prominent role for the pentose-phosphate and Entner-Doudoroff pathways in energy metabolism, and regulatory coupling between carbon catabolism and electron acceptor pathways. In sum, these results indicate that *S. oneidensis* possesses a broader capacity for carbon utilization than previously reported, a view with implications for optimizing its role in microbial fuel cell and bioremediative applications.

Keywords: *Shewanella*; microarray; carbon metabolism.

1. Introduction

Shewanella oneidensis MR-1 is an environmentally ubiquitous, metabolically versatile gamma-proteobacteria with a broad capacity for the respiration of metals. *Shewanella*'s ability to shuttle electrons onto metals – including arsenic and uranium – has made it a model organism for use in microbial fuel cells and environmental remediation applications [10, 17, 23]. In contrast to its well-known utilization of electron acceptors, *Shewanella* has been considered to possess a relatively narrow capacity for utilizing electron donors, preferring simple carbon compounds such as formate, lactate, and other fermentative end products [24]. However, comparative sequence analysis, phenotypic assays, and recent experimental work have suggested that more complex carbon compounds may also drive respiration in this organism [29, 33, 36].

To investigate the pathways involved in the electron donor metabolism of *S. oneidensis*, we measured cell growth and performed whole-genome expression profiling using five substrates as both carbon source and electron donor: a casein-derived mixture of amino acids, the nucleoside inosine, the amino sugar N-acetylglucosamine, and the carboxylic acids pyruvate and lactate. These substrates were chosen because they support robust growth of *S. oneidensis* in the laboratory and are known to exist in the natural environments that *Shewanella* species occupy. N-acetylglucosamine is a monomer of chitin, which is among the largest stores of dissolved organic carbon in the oceans [27]. Free amino acids and inosine are major byproducts in the post-mortem breakdown of marine vertebrate tissue [7, 30]. Finally, lactate is a fermentative end product common to the natural sediments from which *Shewanella* has been isolated, and pyruvate is chemically similar to lactate.

Here we describe expression of chemotactic, transport, and catabolic genes involved in the utilization of these carbon sources, show evidence of transcription factors regulating these genes, and identify the likely metabolic pathways by which these sources are coupled to energy production in the cell.

2. Materials and Methods

2.1. *Experimental Design*

Our experimental design consisted of a two-way layout testing five carbon sources with varying salt and H2O2 levels in a defined minimal medium (Fig. 1C). For each of the carbon sources investigated, three samples were collected. The salt and H2O2 conditions were chosen at levels which did not significantly diminish growth (data not shown), but for which we might be able to detect changes via expression signatures. This two-way layout allowed for the multiple observations of experimental factors without the explicit use of technical replicates.

2.2. *Cell Culture & RNA Isolation*

For RNA samples, *S. oneidensis* MR-1 cells (ATCC strain 700550) were grown overnight to high density in Luria Broth, then washed and diluted to a starting inocolum (OD600 0.05) in a condition-specific minimal media. Cells were then grown for an additional thirty hours at 30 C to achieve sufficient biomass and RNA was extracted. To minimize the effects of metabolic waste products, cells were centrifuged at 1350g for 10 minutes at 4 C, washed, and resuspended in fresh condition-specific media after 12 and 24 hours of growth. Due to differences in growth rates on the carbon sources used, final biomass yields ranged widely (OD600s: inosine 0.22 +/-0.05; N-acetylglucosamine 0.55 +/- 0.02; Casamino 1.95 +/-0.05; lactate 0.38 +/-0.28; pyruvate 0.41 +/-0.15).

The media used for each of the conditions studied was composed of one of five carbon sources (7.5 mM inosine, 7.5 mM N-acetylglucosamine, 0.2% Casamino acids (Difco Laboratories, P/N 223120), 15 mM lactate, or 15 mM pyruvate) crossed with a salt

or peroxide treatment (200mM NaCl or 10uM H2O2), in a background of modified M1 media (3 mM PIPES, 28 mM NH4Cl, 1.3 mM KCl, 4.4mM NaH2PO4-H2O), with micromolar supplements of amino acids, mineral, and vitamins (Table S2). Aeration was achieved via shaking at 220 RPM, and while tests with the oxygen-sensitive dye resazurin indicated presence of oxygen in all wells, the low surface area to volume ratio of our microplates (Whatman, USA, PN 7701-6102) suggests that our cultures were oxygen-limited.

RNA extraction was performed by suspending cells in RNAProtect, followed by isolation using RNEasy spin columns (Qiagen Inc, Valencia, CA), which included an on-column DNAse treatment step to reduce genomic contamination. RNA yields were estimated from UV 260nm/280nm absorbance ratios and normalized to 10ug for all samples. Reverse transcription of RNA to cDNA, cDNA fragmentation and labeling, hybridization to Affymetrix microarrays followed the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). All cell culturing, RNA isolation, and array hybridization steps were performed in parallel, and scanning of hybridized microarrays was performed on the same scanner.

2.3. *Microarray Design & Expression Data Analysis*

The custom microarray we developed for this study is a high-density array manufactured by Affymetrix (Santa Clara, CA) containing 103,797 pairs of perfect-match and single-mismatch 25-mer oligonucleotide probes with a feature size of 11um [16]. provided our chip design file (CDF), which describes array coordinates for probes and their genome targets, in the Supplementary Material.

Probe-level intensity values from Affymetrix CEL files were processed using the RMA algorithm to compute gene expression values [2]. For a given condition, a gene's mean expression was compared against a background mean of all other conditions. Using this difference of means, along with a pooled estimate of variance, the significance of up or down regulation (expressed as P values) was computed according to a two-sample T test statistic. This was repeated for all conditions.

2.4. *Motif Alignment*

Motif discovery was performed by aligning upstream regions of selected genes using the MEME algorithm as implemented by the Microbes Online web site (http://www.microbesonline.org). Visualization of the identified motifs was performed using WebLogo (http://weblogo.berkeley.edu).

3. Results

3.1. *Use of Pentose Phosphate and Entner-Doudoroff Pathways for Growth on Inosine*

The nucleosides adenosine, inosine, and uridine have been identified as the top three most "electrogenic" carbon sources for *S. oneidensis*, on the basis of NADH-

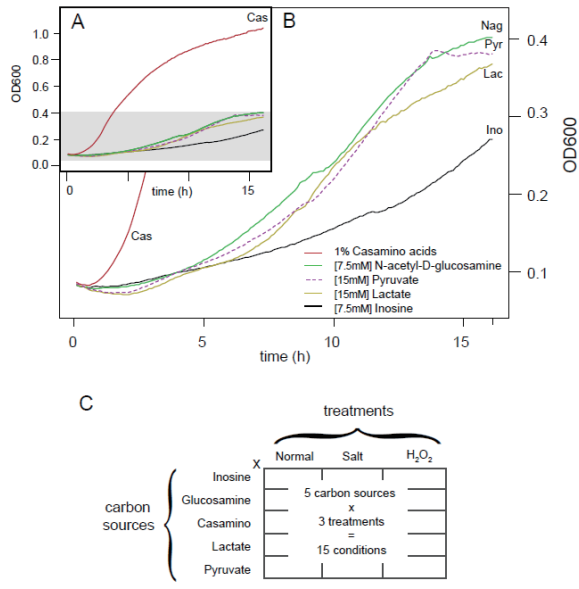

Fig. 1. Sixteen-hour growth profiles for carbon sources, shown at two scales. (A) Growth of Casamino acids (1% or 10 mg/mL) is highlighted in red, showing its rapid growth rate as compared with other carbon sources studied. These slower curves are expanded in panel (B) to resolve the growth rates of N-acetylglucosamine, pyruvate, lactate, and inosine, at concentrations as labeled. Cells were inoculated from overnight LB cultures into a minimal modified M1 media with carbon sources as described in Materials and Methods, and grown in 24-well plates in an aerated, incubated plate reader (Biotek P/N SIAFRTD) at 30 C, with optical density readings (OD600) taken at six-minute intervals. (C) Illustration of the two-way layout experimental design that was used for the fifteen microarrays used in this study.

sensitive dyes which detect electron transport [33]. In this assay, a carbon source is considered more "electrogenic" if its utilization results in more electrons being routed down its electron transport chain.

Previous work has also demonstrated growth on inosine and ribose in other *Shewanella* species [15, 24, 37]. Our growth profiles confirm that inosine supports growth as the sole carbon source in *S. oneidensis*, with a growth rate approximately half that of lactate, the fourth most electrogenic carbon source, at comparable molarities (Fig. 1).

Based on our expression profiles, *S. oneidensis* appears to drive energy production from inosine in several steps: sensing and transport into the cell, release of its ribose base, conversion of ribose to hexose via the pentose phosphate pathway, and finally, cleavage by the Entner-Doudoroff enzymes to generate a triose phosphate

and pyruvate for central metabolism (Fig. 2).

The use of the pentose phosphate cycle for non-oxidative conversion of ribose to hexose is a common strategy in environmental microorganisms [3]. The activity of pentose-phosphate and Entner-Doudoroff pathway enzymes in *S. oneidensis* extracts has previously been demonstrated [22], and recent 13C labeling experiments have established significant fluxes through these pathways during growth on lactate [31], both under aerobic conditions. Our microarray profiles show high constitutive expression of pentose-phosphate pathway enzymes during all growth conditions we assayed (Fig. 2), though no significant differential expression during inosine growth.

In the Entner-Doudoroff pathway, we observed significant upregulation of both the *eda* and *edd* genes, part of the *zwf-pgl-edd-eda* operon (SO2489-SO2486) encoding enzymes for conversion of glucose-6-phosphate to pyruvate and glyceraldehyde-3-phosphate. These products form precursors that can flow into central energy metabolism or be used a substrates for direct generation of NADH via dehydrogenases.

In addition to the activation of these catabolic pathways, growth with inosine also results in differential regulation of several genes involved in nucleotide biosynthesis. For example, the *nrdAB* locus, encoding an aerobic ribonucleoside-diphosphate reductase, which converts nucleotides to deoxynucleotides, showed significant upregulation, reflecting greater flux through this conversion pathway (Table S1). Conversely, six of the ten genes annotated as being in the de novo purine synthesis pathway were significantly repressed (Fig. 2B).

In *E.coli* the purine synthesis pathway is controlled by the transcriptional regulator PurR [20], for which no homolog exists in *S. oneidensis* or in any of the twelve sequenced *Shewanella* genomes currently available (http://img.jgi.doe.gov). Here, as with several of the other pathways discussed, *cis*-regulatory sequences of the top differentially expressed genes were aligned, but no significant motifs were detected. The coordinated repression of these six genes, which are spread across five distinct loci on the genome, could be effected through an as yet unidentified transcription factor, or via a small RNA mechanism (antisense or riboswitch).

3.2. *Growth on N-acetylglucosamine and Chitin-Related Pathways*

In the natural aquatic environments where N-acetylglucosamine is found, marine bacteria are thought to coordinate several activities to facilitate its catabolism: chemotaxis and adhesion to chitin, breakdown into N-acetylglucosamine, transport into the cell, and finally conversion to fructose [14]. Our expression profiles of *S. oneidensis* grown with N-acetylglucosamine indicate several transcriptional programs which activate these cellular functions.

The most significant transcriptional response to growth on N-acetylglucosamine in *S. oneidensis* occurs in the neighborhood of the *nag* operon, encompassing a set of eleven adjacent genes SO3514 thru SO3503 (Fig. 3B). Recent analysis has

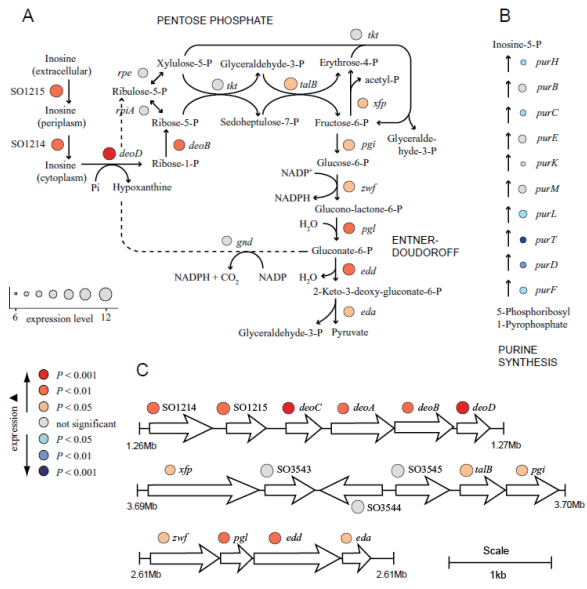

Fig. 2. Expression of enzymes involved in pathways related to inosine metabolism. Red and blue colors indicate up and downregulation, respectively, during growth with inosine; circle circumference represents absolute levels of expression. (A) A liberated ribose moiety from inosine is thought to be converted to fructose-6-phosphate by the non-oxidative branch of the pentose phosphate cycle, and fed into the Entner-Doudoroff pathway. (B) Repression of genes involved in the first steps of purine synthesis, for which inosine-5-phosphate is a key intermediate; for brevity, not all metabolites are shown. (C) Expression of three key gene neighborhoods in their genomic contexts. Refer to Table S1 for full gene descriptions, Enzyme Commission (EC) numbers, and detailed data for these genes.

suggested that this cluster contains the major components of *S. oneidensis* MR-1's chitin metabolism: specifically, these genes encode two permeases specific for chitin oligosaccharides, a chemotactic-response protein, and enzymes involved in converting N-acetylglucosamine into fructose [36]. The extraordinarily high level of expression detected for SO3514 (Fig. 3A and Table S1), the TonB-dependent outer membrane transporter, indicates the highly specific regulation and functional importance of its encoded protein.

The *nag* locus does not, however, contain any genes which govern adhesion and attachment of cells to chitin surfaces, which has been described as a central part of the chitin utilization program in *Vibrio cholerae* [19]. *S. oneidensis* appears to modulate such a response via a locus of five genes (SO0854 thru SO0850) that are coordinately upregulated during growth on N-acetylglucosamine (Fig. 3). Existing annotations for this locus indicate the presence of type IV pili biogenesis domains in SO0853, SO0852, and SO0851, and the presence of multiple repeats characteristic

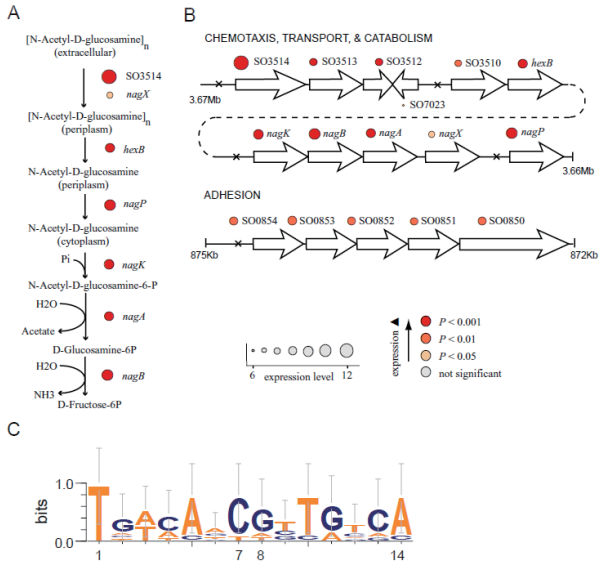

Fig. 3. Expression of enzymes involved in pathways related to N-acetylglucosmaine metabolism. Red and blue colors indicate up and downregulation, respectively, during growth with inosine; circle circumference represents absolute levels of expression. (A) Expression of chemotaxis, transport, catabolic, and adhesion pathways for N-acetylglucosamine. (B) Genomic context of the *nag* operon (top) and the cluster of type IV pili proteins, SO0854-SO0850 (bottom) implicated for a role for attachment to chitin. Black crosses indicate presence of the conserved binding motif described in (C). (C) The 14-base long *cis*-regulatory motif which was found by aligning promoters from the top 15 N-acetylglucosamine upregulated genes, consisting of two palindromic seven-base halves.

of adhesins in SO0850. We therefore suggest that these genes are involved in the assembly and extension of type IV pili for adhesion onto chitin and chitin-derived oligosaccharides.

To investigate whether these two gene neighborhoods are modulated by a common transcriptional regulator we aligned the promoter sequences of the top 15 most significantly overexpressed genes, which included all but one of the 11 nag neighborhood of genes and three of the five type IV pili genes (all P values < 0.01). This alignment revealed a statistically significant 14-base long motif (P value < 0.001) present at six distinct promoter sites, consistent with a motif previously inferred by [36] (Fig. 3C).

Coordinated changes in gene expression in response to external stimuli are typically mediated by two-component systems in bacteria [13], and it is possible that this detected motif may be a target of such a response regulator. Based on its proximity to the *nag* operon, and its homology with a LacI family of regulators, the gene

SO3516 was previously denoted as *nagR* and proposed to encode for a repressor of N-acetylglucosamine metabolic genes [36]. However, though this gene was expressed above background, it did not show a significant change in expression during growth with N-acetylglucosamine (Table S3), suggesting that if SO3516 is a regulator of the *nag* operon, its activation may be mediated non-transcriptionally via allosteric binding of substrate.

3.3. *Biosynthetic and Degradative Pathways Induced by Growth with Amino Acids*

Growth of *S. oneidensis* using amino acids as the sole carbon source has been previously reported [28], and a recently published sequence analysis described several likely pathways for their utilization [29]. Our growth profiles reveal growth rates exceeding all that of the other four carbon sources used in this study (Fig. 1).

Amino acid metabolism is tightly controlled in the cell, and the regulation of transcription represents just one of several modes of control. During growth with amino acids, over 5% of the genome (238 genes) were significantly changed (P value $<$ 0.05).

Here we focus on utilization of the amino acid valine which, by way of having distinct pathways for its degradation and synthesis, offers a clear example of the inverse regulation of these processes. Pathways for valine metabolism share enzymes with those for the other branched-chain amino acids isoleucine and leucine. With respect to valine, we observed three trends: the differential regulation of genes involved in passive and active transport, upregulation of degradative enzymes, and repression of synthesis enzymes.

For transport, two APC Superfamily amino acid transporters (SO0313, SO4565), and an AzlCD-like branched chain amino acid transporter (SO1759, SO1760) were found to be upregulated during growth with amino acids. In addition, we observed repression of an operon annotated to encode an ABC arginine transporter complex (SO1042-SO1044), as well as downregulation of two cation:alanine/glycine transporters of the AGCS family (SO3063, SO3541). The repression of these latter two active transport mechanisms suggests that when concentrations of amino acids are high outside the cell, passive channels may be the primary mode of uptake.

Enzymes involved in the degradation of valine were strongly induced in the presence of amino acids (Fig. 4). The first step of valine catalysis, the removal of the amino group from the amino acids, appears to be effected by a leucine dehydrogenase (SO2638) rather than an aminotransferase as previously predicted [29]. Leucine dehydrogenase is also known to act on isoleucine and valine. New assignments were also made to intermediary steps in the isoleucine and valine degradation pathways based on significant increases in the expression of adjacently located genes (SO1677-SO1683).

The synthesis pathway of valine comprises several multi-component enzymes which catalyze the steps of its formation from pyruvate. As indicated in Fig. 4, these en-

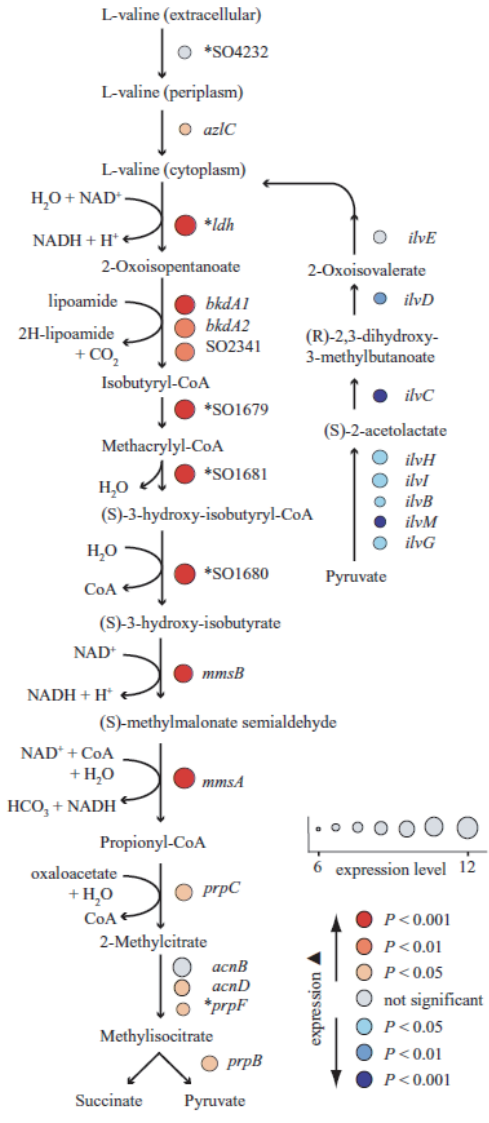

Fig. 4. Differential induction of degradative enzymes and concomitant repression of the biosynthetic pathway for L-valine during growth with an amino acid mixture. Red and blue colors indicate up and downregulation, respectively; circle circumference represents absolute levels of expression. Refer to Table S1 for full gene descriptions, Enzyme Commission (EC) numbers, and detailed data for these genes.

zymes are significantly repressed during growth with amino acids. As with degradative pathways described above, the enzymes of the valine synthesis pathways overlap considerably with those of isoleucine and valine. Thus the observed repression of these enzymes may be due, in sum or in part, to the presence of any of the branched chain amino acids in our amino acid mixture.

4. Discussion

Shewanella's metabolic versatility reflects its diverse environmental ecology. In recent years, *Shewanella* species have been isolated from fresh water and marine sediments around the world, surface waters of the Sargasso sea, hydrothermal vents of the deep Pacific, mollusks and spoiling fish [9, 24, 25, 34, 37]. Reflecting its aquatic lifestyle, many of the preferred electron donors and acceptors for *S. oneidensis* are organic breakdown products of marine tissues and minerals abundant in marine water and sediments.

To successfully compete across these different niches and in shifting nutritive environments, *Shewanella* species must be catabolic and respiratory generalists. For example, *S. oneidensis*' capacity to utilize carbon sources such as inosine and N-acetylglucosamine as electron donors may be an infrequently utilized but beneficial trait, which confers a competitive advantage over other niche organisms.

The pathways and regulation of carbon metabolism in environmental organisms such as *S. oneidensis* differ in several ways from the canonical models derived from *E.coli* and *B. subtilis*. The pentose phosphate and Entner-Doudoroff pathways, for example, appear to play a key role in sugar catabolism, as suggested here and in a recent study of seven phylogenetically diverse bacteria [8]. In addition, the absence of homologs for several transcription factors governing central intermediary metabolism – such the purine biosynthesis repressor PurR – imply that alternative regulatory mechanisms have yet to be discovered. Additional isotopic labeling experiments and further whole-genome expression assays will be necessary to close these and other gaps in our understanding of the metabolic and regulatory networks of *S. oneidensis*.

The interaction of carbon catabolism and respiratory pathways may also impact the efficency and rate of energy conservation in cells. Utilization of some carbon sources can yield more ATP per substrate molecule, but result in slower growth rates [26]. This may partially explain why inosine, the most electrogenic carbon source known for *S. oneidensis*, has the slowest growth kinetics of the carbon sources we tested. How carbon sources regulate the bioenergetic strategies cells use, and how these strategies influence an organism's competitive fitness, are questions that merit further study.

While terminal electron acceptor pathways have been a dominant focus of study to date in *S. oneidensis*, an understanding of carbon metabolism is equally important in defining its ecophysiology. Moreover, the existence of regulatory coupling between catabolic and respiratory pathways suggests novel approaches for optimization of *Shewanella* and other metal-reducing bacteria in microbial fuel cells and bioremedial

applications.

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