

1 **Large scale comparative phenotypic and genomic analyses reveal ecological**
2 **preferences of *Shewanella* species and identify metabolic pathways conserved at**
3 **genus level**

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1 **ABSTRACT**

2 The use of comparative genomics among different microbiological species has
3 increased substantially as sequence technologies become more affordable. However,
4 efforts to fully link a genotype to its phenotype remain limited to the development of one
5 mutant at the time. In this study, we provide a high throughput alternative to this limiting
6 step by coupling comparative genomics to phenotype arrays for five sequenced
7 *Shewanella* strains. Positive phenotypes were obtained for 441 nutrients (C, N, P, and S
8 sources), with N-based compounds being the most utilized for all strains. Many genes
9 and pathways predicted by genome analyses were confirmed with the comparative
10 phenotype assay, and three degradation pathways believed to be missing in *Shewanella*
11 were confirmed. A number of previously unknown gene products were predicted to be
12 part of pathways or to have a function, expanding the number of gene targets for future
13 genetic analyses. Ecologically, the comparative high throughput phenotype analysis
14 provided insights into niche specialization within the five different strains. For example,
15 *Shewanella amazonensis* strain SB2B, isolated from the Amazon River delta, was
16 capable of utilizing 60 C compounds, whereas *Shewanella* sp. strain W3-18-1, from the
17 deep marine sediment, utilized only 25 of them. In spite of the large number of nutrient
18 sources yielding positive results, our study indicated that except for the N-sources they
19 were not sufficiently informative to predict growth phenotypes from increasing
20 evolutionary distances. Our results indicate the importance of phenotypic evaluation for
21 confirming genome predictions. This strategy will accelerate the functional discovery of
22 genes and provide an ecological framework for microbial genome sequencing projects.

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1 INTRODUCTION

2 The *Shewanella* genus is composed of facultative anaerobic bacteria known for
3 their distinctive capability of utilizing a variety of electron acceptors such as NO_3^- , U, Cr,
4 Tc, Pu, and nitroaromatic compounds (13). Members of this genus have also being
5 regarded for their role as drivers of global biogeochemical cycles of C, N, and S in redox
6 interfaces of marine environments (3, 28).

7 Being found in different environments such as salt and fresh waters, sediments,
8 and subsurface formations, it is not surprising that the genus *Shewanella* developed its
9 hallmark respiratory capability of utilizing many different electron acceptors. This
10 diversity of respiratory phenotypes is a reflection of the genetic makeup carried by
11 members of this genus. The sequenced genome of the *Shewanella oneidensis* strain
12 MR-1 (MR-1) showed a large percentage of genes dedicated to the cell's electron
13 transport system including cytochromes, reductases, iron-sulfur proteins, and quinones
14 (12). As revealed by the genome sequencing of 22 additional *Shewanella* species and
15 strains of the same species (10), the genetic diversity carried out by this genus is
16 significant, with less than half of the genes being shared among ten of the sequenced
17 *Shewanella* genomes (21).

18 Recently, several studies have used comparative genomics to systematize the
19 genomic content into two groups: the core genome comprised of genes present in all
20 strains and the accessory genome consisting of unique or strain-specific genes (21, 39).
21 This approach has allowed for putative determination of the total number of genes and
22 operons that might be involved in the ecological fitness of strains subjected to a specific
23 environmental condition (18, 19, 24, 33). It is, however, less clear how this genomic
24 diversity is translated into phenotypic traits and what their implications are to the
25 ecological success of the species. Traditionally, a particular genotype has been linked to
26 a phenotype through the development and characterization of mutants (23). Based on

1 the 862 genes (19.2%) that still remain to be characterized in the genome of the model
2 microorganism *Escherichia coli* strain K12 (36), the above procedure is not only a labor
3 intensive, but also a time-consuming activity.

4 High-throughput phenotype arrays can be used as an alternative approach to
5 expedite the functional characterization of genes. The Biolog assay uses tetrazolium
6 violet to monitor cell respiration, assuming that oxidation of the nutrient source will lead
7 to respiration and, hence to purple dye formation (1). High-throughput phenotype arrays
8 have been extensively used to characterize knockout mutants of single microorganisms
9 (15, 43), but have yet to be tested for comparative analysis of phenotypes in light of
10 genome sequence data (2).

11 In this study, we sought to gain access to the ecology of members of the
12 *Shewanella* genus through a large-scale comparative analysis of phenotypes. Here, we
13 took advantage of five fully sequenced *Shewanella* genomes and compared them to
14 high-throughput phenotype arrays containing 561 nutrient sources. We established
15 genotype-phenotype relationships, expanded the number of genes associated with
16 specific phenotypes, and showed there is a limit in predicting phenotypes with increased
17 phylogenetic distances.

18 19 **MATERIAL AND METHODS**

20 **Strains used in this study.** Microorganisms and their genome accession numbers (in
21 parenthesis) used in this study were *Shewanella oneidensis* strain MR-1 (AE014299,
22 AE14300), *Shewanella* sp. strain MR-4 (CP000446), *Shewanella* sp. strain MR-7
23 (CP000444, CP000445), *Shewanella* sp. strain W3-18-1 (CP000503), and *Shewanella*
24 *amazonensis* strain SB2B (CP000507). Strain selection was based on the following: (1)
25 representation of an evolutionary gradient with strains of the same species and different

1 species, (2) genomes that were curated manually. A description of habitat conditions at
2 the time of sampling is presented in Table 1.

3 **High-throughput phenotypic comparisons.** Phenotype microarray assays were
4 performed at Biolog Inc. (Hayward, CA) as previously described (1). Briefly, *Shewanella*
5 strains were grown on R2A plates and incubated overnight at 22°C. Colony swabs were
6 used to suspend cells in IF-0 GN medium and diluted until 85% of transmittance of the
7 cell suspension was achieved. A volume of 100 µl was added to each plate well. Plates
8 were incubated in the OmniLog reader at 22°C. Monitoring of color change was recorded
9 for 48h for all plates. Only positive results for three replicate plates were reported. For
10 the purpose of this work, a nutrient was defined as being used as the sole source for the
11 element, e.g. when glycine was defined as a N source, another C source was provided
12 in the well to be oxidized, providing electrons for the dye reduction.

13

14 **Gene analysis and pathway reconstructions.** Protein sequences encoded by the five
15 *Shewanella* genomes were analyzed and compared. Function predictions for the gene
16 products were obtained from the *Shewanella* Knowledgebase (17). Many of these
17 predictions resulted from careful manual curation as previously described (17, 35, 37).
18 Further, the gene products were assigned to metabolic pathways according to the
19 MetaCyc schema (5) and the primary literature. In addition, we made use of the
20 orthologous relationships previously determined for ten *Shewanella* strains, including the
21 five strains used in our analysis (21). The sets of orthologs were identified based on their
22 sequence similarity and genome neighborhoods and included genes present in one or
23 more of the *Shewanella* genomes. The cut-off criteria for gene presence or absence was
24 compiled from three different methods: (1) protein-protein pair-wise reciprocal BLAST-P,
25 (2) Pair-wise alignments with PAM score of 100, and (3) reciprocal tBLAST-N (21). The
26 ortholog table for the five *Shewanella* strains is available in Supplementary Material ST2.

1 Metabolic pathway predictions and the complete *Shewanella* ortholog dataset are also
2 available through the *Shewanella* Knowledgebase (17).

3 **Phenotypic and genome clustering analyses.** Data analysis was performed using the
4 kinetic and parametric modules of the software OmniLog v1.2. Tested compounds
5 yielding negative or not reproducible results for all *Shewanella* strains were removed
6 from the analysis. Results from three replicates were averaged and subtracted from
7 control intensity value. Next, substrates with intensity values below the threshold of 10
8 were discarded as a conservative measure for respiration. Phenotypic profiles were
9 converted into a matrix using the following parameters: 1 for values below 50, 2 for
10 values between 50 and 100, and 3 for values above 100. Clustering was performed with
11 the Cluster 3.0 software (6), using the single linkage and Euclidean distance.

12 Genomic clustering for all five strains was calculated based on gene orthologs
13 identified in the genomes. When a gene ortholog was observed in the genome, it was
14 scored as present (one in the matrix), whereas a gene not observed was scored as
15 absent (zero in the matrix). Clustering analysis was carried out using a single linkage
16 scheme and Euclidean distance. Pair-wise average nucleotide identity (%ANI) values
17 between genomes were calculated according to Konstantinidis and Tiedje (20).

18

19 **RESULTS**

20 **Large scale comparative phenotypic analysis.** Five *Shewanella* strains were tested
21 for metabolic diversity with 561 sources of carbon (C), nitrogen (N), sulfur (S), or
22 phosphorus (P). Positive phenotypes for at least one strain were observed for a total of
23 441 sources (Supplemental Material SF1). Among the different substrates tested, 55
24 (92%) of the P, 293 (77%) of the N, 21 (58%) of S, and 67 (35%) of the C sources were
25 utilized by one or more of the strains. The majority of the nutrient sources were used by

1 2-4 of the strains with only 15% and 16% being utilized by all strains or by one of the
2 strains, respectively.

3

4 **Carbon utilization patterns.** Only 15 of the 190 carbon sources tested were utilized by
5 all five strains (Fig. 1A). Growth on six of these substrates (L-lactate, adenosine, inosine,
6 pyruvate, and N-acetyl-D-glucosamine) has been confirmed in other studies (7, 31, 42).
7 The remaining compounds (2'-deoxyadenosine, methyl-pyruvate, uridine, Tween 80,
8 Tween 40, Tween 20, gelatin, and three dipeptides) have not been shown to support
9 growth of the five *Shewanella* strains and remain targets for further experimental studies.
10 Hydrolyses of gelatin and Tween detergents have been previously reported for *S. loihica*
11 strain PV-4 (11) and *S. affinis* strains KMM 3586 and KMM 3587 (14), respectively, and
12 it is possible that these phenotypes are shared by the entire genus. The three di-
13 peptides utilized by all strains had an N-terminal glycine (Gly-Glu, Gly-Pro, Gly-Asp). A
14 fourth dipeptide with a C-terminal glycine (Ala-Gly) was only degraded by SB2B
15 suggesting the need for a separate transporter or peptidase to utilize this dipeptide. The
16 majority of the substrates degraded by all strains enter the central metabolism at the 2 or
17 3 carbon compound level (Fig. 2). This agrees with earlier predictions made in an
18 analysis of the *S. oneidensis* MR-1 (MR-1) genome (37) where many of these pathways
19 were outlined. It was also noted that MR-1 contained a smaller number of iso-enzymes
20 for the degradation of 5 and 6 carbon compounds compared to *Escherichia coli*, an
21 organism that is capable of using a plethora of 5 and 6 carbon compounds. Instead MR-
22 1 was found to encode iso-enzymes for the utilization of 3 carbon compounds, i.e. 3
23 glyceraldehyde dehydrogenases. *Escherichia coli* also contains over 40 PTS systems for
24 import of 4 – 6 carbon carbohydrates while MR-1 only has one such system. These
25 trends hold up for the five *Shewanellas* included in this study. An additional mannose

1 specific PTS system was found on a mobile island in *Shewanella* sp. strain W3-18-1
2 (W3-18-1 from here on).

3 We observed a variation in the growth phenotype for sugar and polysaccharide
4 utilization where each substrate was degraded by one to three strains. Several of the
5 sugars tested included dimers or polymers of glucose subunits (i.e. α -, β -, and γ -
6 cyclodextrin, dextrin, maltose, maltotriose, and sucrose). Since all of the glucose
7 multimers were utilized by *Shewanella amazonensis* SB2B (SB2B), *Shewanella* sp. MR-
8 4 (MR-4) and *Shewanella* sp. MR-7 (MR-7), and not by W3-18-1 or MR-1, it is likely that
9 some of the same enzymes (pathways) and transporters are shared by different strains
10 and involved in the degradation of the above compounds.

11 SB2B showed the most phenotypic versatility for carbon sources when compared
12 to the other species. This strain was able to utilize 60 different substrates, while strain
13 W3-18-1 used only 25. The limited substrate utilization by W3-18-1 includes its inability
14 to utilize the many glucose-based multimers as well as several amino acids. In fact,
15 none of the single amino acids tested were degraded by this strain. When the two most
16 evolutionarily related strains isolated from the Black Sea water column were compared,
17 namely strain MR-4 and MR-7, their carbon utilization pattern showed larger than
18 expected differences. Both strains shared the ability to metabolize 31 carbon substrates
19 with an additional 13 sources being solely utilized by strain MR-4 while strain MR-7 used
20 another 6 substrates. MR-4 and MR-7 have an average nucleotide identity (ANI) of 97%
21 suggesting that they belong to the same species.

22

23 **Nitrogen utilization patterns.** A total of 50 nitrogen sources were utilized by all five
24 strains (a subset of sources is presented on Fig. 1B, Supplementary Material SF1).
25 These substrates were all dipeptides with pronounced preference for amino acids with
26 polar uncharged side chains: serine, threonine, asparagine, and glutamine. Amino acids

1 with electrically charged side chains (positive or negative) or hydrophobic side chains
2 were of limited use for these strains, with exception of alanine and leucine. When
3 analyzing for nitrogen substrates utilized by all *Shewanella* strains but one, the number
4 of substrates increased to 126, with the inclusion of purine bases such as xanthine and
5 adenine.

6 The Black Sea isolates, MR-4 and MR-7, were able to utilize 285 and 242
7 substrates, respectively; followed by strain MR-1 with a pattern of substrate utilization
8 corresponding to 203 nitrogen sources. Inversely proportional to the carbon utilization
9 profile, strain SB2B had the lowest number of positive Biolog phenotypes (81) for
10 nitrogen sources. The low N-source diversity displayed by SB2B reflects its inability to
11 utilize 194 (71%) of the dipeptides and tripeptides, and these made up 89% of the N-
12 sources tested. Furthermore, the only amino acids or amines utilized by SB2B were L-
13 tyrosine and N-acetyl glucosamine.

14

15 **Phosphorus and sulfur utilization patterns.** Adenosine 5'-monophosphate (AMP) was
16 the sole phosphorus source utilized by all five strains. Of the 55 phosphorus compounds
17 degraded by *Shewanella*, 39 were only utilized by MR-7 and MR-1. In fact, these two
18 strains yielded a larger number of positive phenotypes for both the phosphate and sulfur
19 sources tested. MR-7 was able to utilize 52 phosphate- and 15 sulfur- sources, and
20 strain MR-1 used 48 and 19 substrates, respectively (Fig. 1C and 1D). The remaining
21 strains degraded a very limited number of phosphate substrates besides AMP; W3-18-1
22 utilized TMP, AMP, GMP, CMP, UMP, pyrophosphate; while MR-4 utilized
23 phosphogluconic acid and carbamylphosphate. Strain SB2B was able to utilize only TMP
24 in addition to AMP (Fig. 1C). While W-3-18-1 tested positive for 3 of the sulfur
25 compounds (L-cysteine sulfinic acid, glutathione, Glu-Met), MR-4 and SB2B did not
26 utilize any sulfur compounds under the conditions tested (Fig. 1D).

1

2 **Smaller scale comparisons of biolog phenotypes.** We sought to explain some of the
3 Biolog phenotypes from known phenotypes, genome contents, and orthologous
4 relationships of the encoded genes (Supplementary Material ST1). The utilization of N-
5 acetyl-D-glucosamine as a carbon source agrees with the published literature (42).
6 Lactate and pyruvate degradation via acetyl-CoA to acetate or the TCA cycle has been
7 shown with growth experiments (31). Also, the use of nucleotides and nucleosides,
8 including adenosine and inosine, has previously been shown for MR-1 (7), and can be
9 inferred for the other strains based on the presence of genes encoding for the
10 degradation enzymes.

11 According to the high throughput phenotype assay, MR-1 was the only strain
12 unable to utilize the C4 dicarboxylates L-malate, succinate, and fumarate. When these
13 results were observed in light of the genome sequences, we found that the gene
14 encoding the dicarboxylate carrier AbgT was absent from MR-1, but present in the other
15 strains (MR4_3833/MR7_3926/W3181_3971/Sama_3559). Furthermore, an
16 oxaloacetate decarboxylase was absent in MR-1 and present in the other strains
17 (MR4_2984-7/MR7_3066-9/W3181_3133-6/Sama_1054-1). This enzyme
18 decarboxylates oxaloacetate to pyruvate, and may be involved in the conversion of 4C
19 dicarboxylic acids via pyruvate and the gluconeogenesis pathway to 5C and 6C
20 essential metabolic intermediates. MR-1 also tested negative for L-arabinose utilization.
21 An arabinose (and arabinoside) metabolism locus, including a TonB arabinose receptor,
22 an ABC arabinose transporter, and enzymes degrading L-arabinose to xylose-5-
23 phosphate, is found in the genomes of the strains MR-4 (MR4_1977-2001), MR-7
24 (MR7_1997-1974), and W3-18-1 (W3181_1944-1966), but not in MR-1, also observed
25 by (34). Strain SB2B, also with an L-arabinose utilizing phenotype, did not contain the
26 above locus and may use another hitherto unknown pathway to degrade arabinose. Two

1 additional sugars, D-mannose and D-fructose, were utilized by MR-7 and SB2B. A locus
2 containing genes with similarity to the mannose utilization pathway is present in these
3 organisms (MR7_3383-8/Sama_0565-60) with a second mannose transporter and
4 utilization locus detected in SB2B (Sama_0303-4). While fructose can be degraded via
5 the mannose degradation pathway, a transporter specific for fructose was not identified,
6 agreeing with previous results (34).

7 In another example, N-acetyl-D-galactosamine was degraded by MR-4, MR-7,
8 and SB2B. This phenotype agrees with the presence of the *aga* operon for uptake and
9 degradation of N-acetyl-D-galactosamine in these strains (MR4_2530-6/MR7_2597-
10 2603/Sama_1199-3). The Biolog assay also showed degradation of citric acid by the
11 same three strains. Based on a recent paper describing citric acid utilization by
12 *Corynebacterium glutamicum* (4), we identified genes for a citrate sensing two-
13 component regulator and a three-component citrate transporter of the tricarboxylate
14 transporter (TTT) family in MR-4 and MR-7 (MR4_3099-5/MR7_0873-7). Genes
15 encoding these products were not found in the SB2B genome, suggesting that another
16 citrate utilization path was taken in this organism.

17

18 **Expanding testable predictions.** The high throughput genotype-phenotype comparison
19 among different strains allowed us to make targeted predictions for further experimental
20 analysis. For example, degradation of Tween 80 has been shown to involve an outer
21 membrane esterase in *Pseudomonas aeruginosa* (29). While no close homolog to the *P.*
22 *aeruginosa* enzyme was found in the 22 *Shewanella* genome sequences, we did identify
23 candidate genes present in the Tween degrading strains. Specifically, three candidate
24 genes encoding for a surface expressed lipase
25 (SO_2934/MR4_1469/MR7_1535/W3181_1613/Sama_2120), an outer membrane
26 phospholipase (SO_0428/MR4_0432/MR7_3595/W3181_0530/Sama_0379) and a cold-

1 adapted lipase (SO_1994/MR4_2269/MR7_2341/W3181_1692/Sama_2029) were
2 conserved among the strains. The latter gene encodes for a protein with sequence
3 similarity to a cold-active lipase isolated from a deep-sea sediment metagenome (16).
4 We also identified two proton-dependent (oligo)peptide transporters
5 (SO_0002/MR4_3938/MR7_4030/W3181_4066/Sama_2411 and
6 SO_3195/MR4_1313/MR7_1380/W3181_1459/Sama_2266) that might be associated
7 with the use of Gly-Glu, Gly-Pro, or Gly-Asp as carbon sources by *Shewanella*.

8 Phenotype assays yielded positive results for strains MR-1, MR-4, and SB2B
9 tested with the heteropolysaccharide pectin. We searched the genomes for genes
10 present in these three strains and absent in MR-7 and W3-18-1 genomes, and noted an
11 outer membrane Ton-B dependent receptor (SO_1822/MR4_2467/Sama_1252). The
12 involvement of TonB receptors in the uptake of sugars and their derivatives has been
13 shown for MR-1 (42). We also identified a gene coding for a sugar-binding periplasmic
14 protein that may be linked to the degradation of dextrans, maltose, and sucrose in MR-4
15 (MR4_0355), MR-7 (MR7_3671), and SB2B (Sama_3287).

16

17 **Linking genotypic and phenotypic changes.** To gain insight into the phenotypic
18 variation and its relationship to the genome, we plotted the evolutionary distance
19 (defined as the % average nucleotide identity (ANI)) among the five strains and the
20 percent of identical phenotypes (Fig. 3, Supplementary Materials ST2 and ST3). The %
21 rRNA similarity vs. % ANI regression line was selected to represent a conserved trait
22 (correlation coefficient $R^2 = 0.89$). The % conservation for orthologs was also included,
23 and it decreased more sharply with as the evolutionary distance increased ($R^2 = 0.76$).
24 We also observed a correlation between N source utilization and evolutionary distance
25 ($R^2 = 0.86$) (Fig 3). However, no such correlation was detected for the C source
26 utilization ($R^2 = 0.05$). Based on the clustering of the P and S utilization phenotypes (Fig.

1 1C and 1D), it was evident that neither of these phenotypes would be conserved
2 according to their evolutionary distance. Because the number of 294 phenotypes for N
3 sources was significantly higher than the other compounds (67 C, 55 P, and 21 S), we
4 selected sets of 60 N sources randomly to calculate the % similarity vs. % ANI in order
5 to test for sample size bias. These datasets also showed a decrease in % similarity with
6 increasing evolutionary distance suggesting that the N source phenotypic results are not
7 dependent on the number of chemicals compared (data not shown).

8 In order to test whether the observed phenotypic diversity paralleled the gene
9 content diversity, we performed clustering analyses with each one of the datasets (Fig. 4
10 A and B). Similar clustering branches were observed for closely related strains MR-4
11 and MR-7, but the same correspondence was not seen with greater evolutionary
12 distances. The phenotypic diversity for closely related strains was much greater (longer
13 branch lengths in Fig. 4B) than their gene content diversity (shorter lengths, Fig. 4A).

14 15 **DISCUSSION**

16 Historically, the establishment of a direct link between genotype and its
17 phenotype has been performed through the study of mutants. However, the generation
18 of thousands of mutants can be costly and laborious. Here, we explored a different
19 avenue for developing such a link by making use of hundreds of Biolog phenotypes and
20 by taking advantage of predicted genes and pathways through comparative genomics.
21 Owing to the high phenotypic diversity within the *Shewanella* and availability of many
22 sequenced genomes, we selected five isolates representing a gradient of evolutionary
23 distances within the genus for this study.

24 The phenotype assay is not a measure of growth on the different substrates, but
25 rather an indication of respiration when a nutrient source is provided. The presence of a
26 transport system and catabolic pathway for a specific chemical compound would lead to

1 the production of NADH that then reduces a tetrazolium dye (1). Measuring respiration
2 has an advantage over growth assays as the microbial cell metabolic response to a
3 chemical compound is detected, even when growth support is not observed. Hence, in
4 our study respiration is a reflection of the ability to utilize different substrates. Likewise,
5 we used five genome sequences and their gene content to reflect the genetic potential of
6 the *Shewanella* genus, without taking into account the many regulatory levels that
7 determine whether a gene is expressed or whether the protein is synthesized and active.
8 By analyzing the predicted functions of the encoded proteins as well as their presence or
9 absence relative to a given phenotype, we were able to successfully confirm a series of
10 genome predictions towards a specific metabolism and to identify a series of target
11 genes for further genetic analysis.

12 Because the comparative phenotype analysis dealt with different numbers of C,
13 N, P, and S sources, we normalized the utilization profile based on the percent of
14 positive results over the total of number compounds tested. The emerging pattern of
15 phenotypes suggested that *Shewanella* is capable of utilizing a variety of N compounds,
16 (77% of the tested N sources) including several amino acids and dipeptides (Fig. 1). The
17 particular ability to grow on several amino acids was previously observed for strain MR-1
18 (32), but the use of amino acids as a sole N source was not extensively tested in the
19 *Shewanella* genus. This preference for N sources might explain the isolation of different
20 species of this genus from a variety of environments such as dead fish (9), chicken
21 breast (9), clinical samples (41), and marine sediments (26, 27, 40), where DNA and
22 polypeptides are known to accumulate. Despite the large number of N sources tested,
23 only 50, mainly dipeptides (90%), were utilized by all five strains. N-acetyl-D-
24 glucosamine was used by these five strains as both N and as C sources (Fig. 2). While
25 N-acetyl-D-glucosamine previously has been characterized as a carbon and energy
26 source for the *Shewanellae* (42), its use as an N source has not been reported. We

1 identified 15 compounds that could be used both as N and C source by at least one of
2 the strains. Interestingly, while SB2B was the only strain able to utilize L-threonine as a
3 C source, the other four strains utilized it as an N source. Overall, there was a decrease
4 in the number of N compounds shared among the strains as the ANI values increased.
5 Fewer shared compounds might indicate a decrease in ecological niche overlap once
6 speciation has taken place. To address this possibility, first we compared the N
7 utilization profiles for the most closely related isolates, namely MR-4 and MR-7. These
8 *Shewanellae*, with 97.05% ANI, are strains of the same species (21) and shared the
9 largest number of N compounds (227). A comparison of these two strains to *S.*
10 *oneidensis* showed the number of shared N sources decreasing to 187 for MR-4 vs. MR-
11 1 (87.74% ANI) and 175 for MR-7 vs. MR-1 (87.70% ANI). Strains MR-4 and MR-7 came
12 from the same environment, where niches have more similar conditions (Table 1).
13 Accordingly, strain SB2B, which came from an environment differing significantly from
14 the others, had the least number of overlaps in N source utilization with 81 positive
15 phenotypes when compared to the other strains. None of these N sources were uniquely
16 utilized by SB2B. However, upon inspection of the SB2B orthologs (Supplementary
17 Material ST1) we identified a stretch of six genes (Sama_0018 to Sama_0023) absent
18 from the other four strains that could be linked to nitrogen metabolism. Two of these
19 genes, Sama_0022 and Sama_0023, encode a predicted urea carboxylase cleaving
20 urea-1-carboxylate to CO₂ and ammonia, with the possibility of the later being
21 assimilated as a nitrogen source. The other co-localized genes included two membrane
22 proteins and a LamB family protein, but their specific role in nitrogen metabolism
23 remains unknown.

24 When attempting to make in depth connections between the ecology and
25 genomic information of microorganisms, environmental metadata is generally missing or
26 not fully described. Although, not different in our study, the limited habitat description for

1 the *Shewanella* isolates in congruency with the large-scale comparative phenotype
2 approach provided insights into the functional strategies devised by the five strains in
3 their environment. *Shewanella amazonensis* strain SB2B was capable of utilizing a full
4 range of C compounds, 60 out of the 67 tested, varying from well-known growth
5 substrates for *Shewanella* such as pyruvate and lactate to previously unknown
6 compounds such as the Tween series and laminarin. As SB2B was isolated from the
7 coastal muddy waters of the Amazon River, a low salinity environment (Table 1), it is
8 likely exposed to a full plethora of C compounds washed out from the Amazon forest
9 (25). In contrast with the SB2B C utilization profile, strain W3-18-1 yielded positive
10 results for only 25 C compounds. This might be a reflection of the W3-18-1 genetic
11 adaptation to nutrient limitation imposed in the marine sediments of the Pacific Ocean
12 (38).

13 Together, the five *Shewanella* strains selected for this study encode a total of
14 6,790 genes. Approximately 39% of the genes are present in all strains while the unique
15 genes comprise 21 - 40% of the genomes. On average, 550 genes per strain were
16 identified as unique, a number in accordance with the previously reported 468 unique
17 genes when ten *Shewanella* genomes were compared (21). In our high-throughput
18 genotype-phenotype analyses, we were able to link a few of these unique genes to
19 specific phenotypes. In addition, pathways believed to be missing in these *Shewanella*
20 strains (e.g. degradation of galactose, ribose, trehalose) were confirmed using the
21 phenotype assay.

22 It is noteworthy that not all observed phenotypes were explained through the use
23 of comparative genomics. For example MR-7 was the only strain unable to utilize
24 acetate and thymidine as C sources. From the ortholog table, we identified only four
25 proteins that were missing in MR-7 and present in the other four strains; two
26 lipopolysaccharides biosynthesis genes and a transcriptional regulator with an adjacent

1 multidrug transporter. It is not evident how any of these functions could explain the
2 acetic acid and thymidine growth phenotypes. Likewise W3-18-1 is the only strain unable
3 to degrade L-glutamate and L-glutamine, yet no proteins for transport or degradation of
4 either compound could be found among the 137 genes absent from W3-18-1 and
5 present in the other four strains. Glutamate transporters as well as degrading enzymes
6 (i.e. glutamate decarboxylase, glutamate racemase, and mutase) were detected in all
7 five genome sequences. We observed a glutamate racemase (W3181_0949) and a
8 glutamine amidotransferase (W3181_0925) among proteins unique to W3-18-1,
9 suggesting that this organism may have a different strategy for metabolizing glutamine
10 and glutamate. Two possible reasons might explain the above differences. First, there is
11 a large fraction of genes without function predictions (37). Presently 21% of the protein
12 coding genes in the five strains remains of unknown function. Novel enzyme variants
13 may also encode some of the “missing” activities as was uncovered in studies focused
14 on reevaluating the genomic content of *S. oneidensis* strain MR-1 (31, 42). Genome
15 reconstruction allowed identification of novel enzymes for both degradation of L-lactate
16 (lactate dehydrogenase, *LldEGF*) and N-acetyl-glucosamine (glucosamine-6-phosphate
17 deaminase, *NagB* and N-acetylglucosamine kinase, *NagK*). Second, regulation of
18 expression or activity at the gene or protein level affects whether a phenotype is
19 exhibited, leading to weak phenotypic expression or a false negative in our analysis.

20 In order to validate our high-throughput phenotype approach, we compared our
21 results to the recent work describing the reconstruction of *Shewanella* carbohydrate
22 utilization pathways (34). Rodionov *et al.* determined the C degradation pathways for 19
23 *Shewanella* genomes using orthologous relationships, regulon predictions, and growth
24 phenotypes. The increased number of genomes as well as the inclusion of growth
25 phenotypes and regulatory networks allowed for thorough analysis of a subset of C-
26 sources compared to our analyses. Growth phenotype data were obtained for eight of

1 the sugars (arabinose, cellobiose, fructose, glucose, mannose, maltose, N-
2 acetylglucosamine, sucrose) tested in our high-throughput phenotype assay. A
3 comparison between both studies indicated an agreement of 75% of the phenotypes
4 (W3-18-1 was not included in their growth studies). Although the dataset used for
5 comparison contains only a small number of sugars, we did not detect bias with regards
6 to a particular substrate or strain, attesting the validity of our approach. We also found
7 agreement in the utilization of amino acids by MR-1 between the Biolog phenotype and
8 growth (30) for six of the seven amino acids tested in both assays.

9 The five strains selected for this study had varying degrees of relatedness as
10 measured by the ANI. The two closest strains in the dataset, namely MR-4 and MR-7,
11 shared the highest fraction of genes (93%) relative to the other genome pairs (e.g. from
12 80% between MR-4 and MR-1 to 72% between MR-4 and SB2B). However, this high
13 sequence similarity between MR-4 and MR-7 was not reflected throughout the
14 phenotypic results (Fig. 4). These strains were among the most similar for the carbon
15 sources (88%) and nitrogen sources (77% similarity), but they were unable to both utilize
16 any of the 21 S sources tested and could only both use one of the 55 P sources tested.
17 Rather, MR-1 and MR-7 were able to utilize 87% and 95% of the P sources and 90%
18 and 76% of the S sources, respectively. The remaining strains only utilized 14% or less
19 of the P and S compounds. Considering that strains MR-1 and MR-7 have a more
20 distant evolutionary history (87.70% ANI), their similar growth phenotypes for P and S
21 sources might reflect the importance of whole cell networks and expression on
22 regulating ecological fitness under certain environmental conditions. High-throughput
23 phenotypic arrays were not designed to capture the interplay between whole cell gene
24 expression and environmental conditions. While it is clear that the present study
25 succeeds in deciphering novel phenotypic traits from a combination of high-throughput

1 phenotypes and comparative genomics, future work on linking the evolution of the
2 phenotypes to environment fitness will need to include genome wide analyses.

3 The present study addressed the importance of bridging genomes and their
4 counterpart phenotypes. The strategy devised here could accelerate the functional
5 discovery of genes and provide an ecological framework for genome sequencing
6 projects.

7

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16

1 **FIGURE LEGENDS**

2 **Fig. 1.** Two-dimensional map representation of the large scale comparative analysis of
3 nutrient utilization for five *Shewanella* strains. Differences in color shade intensities
4 represent differences in metabolic activity of transformed data as follows: black (no
5 activity was detected), light color (below 50 units), dark color (between 50 and 100
6 units), intense color (above 100 units). The units are arbitrary and represent the
7 calculated difference between the areas of a specific well and the control well.

8

9 **Fig. 2.** Carbon source utilization by five *Shewanella* strains. The substrates and their
10 entry points into central metabolism via key metabolic intermediates are shown.
11 Substrates utilized by all five strains are highlighted in bold. Abbreviations include: PP,
12 pentose phosphate pathway; EDD, Entner-Doudoroff pathway; TCA, tricarboxylic acid
13 pathway; G6P, glucose-6-phosphate; GA3P, glyceraldehyde-3-phosphate; PYR,
14 pyruvate; AcCoA, acetyl-CoenzymeA.

15

16 **Fig. 3.** Phenotypic and genotypic changes over evolutionary time. Five *Shewanella*
17 strains were compared in a pair-wise manner for their similarity at the 16S rRNA (green),
18 number of gene orthologs (purple), nitrogen utilization phenotype (blue), and carbon
19 utilization phenotype (red). The similarity of these traits, measured by % of shared
20 ortholog, positive Biolog phenotypes, or rRNA residues, is plotted relative to the %
21 average nucleotide identity (ANI) of the genome pair. A regression line and the curve
22 fitness (R-value) are shown for each trait. When fewer than 10 symbols are shown,
23 results overlap.

24

25 **Fig. 4.** Cladograms showing genomic and phenotypic comparisons among five
26 *Shewanella* strains. Panels: (A) gene-content clustering based on orthologs identified

1 among the five genomes and (B) phenotypic transformed data clustering based on the
2 presence/absence of respiratory activity for 441 nutrient sources. Scale bars indicate the
3 percent similarity obtained from calculated matrices.

5 **SUPPLEMENTARY MATERIAL**

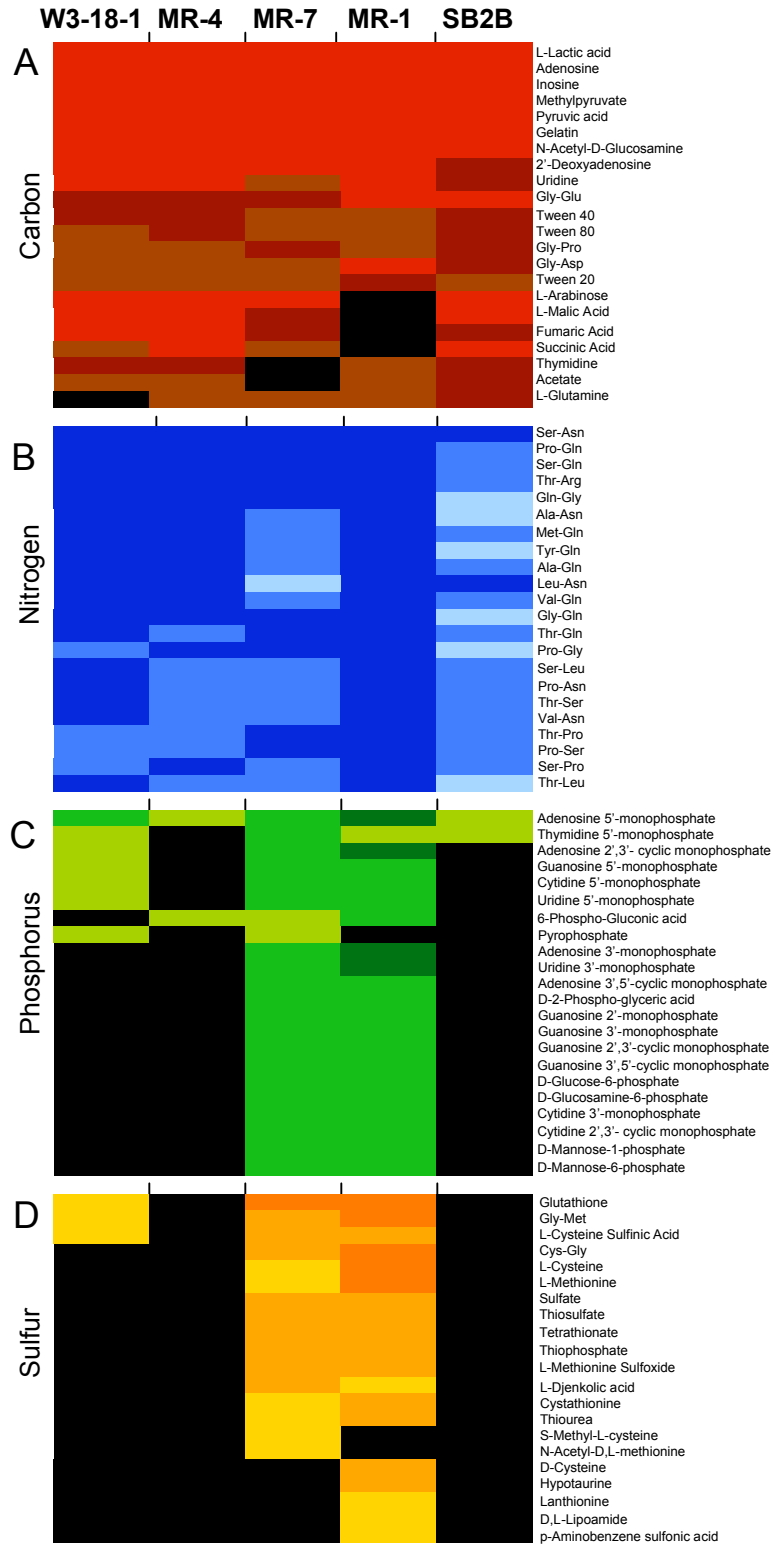
6 1. **Figure SF1.** Complete two-dimensional map of nutrient utilization profile for five
7 sequenced *Shewanella* strains. Only nutrient sources with positive results for at least
8 one strain are depicted: 67 carbon sources (red), 299 nitrogen sources (blue), 55
9 phosphorous sources (green), and 21 sulfur sources (yellow). Differences in color shade
10 intensities represent differences in metabolic activity of transformed data as follows:
11 black (no activity was detected), light color (below 50 units), dark color (between 50 and
12 100 units), intense color (above 100 units).

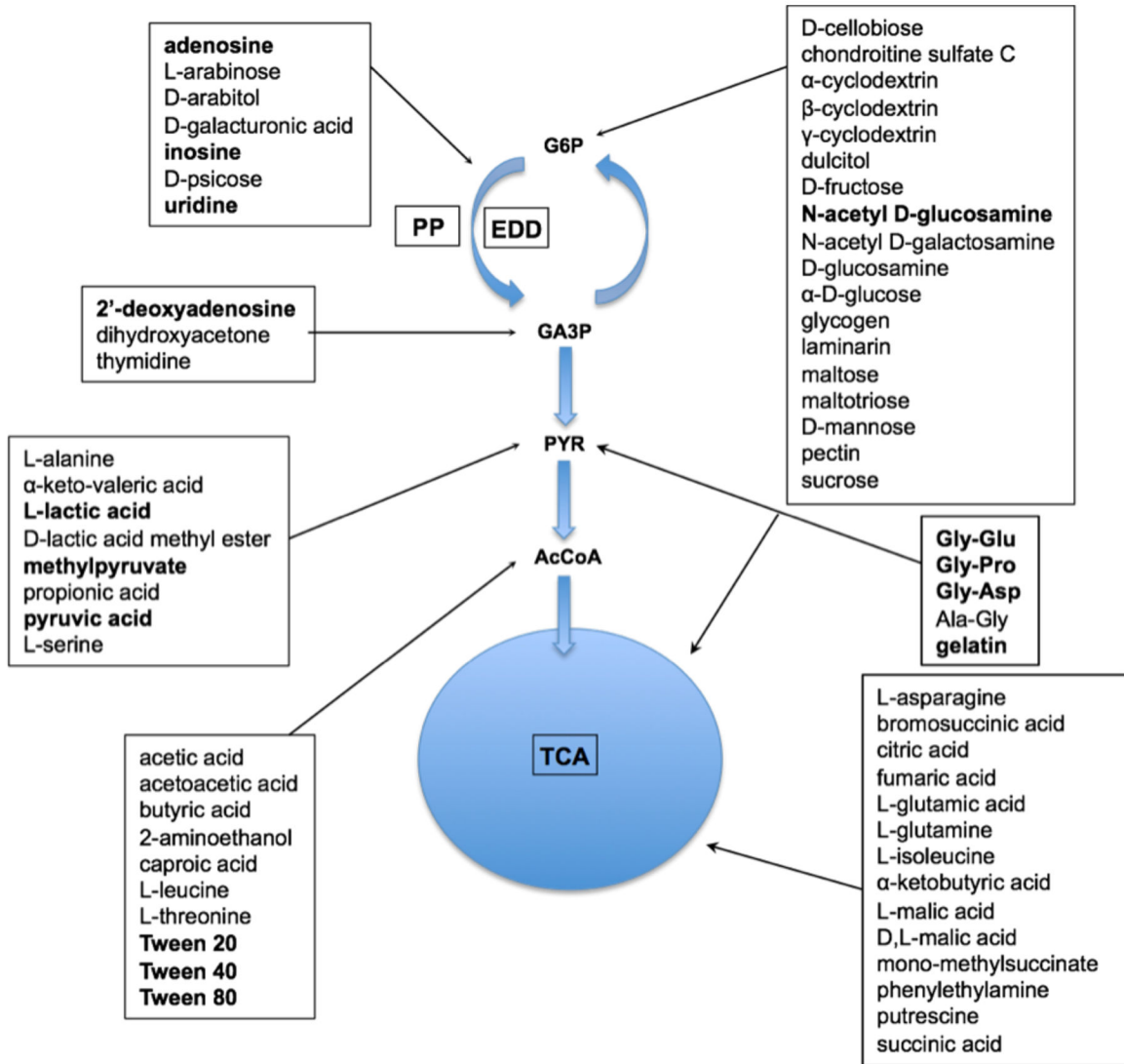
13
14 2. **Table ST1.** Table of *Shewanella* gene products and their orthologous relationships.
15 The complete sets of proteins encoded by the five *Shewanella* strains compared are
16 included in the table. The proteins are labeled with their locus tag, and their orthologous
17 relationships are indicated in the table. Functional descriptions of the gene products are
18 listed as well as the metabolic roles for gene products discussed in the paper.

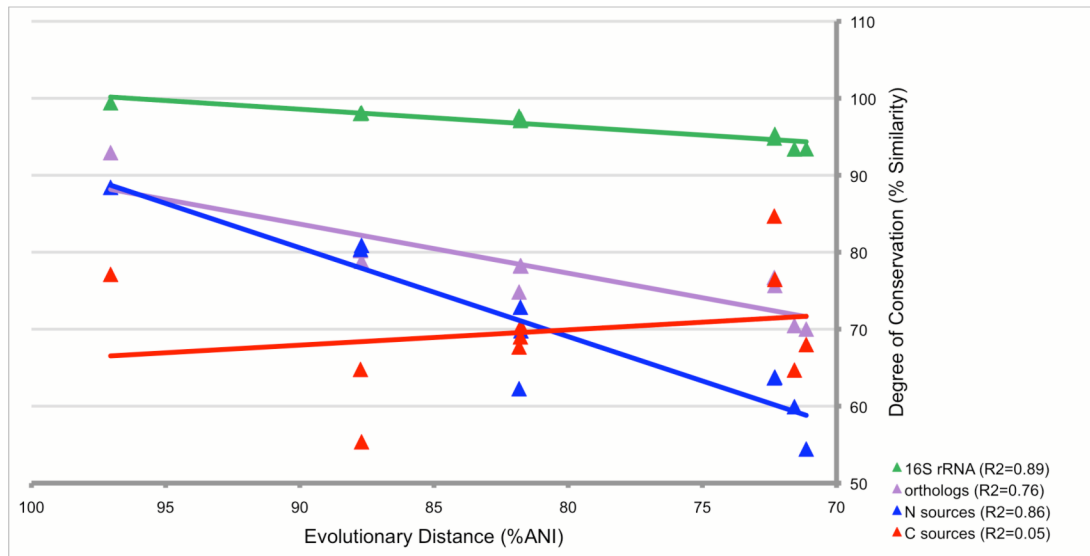
19
20 3. **Table ST2.** Average nucleotide identity values for the strains compared.

21
22 4. **Table ST3.** Calculations of trait similarity across evolutionary distance.

Shewanella strains







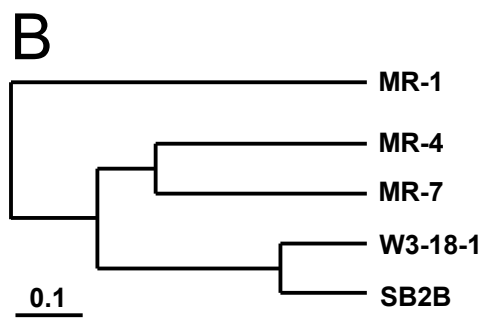
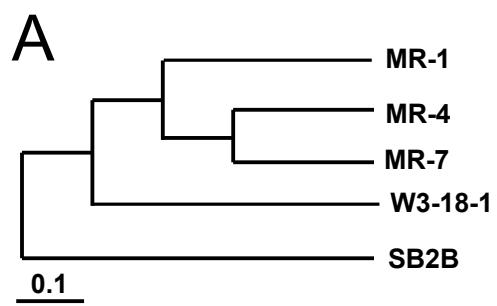


TABLE 1. Description of habitats of isolation and genome information for the *Shewanella* strains used in this study.

Habitat of Isolation	Environmental characteristics	<i>Shewanella</i> species	Genome size (number of genes) ^b	Reference
Lake Oneida (USA) fresh sediment	Anaerobic redox condition, pH 7.5-8.2, 4°C (Winter) and 20°C (Summer), > 100 µM Mn ⁺⁴	<i>Shewanella oneidensis</i> MR-1	5,131,416 (4,745)	27
Amazon River delta (Brazil) sediment	Non-sulfidic, suboxic redox conditions, 26.4-31.5°C, 0.1-1 mM Fe ⁺² , 0.1-0.2 mM Cl ⁻ (low salinity), depth of 1 m	<i>Shewanella amazonensis</i> SB2B	4,306,142 (3,785)	40
Pacific Ocean (USA) marine sediment	Washington coast, 3.4°C, 10 µM Fe ⁺² , 76 µM NH ₄ , depth of 997 m	<i>Shewanella</i> sp. W3-1-18	4,708,380 (4,217)	26, 38
Black Sea sediments	8°C, 5 µM O ₂ , 7 µM NO ₃ , depth of 60 m	<i>Shewanella</i> sp. MR-7	4,799,109 (4,186)	28 ^a
Black Sea water	16°C, 280 µM O ₂ , depth of 5 m	<i>Shewanella</i> sp. MR-4	4,706,287 (4,084)	28 ^a

^a Values were calculated based on reference.

^b Genome size (Mb) and number of predicted genes were calculated as a sum of chromosome and plasmid, when the later was present.