Comparative systems biology across an evolutionary gradient within the *Shewanella* genus

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ABSTRACT

To what extent genotypic differences translate to phenotypic variation remains a poorly understood issue of paramount importance for several cornerstone concepts of microbiology including the species definition. Here, we take advantage of the completed genomic sequences, expressed proteomic profiles, and physiological studies of ten closely related *Shewanella* strains and species to provide quantitative insights into this issue. Our analyses revealed that, despite extensive horizontal gene transfer within these genomes, the genotypic and phenotypic similarities among the organisms were generally predictable from their evolutionary relatedness. The power of the predictions depended on the degree of ecological specialization of the organisms evaluated. Using the gradient of evolutionary relatedness formed by these genomes, we were able to partly isolate the effect of ecology from that of evolutionary divergence and rank the different cellular functions in terms of their rates of evolution. Our ranking also revealed that whole-cell protein expression differences among these organisms when grown under identical conditions were relatively larger than differences at the genome level, suggesting that similarity in gene regulation and expression should constitute another important parameter for (new) species description. Collectively, our results provide important new information towards beginning a systems-level understanding of bacterial species and genera.

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Introduction

Predicting the phenotype of newly isolated organisms based upon the existing knowledge of previously characterized organisms constitutes one of the most fundamental goals of microbiology. Organisms isolated from diverse environments and habitats often have their phenotypic and physiological properties inferred from their evolutionary relatedness, measured by (mainly) the 16S rRNA gene sequence identity or other means (1, 2), to the type strains of known species. Although this practice has been broadly applied in studies of microbial communities, contributing greatly toward advancing microbiology knowledge, its use in this manner is rooted in rather low-resolution experimental methods and procedures (1, 3). The powerful genomic tools now available provide the opportunity for a much more detailed and informative evaluation of the relationship between genetic and phenotypic similarity. Simple questions that remain unanswered or only partially explored such as "to what degree do microorganisms encode and express the same metabolic pathways when grown under identical conditions" and "to what extent are the similarities in expressed pathways determined by the genetic relatedness and/or the (distinct) ecological adaptations of the microorganisms?" can now be answered accurately and quantitatively. Addressing such questions will provide long-needed information to better understand and model the enormous microbial biodiversity that exists on the planet.

To this end, we have analyzed and compared, both at the whole-genome and the whole-proteome levels, ten isolates belonging to the genus *Shewanella*, an important genus in cycling of organic and inorganic materials in the environment (4). These isolates originated from diverse geographic locations and habitats, including fresh and marine water columns, sediments, and subsurface environments (Fig. 1A and Table S1), and carry out a diverse range of metabolic processes (4). Although precise ecological information, e.g., *in-situ* abundance and persistence in time, about each isolate is typically not available, the procedure employed to isolate these strains, i.e., enrichment cultures from a variety of environmental samples for the phenotype or genotype of interest, is similar to common microbiology practice. Accordingly, our analyses with the *Shewanella* strains should be relevant for the questions described above and for broadening our understanding of the interrelationship between genotype, phenotype, environment and evolution. Our results represent the first thorough and system-level assessment of an environmental representative of *Proteobacteria*, an enormously diverse and important group, that can be compared and contrasted to previous assessments of the heavily sampled human pathogens or the ecologically specialized organisms such as the photosynthetic *Prochlorococcus* (5). Such comparisons identified several trends that may apply to other environmentally versatile bacteria besides *Shewanella*.

A continuous genetic gradient within a genus. Phylogenetic analysis of the 16S rRNA gene sequences revealed that the ten *Shewanella* isolates formed a tight cluster, with the intra-cluster sequence identity ranging from 92 to ~100% (Fig. 1B). Hence, these genomes belong justifiably to the same genus according to the most frequently used standards of bacterial taxonomy (2, 6). To gain further insights into the diversity of this group, the Average Nucleotide Identity (ANI) of all pair-wise conserved genes between (any) two genomes, a more sensitive parameter for measuring evolutionary relatedness among closely related genomes than the 16S rRNA gene (7), was employed. The ANI analysis revealed that these genomes form a continuing gradient of genetic relatedness, which was not readily apparent from the 16S rRNA gene analysis (Fig. 1C).

In particular, *S. putrefaciens* strains W3-18-1 and CN-32 as well as *Shewanella* sp. MR-4 and MR-7 are the most closely related pairs, showing ANI values of ~96.5% and ~98.4%, respectively. These values are well above the 95% ANI that corresponds to the 70% DNA-DNA hybridization (DDH) standard frequently used for species demarcation, which is consistent with the experimentally derived DDH values for these organisms (6). Hence, these pairs of genomes sample the sub-species level. The MR-4 and MR-7 genomes show ~92%, ~85%, and ~79% ANI to *Shewanella* sp. ANA-3, *S. putrefaciens* CN-32, and *S. oneidensis* MR-1 genomes, respectively. Thus, these genome pairs represent varied levels of genetic relatedness within the *Shewanella* genus. Finally, all the previously mentioned genomes show ~69.7-72% ANI to *S. frigidimarina* NCIMB400, *S. denitrificans* OS217, *S. loihica* PV-4, and *S. amazonensis* SB2B strains, which represent the four most divergent species sampled within the genus. This gradient provided the opportunity to precisely estimate the number of changes in the genes, pathways and subsystems of the cell over time and as a result of environmental adaptations and selection pressures.

Gene-content variation as a function of evolutionary time and ecology. The ten *Shewanella* isolates have similar genome sizes, varying from 4.3 to 5.3 Mbp (Table S1). Comparative analysis revealed extensive gene content diversity among the genomes. From the 9,782 predicted non-redundant (orthologous genes removed) protein-coding sequences (CDS) annotated in the ten genomic sequences (the pangenome) only ~2,128 (22%, constituting ~54% of the total genes in the genome, on average) were present in all genomes (core CDS set); about 2,965 (30%) were found in at least two genomes (variable CDSs), while the remaining CDS (4,689 or 48%) were strain specific (Fig. 2B and Table S2). Nonetheless, the majority of the variable CDSs were found to be specific to clades, i.e., the MR or *S. putrefaciens* clades (Fig. 1B), while a smaller fraction had a more sporadic distribution among the strains (see the similarity between the gene content tree and the phylogeny of the genomes in Fig. 3). Accordingly, the overall extent of CDS-content similarity showed a very strong linear decrease with increasing evolutionary distance between the genomes compared ($R^2 > 0.9$, see Fig 1C), which is consistent with results reported previously based on other bacterial groups (7). The strong linear trend suggests that, despite the extensive gene diversity and apparent genome fluidity, the genotypic similarity of bacteria may be generally predictable from their evolutionary relatedness.

Although a tight relationship between shared CDS-content and evolutionary relatedness was observed, several significant departures (outliers) from this main trend were also noted and were most likely attributable to ecological adaptations. For instance, the two most closely related genomes based on ANI, CN32 and W3-18-1 (98.4% ANI), showed substantially more CDS-content differences compared to what was expected based on their small evolutionary divergence (see regression trendline in Fig. 1C) or compared to the more distantly related (96.4% ANI) pair of MR-7 and MR-4 (~530 vs. ~430 CDSs, respectively, not counting CDS on mobile elements; see Table S2). CN-32 and W3-18-1 were isolated from more diverse environments (deep-subsurface sandstone vs. marine sediment, respectively) compared to MR-4 and MR-7 (5m vs. 60m depth in the Black Sea, respectively). Hence, it is likely that genetic adaptations specific to these environments account for the larger gene content differences observed in the former strains relative to the latter ones. In agreement with the latter interpretation, CN-32-specific genes included several genes that might be important for survival in the subsurface environment such as an arsenate reductase, copper resistance system, heavy metal efflux pump, and a polysaccharide biosynthesis cluster.

Similarly, *S. denitrificans* strain OS217 is as divergent as three other isolates (strains PV-4, NCIMB400, and SB2B) are from the remaining six *Shewanella* isolates in our collection (e.g., Fig. 3D). Yet, the OS217 genome contained substantially more strain-specific genes and showed the greatest loss of "core-like" CDSs (i.e., CDSs present in all other *Shewanella* genomes) compared to the genomes of PV-4, NCIMB400, or SB2B (Table S2). For instance, the core set increased by 265 genes when OS217 was removed from the analysis compared to fewer than 60 genes when PV-4, NCIMB400 or and SB2B were individually removed. Our genomic, physiological (e.g., Table S5), and proteomic data collectively suggests that strain OS217 has undertaken a unique evolutionary path, possibly driven by the loss of the three menaquinone biosynthetic gene clusters (*menDHCE*, *menF*, *menB*) common to the other *Shewanella* strains and resulting in inability to exploit strictly anaerobic habitats. These results are also consistent with previous findings suggesting that strain OS217 is a specialized denitrifier (4) and with the longstanding observation that respiratory denitrification is not found in organisms that are strong fermentors (8). These findings may indicate that more extensive genetic changes are involved for an organism to diverge to the opposite physiology. Lastly, the (outlier) pairs of genomes with a higher percentage of shared genes than the average, i.e., CN32 or W3-18-1 vs. MR4 or MR7 (Fig. 1C), are attributable to the substantially smaller size of these genomes (i.e., 4.6-4.7 Mbs) relative to that of the rest of the genomes (i.e., ~5.2 Mbp, see Table S1) rather than to more similar ecological adaptations (the number of shared orthologs and mobile gene content in these pairs is comparable to that of other pairs).

Processes contributing to gene-content variation. To provide further quantitative insights into the processes contributing to gene-content variation, the genes that differed in pair-wise whole-genome comparisons were assigned to five major functional categories and the percentage of genes in each category was evaluated against the genetic relatedness of the two genomes compared. The five categories were: i) pseudogenes, denoting genes predicted to encode insertions, deletions, or sequence alterations that would result in premature termination of the encoded protein, ii) IS/Tn, denoting insertion sequences or transposons, iii) mobile islands, denoting runs of neighboring genes (genomic islands) that included integrase genes, iv) other, denoting all other unique genes, including genomic islands that do not contain clear evidence of being mobile, and v) hypothetical or conserved hypothetical, denoting the fraction of the genes in category (iv) that had no detectable homolog in any of the fully sequenced genomes except in other *Shewanella* genomes (Table S3). Our results revealed that mobile islands and insertion elements dominated the gene-content differences among genomes of the same species but their contribution gradually decreased in comparisons among genomes of increasing evolutionary divergence at the expense of genes in the "other" category (Fig. 2A). These findings are consistent with rapid turnover of mobile islands over short evolutionary scales. Further, the majority (>75%) of the genes in the "other" category were typically found in clusters of ~5 to ~40 genes, reflecting presumably their "mobile island" origin. These findings are consistent with preferential deletion of the mobility/transposition genes (presumably due to negative selection) in the course of evolution and retention of only the potentially ecologically important genes of mobile islands. Therefore, the *Shewanella* organisms evaluated here appear to acquire most of their new functions as follows: acquisition of mobile islands followed by selection for the islands carrying ecologically important genes and finally loss of the mobile and ecologically unimportant genes.

The *Shewanella* pangenome and conserved gene core. Comparative analysis of the ten *Shewanella* genomic sequences revealed that sampling of the genus pangenome remained unsaturated (Fig. 2B, blue bars); this result was attributable to the large number (468, on average) of strain-specific genes. Only 10% to 25% of the latter genes, depending on the genome evaluated, found a homolog in a genome outside the *Shewanella* genus when queried against all bacterial genomes available at the end of 2008, indicating the great potential for discovering novel genes with more *Shewanella* strains sequenced. The number of new genes per genome is an order of magnitude higher than those calculated for highly specialized human pathogens (9) but significantly lower than that of the opportunistic pathogen *Escherichia coli (7)*. It must be pointed out, however, that these pan-genome calculations are not directly comparable and should be interpreted with caution. For instance, the average ANI value among all pairs of *Shewanella* genomes is ~76%, which is significantly lower than that within the *E. coli* group (~96%), and there appears to be a strong positive correlation between the amount of novel genes carried in a genome and the (higher) degree of evolutionary divergence of the genome, regardless of the effect of ecology or environmental adaptation (Fig. 1C and in (7)). On the other hand, the prophage content of the *E. coli* genomes is substantially higher than that of the *Shewanella* ones (10-20% vs. 0-5%, respectively), and this accounts for much of the difference observed. When the groups were adjusted for comparable intra-group diversity, by including selected *Salmonella* (~82% ANI to *E. coli*) and *Yersinia* (~72% ANI to *E. coli*) genomes together with *E. coli* ones and with prophage genomes removed from the analysis, the gene diversity observed within the enterics was comparable to that of the *Shewanella* (Fig. 2B). Therefore, the evaluation of these two important groups suggests that sequencing of any new organism, as long as the organism belongs to a versatile genus and has a different ecological history relative to the previously sequenced members of the genus, should be expected to expand substantially the pangenome of the genus.

Both the *Shewanella* and the enterics core gene sets were highly enriched in translational, transcriptional, DNA replication, and central metabolism genes and overlapped extensively (~50% of the genes were shared between the two cores). *Shewanella*-specific core functions were associated mainly with metabolic pathways, as well as chemotaxis and sensory-transduction processes. Using the BioCyc pathway schema (10), 104 pathways were identified as being common to all *Shewanella* genomes, including pathways for energy metabolism, synthesis of building blocks (amino acids, cofactors, fatty acids, and nucleotides), and for degradation or inter-conversion of metabolites and all but two amino acids and metabolites (Fig. S2, and Table S4). A common trait of the *Shewanella* strains appears to be the use of the pentose phosphate and Entner-Doudoroff pathways for hexose degradation. This is based on the lack of the enzyme 6-phosphofructokinase (Pfk; the most important regulatory enzyme of the canonical glycolysis pathway), initially observed in previous gene expression studies of MR-1 cultures (11). Members of the *Shewanella* genus also have fewer phosphotransferase system (PTS) transporters than usually encountered in proteobacterial genomes. Whether there is a connection between the reduced PTS and lack of Pfk is not clear, but it is possible that the lower level of phosphoenolpyruvate (PEP) synthesized as a result of not using the glycolytic pathway may render the PEP-dependent PTS system inefficient.

When the core was defined as the genes present in all but one of the 10 genomes, the dataset increased by 411 protein coding genes (265 when OS217 was excluded from the analysis), corresponding to, on average, 12-14% of the *Shewanella* genome (Table S2). These findings suggest that gene loss, including loss of genes that are apparently indispensable for the majority of the strains of a species, might be a successful strategy for fast evolution and environmental adaptation. A representative example of strain-specific adaptations related to a group core function, which involved considerable gene deletion and/or gene acquisition, is given below. All *Shewanella* strains except for *S. denitrificans* OS217, which shows limited anaerobic growth capabilities presumably due to gene loss during the process of ecological specialization (discussed above), were able to reduce several metals and metalloids (Table S5), a well-known characteristic of the genus (12). The main metal reductase locus, encoded by *mtrCAB* genes, is virtually identical for the nine strains but the adjacent loci vary, reflecting evolutionary history and possibly metal respiratory specialization (4). These dissimilarities explained some, but not all, of the variation in metal respiration among strains observed during our growth experiments. For example, although their *mtr* locus and flanking genes are identical, strain CN-32 was able to grow on lactate (20 mM) when six different metals or metalloids were used as electron acceptors, whereas strain W3-18-1 only grew with Fe, Mn, and Se, under the conditions tested. These results may reflect differences in the upstream pathways to metal reduction between the two strains and underscore the need for more research to understand better the details of the metal respiration cascade.

Gene presence vs. expression as a function of time and ecology. Transcriptome comparisons have shown that gene expression rather than gene content differences, occurring either at different times and/or tissues, are mainly responsible for the differential development of eukaryotic organisms, e.g. human and chimpanzees (13), and the adaptive evolution of natural populations (14). It follows that, in addition to the number of shared genes, gene expression constitutes an important factor determining phenotypic similarity (or dissimilarity). While the latter applies presumably to bacteria as well, systematic assessments of the role of gene expression on the phenotypic differences observed among closely related organisms are lacking.

To begin exploring this issue, the ten strains were grown under identical batch-culture conditions to obtain their whole-cell proteome profiles and contrast the profiles against the evolutionary relatedness among the strains. Overall, the degree of similarity in proteome profiles was congruent with the evolutionary relatedness among the strains, i.e., the fraction of orthologous proteins detected to be expressed in the cultures was higher in closely related strains than in more divergent strains. However, the differences in expressed proteins among the strains were consistently larger than their differences at the gene-content level when gene expression and gene content were assessed for the same 4,300 (reference) genes found in the MR-1 genome (compare branch lengths in Fig. 3B and 3C), which minimized the effect of gene- or strain- specific variations in the measurements. More surprisingly, the same pattern was observed even when gene expression was assessed for the core genes only (Fig. 3A, Fig. S2), which circumvented the dependency of the proteome profiles on the underlying gene-content differences in the previous comparisons. These results were attributable to a high number of proteins expressed by one or a few, but not all, of the strains possessing the corresponding gene, with proportions that varied from 1.9 to 2.6 times more than those proteins expressed by all strains possessing the corresponding gene (Table S6). For instance, although twenty percent (556 genes) of the core proteins were expressed by all strains, a substantially larger fraction of core proteins (993 or 36%) were expressed by one or more (but not all) strains. While some of these differences may be due to higher noise in the proteomics data relative to the genomics data, we believe that many of these differences are biologically relevant due to the high reproducibility (>80%) of proteomics measurements on batch cultures like the ones used in the present study (15), our high stringency in processing and analyzing the proteomics data (see methods), and the fact that very similar results were found when a subset of five specific regions of traditional 2-dimension protein gels were overlaid and compared for absence or presence of protein spots (Fig. S3). Finally, proteins characteristic of the stationary growth phase, such as the RpoS sigma factor (16), were not detected in the expressed proteomes, suggesting that all of our cultures were sampled at their exponential growth phase.

Our findings revealed that although strains CN-32 and W3-18-1 are significantly more closely related than are strains MR-4 and MR-7 [e.g., a 2% higher ANI value translates to substantially higher gene-content and evolutionary relatedness, as we and others have shown (7)], they showed comparable differences in expressed proteins compared to the latter strains for the same genes analyzed (Fig. 3). These findings could therefore be attributable to a higher degree of environmental/ecological adaptations (which may have altered metabolic and regulatory networks) in the CN-32/W3-18-1 pair relative to the MR-4/MR-7 one. Similarly, *S. denitrificans* OS217, which appeared to be the most ecologically specialized organism of the set, also showed the most unique proteomic profile (Fig. 3). The larger gene expression differences observed for OS217 and CN-32/W3-18-1 than anticipated based on their evolutionary divergence alone echoes the results described above based on the gene-content analysis. Further, the largest fraction (44%) of the proteins detected in the protein profiles was strain-specific and included many non-hypothetical proteins such as outer membrane proteins, TonB-dependent receptors, proteases, restriction-modification enzymes, glycosylases, and polysaccharide biosynthesis enzymes. Most of these proteins can be linked to metabolic fitness or interaction with the environment, and hence could possibly underlie important physiological and/or regulatory differences among the strains. The extensive variability in core proteins and the high number of strain-specific proteins expressed under identical growth conditions indicates a multifaceted and highly dynamic control of whole genome expression. Collectively, our proteomics analyses suggest that changing this control appears to represent a particularly important mechanism, in addition to gene acquisition or loss, for fast adaptation in changing and diverse environments. Consistent with these conclusions, the first mutations observed in experimentally evolved *E. coli* strains for 20,000 generations under laboratory conditions involved regulatory genes and networks (17).

Compartmentalized microbial evolution. In order to characterize which cellular functions evolve faster in the *Shewanellae*, the percent conservation of selected functional gene categories (see methods for details) was evaluated against the evolutionary relatedness among the strains compared (measured by % ANI). As evolutionary distance increased, the % conservation of all categories decreased, but the extent of decline (i.e., the slope) differed, presumably reflecting the varied selection pressures on the corresponding genes. The analysis revealed the following order: pathways were substantially more conserved than individual orthologs, orthologs more conserved than transcriptional regulators, sensing and respiration genes, and expressed proteins (Fig. 4). The most rapidly changing individual functions, both in terms of gene presence/absence and sequence conservation, were TonB-dependent outer membrane receptors followed by methyl-accepting chemotaxis proteins, transcription regulators and cytochromes.

These results are consistent with our previous findings and suggest that genomic and regulatory changes in sensing mechanisms represent the first line of adaptive response to different redox conditions. Experimentally determined anaerobic growth characteristics such as biomass produced and electron acceptors used (Table S5) were also very different among the *Shewanella* strains and ranked among the fastest changing functional entities (Fig. 4). A growth phenotype encompasses the sensing of a substrate, expression of relevant regulators, transporters and enzymes, in addition to physiological parameters related to the change in growth conditions. These potential sources for additional variation among the strains may explain why the growth phenotype is significantly less conserved compared to pathways, orthologs, and protein expression patterns.

Summary and perspectives for the future

Microbiologists have been primarily focused on comparisons among either very closely related strains of the same species or distantly related species in order to advance understanding of the microbial life on Earth. The ten *Shewanella* genomes studied here were selected to represent a range of evolutionary distances, providing for a more unconstrained view of microbial diversity and evolution. Comparisons among these genomes revealed that the *Shewanella* genus is genomically and more so proteomically diverse. Although a high degree of variation in protein expression profiles was anticipated among distantly related species, the variation observed among strains of the same species was comparatively much larger than expected, given also the single growth condition used (Fig. 3 & 4). It also appears that, in some cases, the variation in expressed proteomes correlated positively with the extent of environmental adaptation (specialization). These findings have important implications for the correspondence between genotype and phenotype and hence, for the bacterial species concept. The evolutionary and functional gradients reported here also suggested that specialization might occur over a very short time span, much shorter compared to what corresponds to the current species standards. Specialization appeared to take place primarily through changes at the regulatory level and through the high plasticity and fluidity of the *Shewanella* chromosomes (e.g., Fig. 4).

The power of "omics" compared to traditional approaches to unravel organism's environmental/ecological adaptations and make robust predictions about the similarity (or difference) in phenotypic traits among organisms was also highlighted by our analyses. The literature as well as our experimentally derived physiological and growth data could not easily distinguish between most of the strains used in this study or (even) define general properties for the major clades represented by these strains. This was also reflected in the very low correlation obtained between anaerobic growth characteristics (Table S5) and the evolutionary relatedness of the strains compared. In contrast, genomic and proteomic data correlated well with the phylogeny of the strains and identified congruently strain-specific adaptations that might be linked to speciation for several of the strains studied. These results further corroborate the notion that it is time to start replacing the traditional approaches for defining diagnostic phenotypes for (new) species or clades with omics-based procedures.

Distinguishing the effect of ecological adaptation from that of evolutionary divergence alone represents the most limiting factor in increasing the power of our predictions on phenotype based on the genotype. Towards this direction, studying the extent of variation among members of the same natural population, i.e., among organisms with very similar environmental adaptations, and contrasting it to the levels of variation detected in this study with diverse organisms will allow for fruitful conclusions. The trendlines obtained in this study (Fig. 1C & Fig. 4) also provide a reference for comparing organisms of narrower (or broader) metabolic versatility than the *Shewanellae*. Further, although the growth conditions used in this study were very limited, they remain artificial compared to the environmentally relevant conditions and hence, may represent different stresses for each isolate evaluated. Replicate experiments and experiments performed with continuous cultures (chemostats) are currently underway in order to provide further quantitative insights into the role of variation in gene expression. Finally, a major limitation remains in that, despite the dedicated efforts of numerous laboratories, many of the genes in the genome have not been experimentally characterized and their physiological role is unknown. Continuing the efforts to establish function to as many genes in the genome as possible is critical for a thorough understanding of a bacterium that could serve as a model for versatile environmental bacteria.

Regardless of these limitations, the results presented here constitute important information towards better modeling the correspondence between genotype and phenotype and provide directions and testable hypotheses that will bring us one step closer to systems-level understanding of microbial species and populations.

Material and Methods

The organisms used in this study, their genomic features, gene-content, and accession numbers of the versions of the genomic sequences used in the study are provided in Table S1. Orthologs were identified for the ten *Shewanella* genomes by a combination of three methods: i) protein-protein pair-wise reciprocal BLAST (blastp) (18), ii) reciprocal protein-genomic sequence best match (tblastn), and iii) Darwin pair-wise best hit (19). Genes found in plasmids or mobile elements were excluded from ortholog and proteome comparisons among the strains. The degree of conservation of cellular functions or traits between two strains (e.g., Fig. 4) was determined as follows. I) For orthologs, transcriptional regulators, TonB receptors, MCPs, and cytochromes: all genes in the genome assignable to each of these categories were determined based on the gene annotation and the number of orthologous genes shared between two strains for each category (according to Table S2) was divided by the total number of genes assignable to the category for each strain. The two values were averaged to provide the values used in figure 4. II) A total of 163 unique pathways were identified in the ten *Shewanella* genomes according to the BioCyc pathway schema (http:/biocyc.org). The number of shared pathways between the strains, as a fraction of the total pathways carried by a strain, was determined based on the presence/absence of the corresponding pathway genes. III) For proteomes and anaerobic growth, the number of orthologous proteins expressed (Table S6) and metal/metalloids respired (Table S5) by both strains in a pair was divided by the total number of (non-redundant) proteins expressed and metal/metalloids respired by either strain, respectively. The use of "total traits counted for both strains" as the denominator (as opposed to "counts for one strain") provided also for more direct comparisons to the sequence-based traits (I and II above) because otherwise the latter traits would have been penalized relatively higher due to the high number of "auxiliary" genes, which remained un-expressed under the simple growth conditions tested. For proteomics analysis, cultures were grown aerobically in Tryptic Soy Broth to final Optical Density, OD=0.5. Cells were lysed, proteins extracted and digested with trypsin, and the resulting peptides analyzed by mass spectrometry as previously described (20), with the only exception that filtering of the data was performed as described in (21). Two-dimensional proteomic gels were carried out as described previously (15). A detailed description of materials and methods is included in the supplementary material.

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REFERENCES

1. Stackebrandt E*, et al.* (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* 52(3):1043-1047.
2. Brenner D, Staley J, & Krieg N (2001) *Bergey's manual of systematic bacteriology* (Springer-Verlag, New York) 2nd Ed pp 27-31
3. Vandamme P*, et al.* (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* 60(2):407-438.
4. Fredrickson JK*, et al.* (2008) Towards environmental systems biology of Shewanella. *Nat Rev Microbiol* 6(8):592-603.
5. Kettler GC*, et al.* (2007) Patterns and implications of gene gain and loss in the evolution of Prochlorococcus. *PLoS Genet* 3(12):e231.
6. Goris J*, et al.* (2007) DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 57(Pt 1):81-91.
7. Konstantinidis KT, Ramette A, & Tiedje JM (2006) The bacterial species definition in the genomic era. *Philos Trans R Soc Lond B Biol Sci* 361(1475):1929-1940.
8. Tiedje JM (1988) *Ecology of denitrification and dissimilatory nitrate reduction to ammonium* (John Wiley and Sons, New York) pp 179-244.
9. Medini D, Donati C, Tettelin H, Masignani V, & Rappuoli R (2005) The microbial pan-genome. *Curr Opin Genet Dev* 15(6):589-594.
10. Caspi R*, et al.* (2008) The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. *Nucleic Acids Res* 36(Database issue):D623-631.
11. Driscoll ME*, et al.* (2007) Identification of diverse carbon utilization pathways in Shewanella oneidensis MR-1 via expression profiling. *Genome Inform* 18:287-298.
12. Hau HH & Gralnick JA (2007) Ecology and biotechnology of the genus Shewanella. *Annu Rev Microbiol* 61:237-258.
13. Enard W*, et al.* (2002) Intra- and interspecific variation in primate gene expression patterns. *Science* 296(5566):340-343.
14. Oleksiak MF, Churchill GA, & Crawford DL (2002) Variation in gene expression within and among natural populations. *Nat Genet* 32(2):261-266.
15. Elias DA*, et al.* (2008) The influence of cultivation methods on Shewanella oneidensis physiology and proteome expression. *Arch Microbiol* 189(4):313-324.
16. Lange R & Hengge-Aronis R (1991) Identification of a central regulator of stationary phase gene expression in Escherichia coli. . *Mol. Microbiol.* (5):49-59.
17. Philippe N, Crozat E, Lenski RE, & Schneider D (2007) Evolution of global regulatory networks during a long-term experiment with Escherichia coli. *Bioessays* 29(9):846-860.
18. Altschul SF*, et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389-3402.
19. Gonnet GH, Hallett MT, Korostensky C, & Bernardin L (2000) Darwin v. 2.0: an interpreted computer language for the biosciences. *Bioinformatics* 16(2):101-103.

20. Fang R*, et al.* (2006) Differential label-free quantitative proteomic analysis of Shewanella oneidensis cultured under aerobic and suboxic conditions by accurate mass and time tag approach. *Mol Cell Proteomics* 5(4):714-725.

21. Washburn MP, Wolters D, & Yates JR, 3rd (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* 19(3):242-247.

22. Bruen TC, Philippe H, & Bryant D (2006) A simple and robust statistical test for detecting the presence of recombination. *Genetics* 172(4):2665-2681.

FIGURE LEGENDS

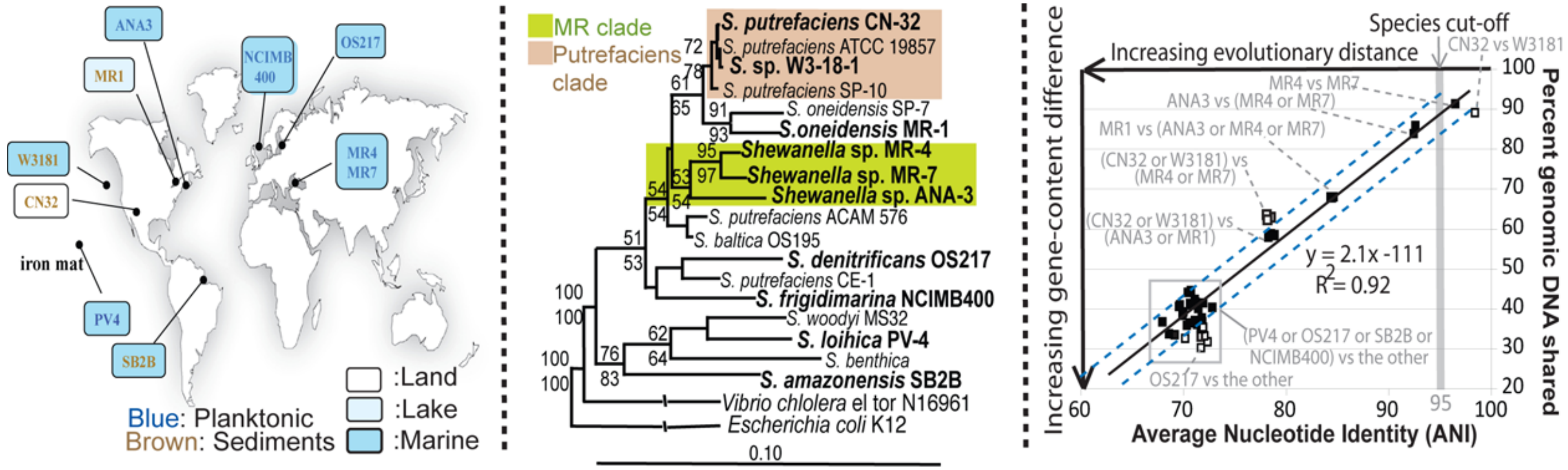
Figure 1. The ten *Shewanella* genomes used in this study and their evolutionary gradient. The geographic origin (**A**) and the 16S rRNA-based phylogenetic tree (**B**) of the ten genomes (in bold) are shown. The scale represents the number of substitutions per position and the numbers above and below the nodes represent the bootstrap support from 1,000 re-samplings using parsimony and maximum likelihood methods, respectively. Bootstrap values below 50 were omitted. A continuous genetic gradient was formed (**C**) when the fraction of the total genes in the genome shared between two genomes (y-axis) was plotted against the ANI of the shared genes between the two genomes (45 comparisons in total are shown). Dashed blue lines represent the 90% prediction intervals of the regression line; thus, open squares identify the outlier pairs of genomes observed (discussed in the text).

Figure 2. The *Shewanella* pangenome. ***A: Contribution of different categories of genes to the pangenome as a function of ANI***. The genes that differed in all pair-wise whole-genome comparisons among the ten *Shewanella* genomes (45 comparisons in total) were assigned to five major functional categories (graph legend). The number of genes in each category, expressed as a fraction of the total genes that differed between the two genomes (y-axis), is plotted against the genomic ANI value of the two genomes compared. Individual data-points representing each comparison have been removed for clarity; only trendlines representing the mean and bars representing one standard deviation from the mean are shown instead. ***B: Comparisons to the enterics pangenome***. The number of genes that remained conserved (y-axis) with the inclusion of more genomes in the analysis is plotted against the number of genomes (x-axis) used (light colors). The total number of non-redundant unique genes in all genomes used is also shown (dark colors). Bars represent one standard deviation based on 10 random combinations in adding the genomes to the analysis.

Figure 3. Genome vs. proteome comparisons among nine *Shewanella* strains. The protein profiles of nine *Shewanella* strains were compared based on the 2,128 core genes (**Panel A**) and the 4,300 genes found in the genome of strain MR-1 (**Panel B**) for gene expression, and the nine strains were subsequently clustered based on their overall similarity in the expression patterns of these two gene sets as follows: For each gene set, a full (all genes by all genomes) 0/1 matrix was built, with 1 denoting expression (defined as the detection of at least 2 unique peptides per protein) and 0 denoting no expression of the corresponding protein; the derived matrices were clustered as described in the supplementary material and the resulting cladograms are shown. Similarly, the nine strains were also clustered based on the presence/absence of the 4,300 MR-1 gene orthologs in their genome (sequence comparisons, **Panel C)**. A maximum likelihood phylogenetic tree of the concatenated alignment of 1,507 single-copy core genes that had no detectable signal for recombination by Phi Test analysis (22) is also shown (**Panel D**). Scale bars represent percent similarity in the derived matrices for panels A, B, and C; and number of substitution per site for panel D.

Figure 4. Modeling bacterial genotypic and phenotypic conservation across an evolutionary gradient. The presence of orthologous proteins, TonB outer membrane receptors, cytochromes, methyl-accepting chemotaxis proteins (MCPs), transcriptional

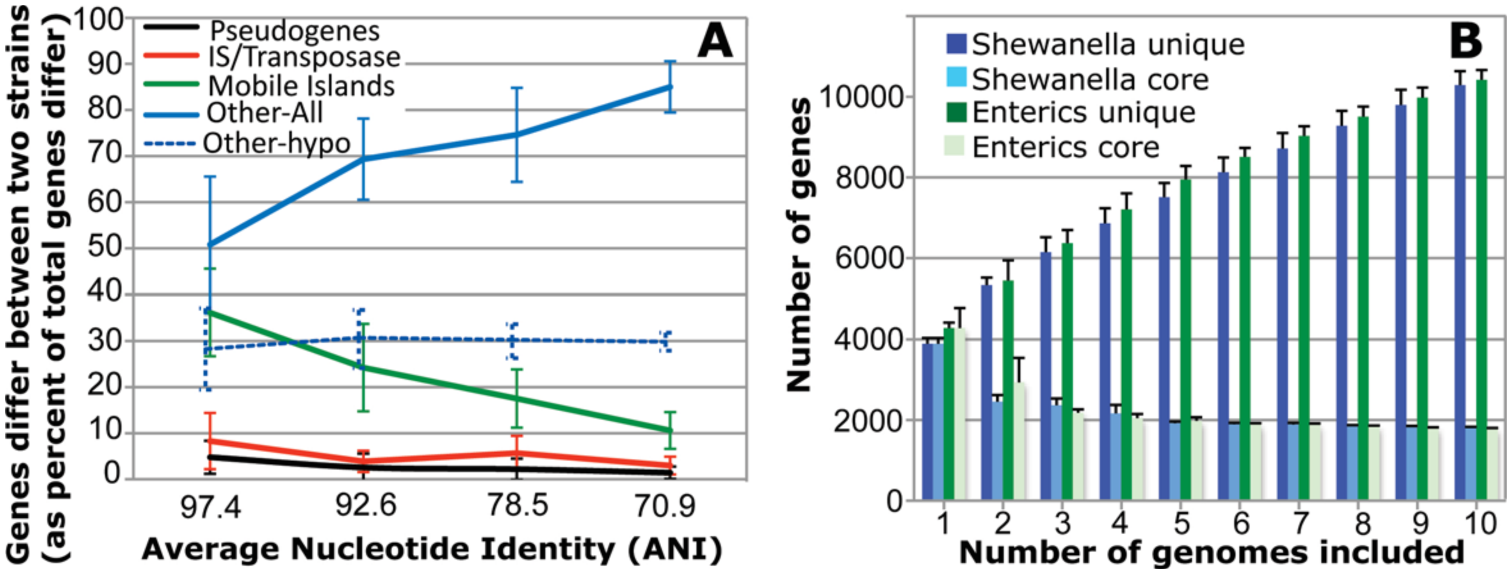
regulators, metabolic pathways, protein expression patterns, and reduction of metal or metalloids (anaerobic growth) was determined for the ten *Shewanella* strains (see methods). Each of the traits was compared among the *Shewanella* strains in a pair-wise manner (45 comparisons in total). The fraction of shared traits was determined for each pair of strains and plotted against the average nucleotide identity (ANI) of the respective strain pair. The inserted graph depicts the relationships between conservation of the traits and evolutionary distance using linear regression trendlines adjusted to intersect with the x and y-axis at 100%. The r-squared values of the regressions are also shown (figure legend).

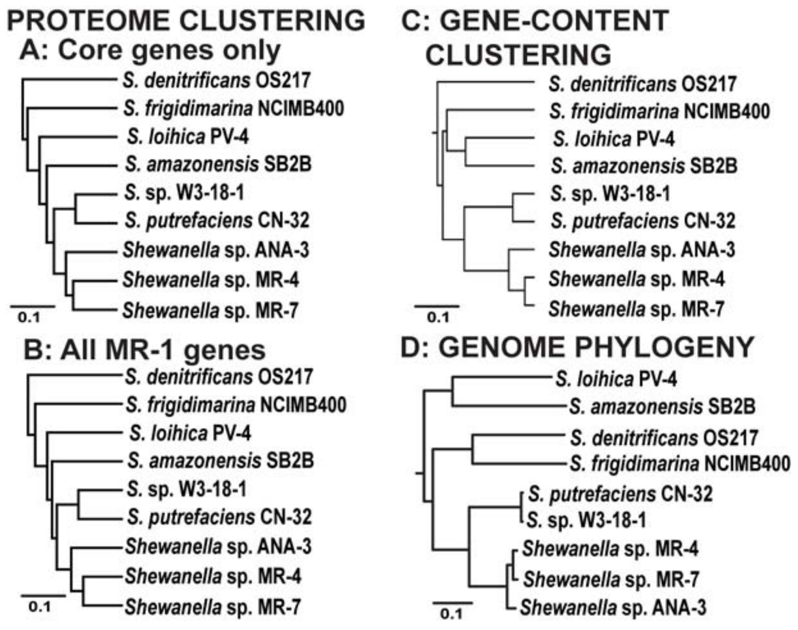


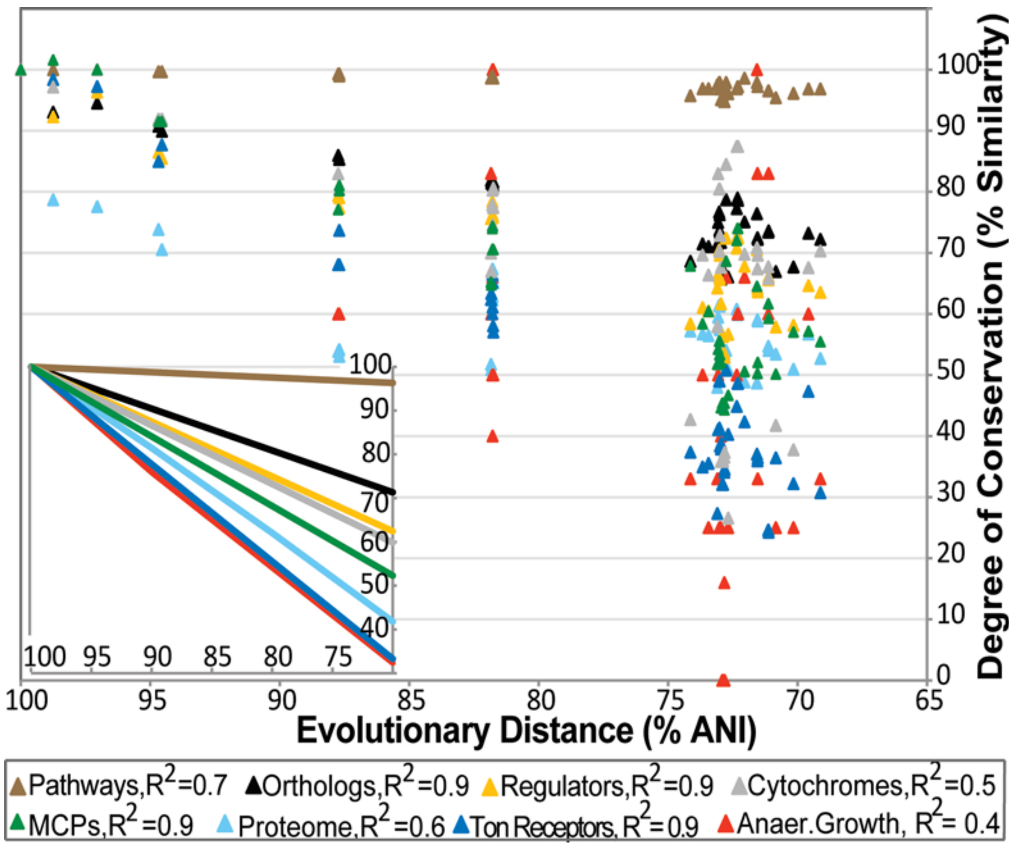
A. The Geographic origin

B. The 16S rDNA tree

C. An evolutionary gradient


A
Pseudogenes
IS/Transposase
Mobile Islands
Other-All
Other-hypo
Genes differ between two strains
(as percent of total genes differ)
100
90
80
70
60
50
40
30
20
10
0
97.4
92.6
78.5
70.9
Average Nucleotide Identity (ANI)
B
Shewanella unique
Shewanella core
Enterics unique
Enterics core
Number of genes
10000
8000
6000
4000
2000
0
1
2
3
4
5
6
7
8
9
10
Number of genomes included


PROTEOME CLUSTERING
A: Core genes only
S. denitrificans OS217
S. frigidimarina NCIMB400
S. loihica PV-4
S. amazonensis SB2B
S. sp. W3-18-1
S. putrefaciens CN-32
Shewanella sp. ANA-3
Shewanella sp. MR-4
Shewanella sp. MR-7
0.1
B: All MR-1 genes
S. denitrificans OS217
S. frigidimarina NCIMB400
S. loihica PV-4
S. amazonensis SB2B
S. sp. W3-18-1
S. putrefaciens CN-32
Shewanella sp. ANA-3
Shewanella sp. MR-4
Shewanella sp. MR-7
0.1
C: GENE-CONTENT CLUSTERING
S. denitrificans OS217
S. frigidimarina NCIMB400
S. loihica PV-4
S. amazonensis SB2B
S. sp. W3-18-1
S. putrefaciens CN-32
Shewanella sp. ANA-3
Shewanella sp. MR-4
Shewanella sp. MR-7
0.1
D: GENOME PHYLOGENY
S. loihica PV-4
S. amazonensis SB2B
S. denitrificans OS217
S. frigidimarina NCIMB400
S. putrefaciens CN-32
S. sp. W3-18-1
Shewanella sp. MR-4
Shewanella sp. MR-7
Shewanella sp. ANA-3
0.1


Degree of Conservation (% Similarity)
0
10
20
30
40
50
60
70
80
90
100
Evolutionary Distance (% ANI)
100
95
90
85
80
75
70
65
100
90
80
70
60
50
40
100
95
90
85
80
75
Pathways,R^2=0.7
Orthologs,R^2=0.9
Regulators,R^2=0.9
Cytochromes,R^2=0.5
MCPs,R^2=0.9
Proteome,R^2=0.6
Ton Receptors, R^2=0.9
Anaer.Growth, R^2= 0.4