SYSTEMS MICROBIOLOGY

Towards environmental systems biology of *Shewanella*

James K. Fredrickson, Margaret F. Romine*, Alexander S. Beliaev*, Jennifer M. Auchtung‡, Michael E. Driscoll§, Timothy S. Gardner§, Kenneth H. Nealson||, Andrei L. Osterman¶, Grigoriy Pinchuk*, Jennifer L. Reed#, Dmitry A. Rodionov¶, Jorge L. M. Rodrigues**, Daad A. Saffarini‡‡, Margrethe H. Serres§§, Alfred M. Spormann||||, Igor B. Zhulin¶¶ and James M. Tiedje‡*

Abstract | Bacteria of the genus *Shewanella* are known for their versatile electron-accepting capacities, which allow them to couple the decomposition of organic matter to the reduction of the various terminal electron acceptors that they encounter in their stratified environments. Owing to their diverse metabolic capabilities, shewanellae are important for carbon cycling and have considerable potential for the remediation of contaminated environments and use in microbial fuel cells. Systems-level analysis of the model species *Shewanella oneidensis* MR-1 and other members of this genus has provided new insights into the signal-transduction proteins, regulators, and metabolic and respiratory subsystems that govern the remarkable versatility of the shewanellae.

Dissimilatory
An enzymatic reaction in which a compound is oxidized or reduced but is not assimilated or incorporated into cells for the purposes of biosynthesis during, for example, respiration.

Electron acceptor
An oxidant used during cellular respiration.

**Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington 99352, USA. ‡Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824, USA. Correspondence to J.K.F. and J.M.T.*
e-mails: jim.fredrickson@pnl.gov; tiedjej@msu.edu
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The modern-day *Shewanella* era began in 1988 with the publication of the description of *Shewanella oneidensis* MR-1 (formerly *Alteromonas putrefaciens*), which was capable of dissimilatory metabolism of manganese and iron oxides[1]. Among the notable properties of *S. oneidensis* MR-1 was its ability to transfer electrons to solid metal oxides and its remarkable anaerobic versatility (it can use more than ten different electron acceptors). It also produced sulphide from thiosulphate (an almost diagnostic trait for the *Shewanella* group) and reduced elemental sulphur, a property that is not commonly associated with facultative aerobic bacteria. Isolation of similar microorganisms soon followed: abundant populations were identified at redox interfaces in the Black Sea[2] and many other marine[3,4] and non-marine[5–7] shewanellae were found to be iron and manganese reducers. In fact, shewanellae had been in culture collections since the middle of the twentieth century, but were identified as *Pseudomonas* (or *Alteromonas*) *putrefaciens*[5–8]. What was new was the discovery of its reductive capacities, although there were many clues to its dissimilatory abilities in several publications before its recognition as *Shewanella* in the late 1980s[6,8–11].

It was soon realized that the versatility of *Shewanella* spp. extended to other 'non-standard' metal electron acceptors, such as uranium [U(VI)][3], chromium [Cr(VI)][12], iodate[13], technetium[14], neptunium[15], plutonium[16], selenite[17], tellurite[17] and vanadate[18], and even the reduction of nitroaromatic compounds by some strains[19,20]. These diverse metabolic capabilities provide shewanellae with considerable potential for the remediation of environments that are contaminated with radionuclides[21]. Furthermore, our analyses of the genome sequences of various strains suggest that, collectively, the shewanellae can use a broad range of carbon substrates. Given their widespread distribution in environments where organic matter is actively degraded, shewanellae should also prove useful as model organisms for obtaining systems-level insights into carbon-cycling processes.

Shewanellae are common members of complex communities in aquatic and sedimentary systems that are chemically stratified on a permanent or seasonal basis[22]. To be competitive in such environments and to respond to the available resources, shewanellae must have robust sensing and regulatory systems. Therefore, genome-centric approaches are being used to study these systems in order of increasing complexity, from individual genes and operons to populations and communities. Because most studies have used genetics and genomics to understand metabolism and regulation, initial systems analyses have focused on *S. oneidensis* MR-1, the first genome of the shewanellae to be sequenced[23]. These analyses have used complementary top-down and bottom-up approaches. In top-down investigations, bioinformatics-based

Author addresses

[§]Program in Bioinformatics, Boston University, Boston, Massachusetts 02215, USA.
[‖]Department of Earth Sciences, University of Southern California, Los Angeles, California 90089, USA.
[¶]Burnham Institute for Medical Research, La Jolla, California 92037, USA.
[#]Department of Chemical and Biological Engineering, University of Wisconsin, Madison, Wisconsin 53706, USA.
[**]Department of Biology, University of Texas, Arlington, Texas 76019, USA.
[‡‡]Department of Biological Sciences, University of Wisconsin, Milwaukee, Wisconsin 53211, USA.
[§§]Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, Marine Biological Laboratory, Woods Hole, Massachusetts 02543, USA.
[‖‖]Departments of Biological Sciences, Chemical Engineering, Civil and Environmental Engineering, and Geological and Environmental Sciences, Stanford University, Stanford, California 94305, USA.
[¶¶]Joint Institute for Computational Sciences, The University of Tennessee — Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA.

genome functional predictions are made using a range of tools and resources (TABLE 1); high-throughput expression analyses and functional genomics also uncover key genes, as well as metabolic and regulatory networks. The bottom-up component uses genetic, physiological and biochemical approaches to test or verify predictions made by the top-down approaches. The coupling of top-down and bottom-up approaches provides a powerful way to describe the function and regulation of metabolic subsystems and, ultimately, to link these subsystems to descriptions of signal-transduction and transcriptional regulatory systems.

Although research efforts have largely focused on *S. oneidensis* MR-1, investigations of shewanellae are being extended to other members of the genus and are taking advantage of the availability of genome sequences from 23 other *Shewanella* strains that are either complete or in progress (TABLE 2). This has allowed the use of comparative genomics to gain insight into the evolution and environmental adaptations of members of the genus. The availability of such a large number of related genome sequences provides the basis for improving our systems-level analysis and understanding of *S. oneidensis* MR-1 and for developing a systems-level understanding of *Shewanella* as a genus.

In this Review, we describe the approaches and insights into *Shewanella* spp. that are being gained at the systems-level and particularly focus on the sensing, regulation and metabolic subsystems within the model organism *S. oneidensis* MR-1. We then broaden our discussion to investigations of *Shewanella* as a genus by using sequenced strains to probe evolution and speciation of this ecologically successful group of organisms. As the field of *Shewanella* systems biology is still in its infancy, we include new information that is primarily based on the analysis of multiple *Shewanella* genome sequences, as well as a review of recent publications. Although the focus of this Review is the *Shewanella* genus, our approach of using genomics and modelling to develop predictions and then experimentally evaluate these predictions can readily be applied to other microorganisms for which multiple genome sequences of closely related species are available. It should also be noted that although we provide specific examples if available, not all of the predictions and conjectures reported here have been tested experimentally.

One-component system
A regulatory protein that combines both sensory and regulatory capabilities that usually reside in two distinct domains. The repressor of the lactose operons (LacI) and the catabolite activator protein of *Escherichia coli* are classical examples.

Two-component system
A regulatory system that is typically composed of two proteins, a sensor histidine kinase and a cognate response regulator. EnvZ and OmpR of *Escherichia coli* are classical examples.

PAS domain
A ubiquitous sensory domain that is found in many one-component and two-component regulatory systems in prokaryotes, as well as in regulatory proteins in eukaryotes. The PAS domain was named after three proteins that it was found in: Per (period circadian protein), Arnt (receptor nuclear translocator protein) and Sim (single-minded protein).

Developing systems-level understanding

Sensing, signal transduction and regulation. Signal-transduction regulatory systems link extracellular and intracellular cues to cellular responses through networks that consist of receptors, transmitters of information and regulators. Because of the ability of *Shewanella* species to thrive in a range of carbon-resource and redox environments, metabolic and regulatory plasticity seems to be one of the keys to the ecological success of the species. Indeed, analysis of the *S. oneidensis* strain MR-1 genome sequence[23] identified 211 one-component systems and 47 two-component systems, which supports the proposed complexity of the energy-conversion pathways in this organism. Most of our information on sensing and regulation in *Shewanella* spp. has come from computational predictions, which, in turn, provide targets for experimental validation and further understanding of signal-transduction complexity. One tool that is useful for the study of these systems is the microbial signal-transduction (MiST) database[24] (TABLE 1), which contains detailed information on predicted signal-transduction genes and proteins from hundreds of microbial genomes and has allowed *S. oneidensis* MR-1 to be compared with *Escherichia coli* and other model organisms.

One- and two-component systems. Protein-sequence analyses combined with cross-genome and inter-genome comparisons revealed that three classes of signal-transduction proteins are significantly enriched in *S. oneidensis* MR-1 compared with most other sequenced gammaproteobacteria: chemotaxis proteins, sensors that contain PAS domains (the ubiquitous sensory domain that has been implicated in detecting environmental signals, such as light, oxygen and chemicals that affect the redox potential[25]) and modulators of second-messenger cyclic nucleotide signalling.

In contrast to *E. coli*, which uses a single set of chemotaxis genes and only five dedicated chemoreceptors[26], *S. oneidensis* MR-1 is predicted to have a more complex chemotaxis system that includes 3 sets of chemotaxis genes and 27 chemoreceptors. This suggests that for *Shewanella* spp., active exploration of the environment by navigation to niches that are favourable for metabolism and growth[27] is vital. One of these systems has been shown to be essential for energy taxis in *S. oneidensis* MR-1, as inactivation of the *cheA* gene, which is located in the third chemotaxis cluster, abolished the chemotactic response to anaerobic electron acceptors[28], whereas genome-sequence analysis suggests that a separate *che* gene system is degenerate[29]. These results are consistent with earlier physiological studies, which suggested that *Shewanella* spp. demonstrate strong chemotactic behaviour towards electron-acceptor substrates rather than sugars and amino acids[30]. Notably, *S. oneidensis* MR-1 also has more than twice as many PAS domain-containing receptor proteins as *E. coli*. Significant enrichment in PAS domains indicates that these sensors might be involved in the adaptation of *Shewanella* spp. to redox-stratified environments.

Table 1 | **Resources for bioinformatics of *Shewanella* spp.**

Resource; web page	Description	Ref.
SEED; http://theseed.uchicago.edu/FIG/index.cgi	Web resource that provides an environment for defining cellular subsystems from comparative genome-sequence analysis	67
IMG; http://img.jgi.doe.gov/cgi-bin/pub/main.cgi	Web resource for comparative analysis of genomes	108
MiST; http://genomics.ornl.gov/mist/	Web resource for analysis of signal-transduction proteins	24
BioCyc; http://biocyc.org/	Software environment for creating metabolic pathway databases	81
M^{3D}; http://m3d.bu.edu/cgi-bin/web/array/index.pl?section=home	Web resource for analysing and retrieving gene-expression data for *Shewanella oneidensis* MR-1	44

Cyclic nucleotide signalling. Cyclic nucleotide signalling is important for many cellular processes in both eukaryotic and prokaryotic cells[31,32]. Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is emerging as an important intracellular signalling molecule that controls diverse functions, such as cellulose synthesis, biofilm formation, motility and bacterial virulence in gammaproteobacteria[33,34]. c-di-GMP is synthesized by diguanylate cyclases and hydrolysed by phosphodiesterases; these proteins can be identified by the presence of domains with the canonical amino acid sequence motifs GGDEF or EAL, respectively[34]. In contrast to transcriptional regulators, there is poor correlation between the number of GGDEF-domain- and EAL-domain-containing proteins per genome and the size of a bacterial genome, which suggests that these proteins are involved in the post-transcriptional and translational control of cellular processes[35]. Computational analysis predicts that the *S. oneidensis* MR-1 genome contains 51 diguanylate cyclases, 27 phosphodiesterases, and 20 hybrid diguanylate cyclase or phosphodiesterase proteins, which places *S. oneidensis* MR-1 into a group of sequenced microorganisms with the highest normalized number of c-di-GMP signalling proteins. This observation reveals the increased importance of post-transcriptional control of cellular processes by numerous environmental signals in *Shewanella* spp., compared with other model organisms.

In many bacteria, cyclic AMP (cAMP), another important cyclic nucleotide, is needed for activation of the cAMP receptor protein (CRP), which is best known for its role in the regulation of carbon metabolism, although it can also regulate antibiotic production, phototrophic growth and pathogenesis[36]. In *S. oneidensis*, however, CRP has also been linked to the regulation of anaerobic respiration[37], whereas in other bacteria, anaerobic growth is governed by the redox-sensing protein FNR (fumarate nitrate-reduction regulator)[38]. Interestingly, the *S. oneidensis* electron-transport regulator A (EtrA), which shares a high degree of amino acid sequence identity with the *E. coli* FNR protein (73.6%)[39], is not essential for anaerobic growth and reduction of electron acceptors by MR-1 (REFS 40,41). By contrast, *S. oneidensis* MR-1 *crp* mutants are deficient in the anaerobic reduction of Fe(III), Mn(IV), fumarate, nitrate and dimethyl sulphoxide (DMSO)[37]. The mechanism of CRP activation under anaerobic conditions is unknown, as CRP lacks obvious redox-sensing domains and therefore is not expected to respond to redox changes. Detection of anaerobic fumarate reductase activity in aerobic cultures supplemented with cAMP suggests that the control of CRP activity under anaerobic conditions might be due to the regulation of adenylate cyclase activity. The genome of *S. oneidensis* MR-1 encodes three putative adenylate cyclase genes, including *cyaA* and *cyaC*, both of which are required for anaerobic growth with fumarate, Fe(III) and DMSO as electron acceptors[42]. Given that redox-stratified environments are subject to spatial and temporal variations in the type and concentration of electron-acceptors that are present, the functional switch between FNR and CRP in *Shewanella* spp.[37,40] might represent a regulatory adaptation to the dynamic shifts in external redox conditions. Regulation by cAMP and CRP could therefore provide the organism with the flexibility that is needed for fine-tuning of metabolic pathways. Analysis of additional *Shewanella* species will reveal whether regulation by cyclic nucleotide signalling is a hallmark of this genus.

Transcriptional regulatory networks. Systems-level approaches have been useful in the elucidation of some of the mechanisms that are involved in growth transitions in different environments. For example, a recent study used global transcriptome analysis to investigate the gene-expression changes that occur under elevated oxygen concentrations, a condition that causes autoaggregation (cell–cell adhesion) of *S. oneidensis* MR-1 cells[43]. These results demonstrated that expression of a large number of genes which encode surface-attachment factors and anaerobic respiratory proteins increased in aggregated cells from aerobic cultures and that aggregation was governed through coordinate regulation by the RpoS, SpoIIA and CRP transcription factors[43]. Aggregate formation in *S. oneidensis* MR-1 could serve as an alternative or as an addition to biochemical detoxification to reduce the oxidative stress that is associated with the production of reactive oxygen species during aerobic metabolism and facilitate the development of hypoxic conditions within the aggregate interior[43].

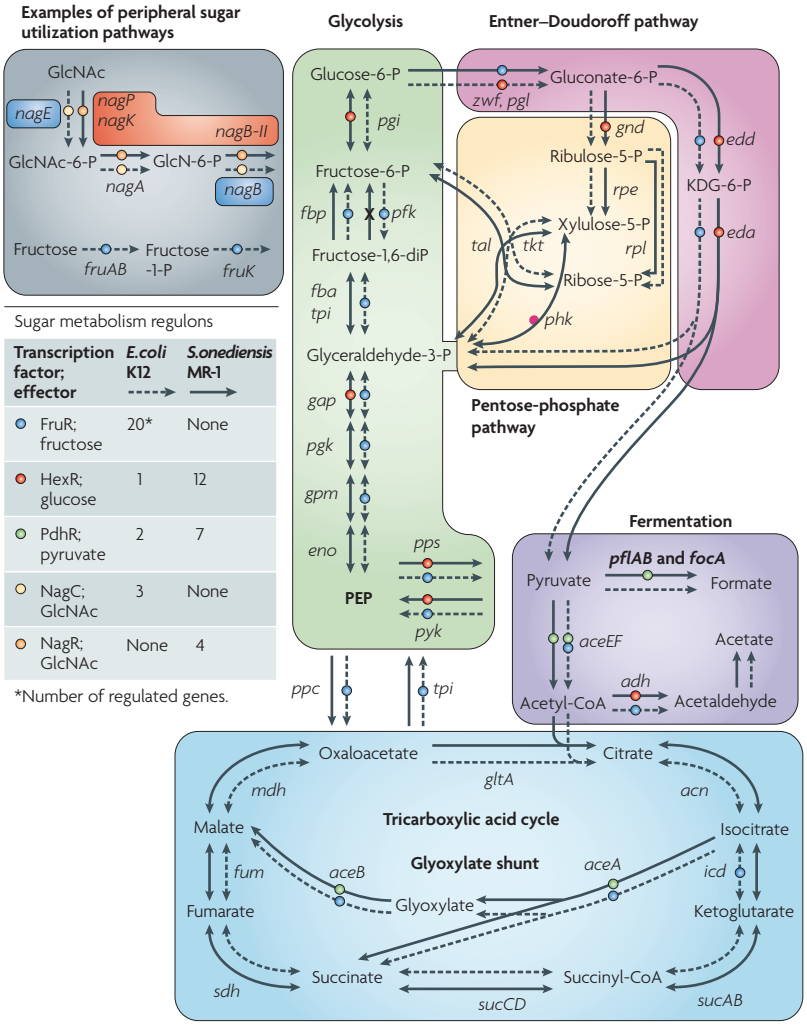
Diguanylate cyclase
An enzyme that synthesizes cyclic diguanylic acid and typically contains the canonical amino acid motif GGDEF.

Table 2 | **Sequenced genomes of *Shewanella* spp. and their habitats**

Species or strain	Geographical origin	Isolation-site characteristics	Ref.
Shewanella amazonensis SB2B*	Amapa River, Brazil	Sediment; suboxic redox conditions; 1 m	109
Shewanella baltica OS155	Baltic Sea	Sea-water; oxic zone; 2 ml per litre of oxygen; 90 m	110
S. baltica OS185	Baltic Sea	Sea-water; oxic–anoxic interface; 120 m	110
S. baltica OS195	Baltic Sea	Sea-water; anoxic zone; 140 m	110
S. baltica OS223	Baltic Sea	Sea-water; oxic–anoxic interface; 120 m	110
Shewanella benthica KT99	Tonga-Kermadec Trench	Deep sea; high-pressure zone; 9,000 m	111
Shewanella denitrificans OS217*	Baltic Sea	Sea-water; oxic–anoxic interface; 120 m	82
Shewanella frigidimarina NCIMB 400*	Coast of Aberdeen, United Kingdom	Sea-water; North Sea	112
Shewanella halifaxensis HAW-EB4	Halifax Harbor, Nova Scotia, Canada	Sediment; munitions dumping area; 215 m	113
Shewanella livingstonensis Ac10	Coast of Antarctica	Sea-water	114
Shewanella loihica PV-4*	Hawaiian Sea mount, United States	Iron-rich mat; hydrothermal vent; 1,325 m	115
Shewanella oneidensis MR-1*	Lake Oneida, New York, United States	Sediment; anaerobic; Mn(IV) reduction	1
Shewanella pealeana ANG-SQ1	Woods Hole Harbor, Massachusetts, United States	Squid nidamental gland	116
Shewanella piezotolerans WP3	West Pacific site WP (142°E, 8°N)	Sediment; under 1,914 m of water	117
Shewanella putrefaciens CN-32*	Albuquerque, New Mexico, United States	Subsurface; shale sandstone; 250 m	5
S. putrefaciens 200	Alberta, Canada	Crude-oil pipeline	6
Shewanella sediminis HAW-EB3	Halifax Harbor, Nova Scotia, Canada	Sediment; 50 nautical miles from shore; 215 m	19
Shewanella sp. ANA-3*	Woods Hole, Massachusetts, United States	Brackish water; arsenic-treated wooden pier	118
Shewanella sp. MR-4*	Black Sea	Sea-water; oxic zone; 16°C; 5 m	2
Shewanella sp. MR-7 *	Black Sea	Sea-water; anoxic zone; high NO_3; 60 m	2
Shewanella sp. W3-18-1*	Washington coast, Pacific Ocean	Marine sediment; under 997 m of oxic water	119
Shewanella violacea DSS12	Ryuku Trench, Philippine Sea	Sediment; 5,110 m	120
Shewanella woodyi MS32	Strait of Gibraltar, Mediterranean Sea	Detritus; 370 m	121

*First ten sequenced strains used for core genome analysis.

Systems-level understanding also requires the application of high-throughput methods that provide a holistic view of the system. One approach that is being used to generate a genome-wide transcriptional regulatory network (TRN) for *S. oneidensis* MR-1 is the application of a mutual information-based algorithm that is known as context likelihood of relatedness (CLR[44]). CLR is a network inference method that uses transcriptional profiles of an organism across a diverse set of conditions to systematically determine transcriptional regulatory interactions. CLR was applied to a compendium of 245 microarrays to generate a regulatory-network interaction map of *S. oneidensis* MR-1. The most densely interconnected region of the resulting network contains three regulators of unknown function that are linked to genes which are associated with several well-studied respiratory subsystems. Among these genes are *omcA-mtrCAB*, which encode a set of outer-membrane-associated proteins that are essential for the reduction of iron, manganese and other metals in *S. oneidensis* MR-1 (REFS 45–48). Mutation of one regulator resulted in growth deficiency with several electron acceptors, including oxygen, whereas mutation of another regulator resulted in alterations in the ability of electron acceptors to reduce sulphur, thiosulphate or sulphite (D.A.S., unpublished observations). This demonstrates how this approach can be used to determine the function of previously uncharacterized genes. Follow-on experiments with these mutants are currently under way and could provide important new insights into the regulation of metal reduction in *S. oneidensis* MR-1, an elusive topic that is not yet fully understood[49].



Sugar metabolism regulons

Transcription factor; effector	*E.coli* K12 ⟶	*S.onediensis* MR-1 ⟶
FruR; fructose	20*	None
HexR; glucose	1	12
PdhR; pyruvate	2	7
NagC; GlcNAc	3	None
NagR; GlcNAc	None	4

*Number of regulated genes.

Figure 1 | **Rewiring of transcriptional regulatory networks in the central carbon metabolism of *Shewanella oneidensis* MR-1.** In the displayed fragment of metabolic reconstruction, biochemical transformations in *Escherichia coli* K12 and *S. oneidensis* MR-1 are shown by dashed and non-dashed arrows, respectively. Genes that encode the respective enzymes in the network are referred to by their commonly used names, except for those that are subject to non-orthologous replacement (for example, the *nag* operon[52]). Transcriptional regulation by the transcription factors that that have been detected in *E. coli* or inferred in *S. oneidensis* by comparative genomic analysis of transcription-factor binding sites[50] is marked by colour-coded dots (as described in the table). GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; KDG, 2-keto-3-deoxy-6-phosphogluconate; PEP, phosphoenolpyruvate; P, phosphate.

The comparative genomic approach for TRN reconstruction combines genome-context analysis with the tentative identification of transcription-factor binding sites in groups of related genomes. Various bioinformatic and experimental studies have demonstrated the power of this approach in the analysis of complex regulatory networks and their evolution in bacteria (reviewed in REF. 50). Comparative analysis of 13 *Shewanella* spp. genomes resulted in a reconstruction of TRN in *S. oneidensis* MR-1 that included 60 transcription factors, approximately 400 transcription-factor binding sites and more than 1,000 target genes. Only half of these transcription factors were defined as orthologues of transcription factors that had previously been characterized in *E. coli*. Several novel regulons were identified, including those that control pathways of degradation of branched-chain amino acids, fatty acids and various carbohydrates. The predicted novel NAD metabolism regulon NrtR was experimentally validated for *S. oneidensis* MR-1 (REF. 51).

Comparison of the tentatively reconstructed *S. oneidensis* MR-1 TRN with the established TRN in *E. coli* revealed striking differences in the regulatory strategies that are used by these two gammaproteobacteria, including the expansion and shrinking of regulons, as well as cases in which non-homologous regulators were used to control equivalent pathways or homologous regulators were used to control distinct pathways. Most remarkably, in one case, TRN rewiring for central carbohydrate utilization pathways was observed (FIG. 1). In *E. coli* K-12, the global regulator FruR controls central carbon metabolism and fructose utilization, whereas the local regulator HexR controls only the *zwf* gene. By contrast, the FruR regulon is absent from *Shewanella* spp., whereas the comparative genomic reconstruction of the HexR regulon predicts a global regulatory role of HexR for genes that are involved in central carbohydrate metabolism. Comparative analysis of candidate PdhR binding sites predicts that the pyruvate-responsive regulon, which solely controls pyruvate dehydrogenase (*pdhR*) in *E. coli*, undergoes radical expansion to include the glyoxylate shunt and formate fermentation genes in *Shewanella* spp. Reconstruction of local regulons for sugar catabolic pathways in *Shewanella* spp. revealed that they are substantially different from *E. coli*. For example, the *N*-acetylglucosamine utilization pathway in *E. coli* is regulated by the NagC repressor, whereas a non-homologous NagR regulator was predicted to control this pathway in *Shewanella* species[52].

Given that signal transduction and transcription regulatory systems control most cellular functions, understanding the mechanisms and levels of control by these systems is essential for building a comprehensive systems-level model of *S. oneidensis* MR-1 and its related species. Here, we have highlighted the progress that has been made towards this goal. However, insights into the molecular mechanisms of energy taxis and chemosensory behaviour of *S. oneidensis* MR-1 and their contribution to the ecophysiology of this organism also benefit both our fundamental understanding of energy metabolism by bacteria and practical applications by laying the groundwork for the manipulation of strains to improve capabilities for metal reduction, carbon metabolism and electrochemical activity in biofuel cells.

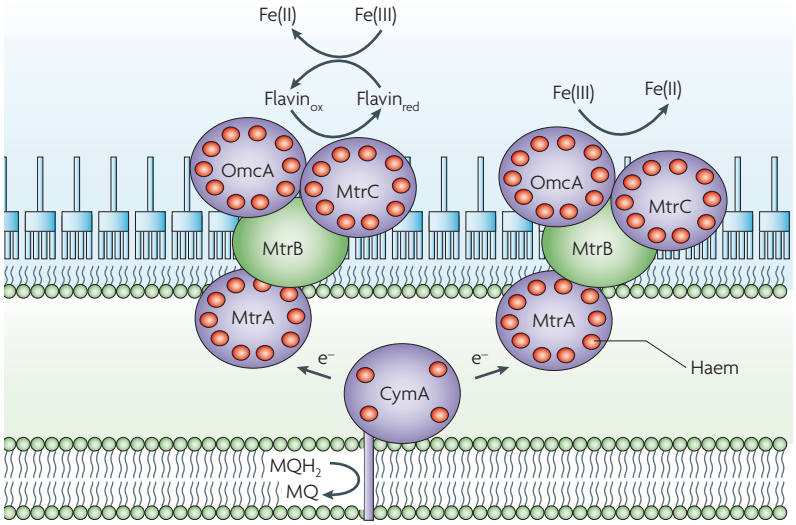
Metabolic networks

The ability of *Shewanella* spp. to compete and thrive in chemically stratified and redox-stratified environments is also reflected by the organization and flexibility of energy-generating and biosynthesis networks that include electron transport and central and peripheral carbon-metabolism pathways.

Electron-transport pathways. Analysis of the *S. oneidensis* MR-1 genome sequence predicts a highly diverse electron-transport system that includes 42 putative *c*-type cytochromes[53]. *S. oneidensis* MR-1 is known to be

Regulon
A set of genes that is controlled by a common transcription factor or regulatory RNA. A regulon usually includes genes that are implicated in a common cellular subsystem or pathway.

Box 1 | **Extracellular electron transfer**



The revelation that *Shewanella* are capable of 'solid-state electron transfer' to solid metal oxide substrates led Kim and colleagues[94] of the Korean Institute of Science and Technology to use these microorganisms in microbial fuel cells, which he has been studying in his laboratory for several years. As he suspected, it was possible to obtain good yields of current in the absence of an exogenous electron shuttle (mediator)[94,95], and subsequent investigations by many laboratories have led to high power yields[96]. Although the mechanism by which *Shewanella* donate electrons to the anode of the fuel cell or to the surface of metal oxides has not been fully elucidated, genetic[46,47,97] and biochemical investigations are generally consistent with the involvement of the multi-haem *c*-type cytochromes MtrC (also known as OmcB) and OmcA (see the figure). These cytochromes are lipoproteins[98] that are associated with the outer membrane and are translocated across the outer membrane by the type II protein-secretion pathway[99–101], where they are exposed to the extracellular environment[102] and can come in direct contact with mineral or electrode surfaces. *In vitro*, these proteins bind and reduce solid-phase metal oxides[103,104]. The haems within MtrC titrate over a broad potential from approximately +100 to –500 mV (standard hydrogen electrode (SHE)) and electron paramagnetic resonance spectra were consistent with the presence of magnetically spin-coupled, low spin *c*-haems[105]. The electrochemical properties of MtrC haems (for example, alignment, electrostatic environment and solvent exposure) are compatible with direct, intermolecular electron exchange to solid surfaces. *Shewanella oneidensis* MR-1 and other *Shewanella* strains have also been shown to secrete flavins into the medium, which can potentially shuttle electrons from cytochromes on the cell surface to external electron acceptors[106,107].

dependent on *c*-type cytochromes for the maintenance of respiratory versatility, as mutants that lack the functional cytochrome *c* maturation genes *ccmC*[54] or *ccmB*[55] are completely compromised in their ability to respire anaerobically. Together with the *c*-type cytochromes, periplasmic (MtrA and DmsE) and outer-membrane (MtrC and OmcA) decahaem *c*-type cytochromes, an inner-membrane tetrahaem cytochrome *c* protein (CymA), and an outer-membrane protein (MtrB or DmsF) have been linked to metal reduction[45–47,56,57] as mediators of extracellular electron transport to Fe(III) and Mn(IV) oxides and to DMSO[58]. These cytochromes and their associated proteins have a key role in extracellular electron transfer from *S. oneidensis* MR-1 to metal oxides and carbon electrodes in microbial fuel cells (BOX 1). As for the remaining *c*-type cytochromes, only a small number can be assigned to systems that are analogous to those characterized in other organisms and few of these systems have been experimentally validated, with the exception of those that are involved in the respiration of fumarate[59,60], nitrate[61], DMSO[58] and trimethylamine *N*-oxide (TMAO)[62]. A substantial number of the *S. oneidensis* MR-1 *c*-type cytochromes are induced by thiosulphate and nitrate[49], but additional work is required to understand the complex anaerobic electron-transport networks in *Shewanella* spp.

Carbon-metabolism pathways. Before the genome of *S. oneidensis* MR-1 was sequenced, this organism was thought to use a restricted range of substrates as carbon and energy sources, such as formate, lactate and pyruvate[63]. However, the genome annotation suggests that *S. oneidensis* MR-1 encodes multiple pathways for the catabolism of C_{1-3} organic acids, fatty acids, amino acids, peptides and nucleotides[64], which reflects its ecological role as a consumer of organic-matter breakdown products. These predictions are supported by our results from physiological experiments[65] and microarray analysis[66] and demonstrate how *S. oneidensis* MR-1 can use a range of amino acids, carboxylates, sugars and nucleosides as sole carbon and energy sources and an even greater range of amino acids, dipeptides and tripeptides as sole nitrogen sources.

To identify the genetic basis of these capabilities, a subsystems-based approach that was implemented in the SEED genomic platform[67] (TABLE 1) has been used to systematically map candidate components of these catabolic pathways and then validate selected predictions using genetic, biochemical and physiological experiments. This approach led to the discovery of the operon in *S. oneidensis* MR-1 that is responsible for the uptake and degradation of *N*-acetylglucosamine, the primary component of chitin. Predicted functions of two previously uncharacterized enzymes (encoded by *nagBII* and *nagK*) were experimentally validated and an entirely novel pathway for *N*-acetylglucosamine utilization was discovered[52].

Metabolic model of* S. oneidensis *MR-1. In addition to metabolic reconstruction, genome-scale models account for components and component interactions in biological networks, and allow *in silico* analysis of metabolism as well as the collection and analysis of metabolic and genomic data[68]. A constraint-based[69] metabolic model of *S. oneidensis* MR-1 has been built on the basis of the current genome annotation and primary literature (J.L.R., unpublished observations). The model includes 713 metabolic reactions, 780 genes and 623 metabolites, and has been used in various computational analyses, including the prediction of growth phenotypes. For example, 33 compounds were predicted by the model to support aerobic growth as sole carbon and energy sources; 14 of these predictions have been experimentally validated (for example, lactate, pyruvate and *N*-acetylglucosamine[52,66]), whereas others (such as cytidine) await experimental validation.

An integrative approach that combines bioinformatics, physiological assays and modelling was used to investigate formate metabolism by *S. oneidensis* MR-1.

Previous work[70] suggested that *S. oneidensis* MR-1 can grow on formate as a sole source of carbon and energy. In addition, a pathway for formate assimilation that combines specific isocitrate lyase serine and central carbon-metabolism-pathway components was proposed on the basis of measured enzymatic activities[70]. Our metabolic modelling assessment predicts that *S. oneidensis* MR-1 can only use formate as a sole source of carbon and energy under anaerobic conditions if pyruvate-formate lyase is used in the reverse direction (acetyl-CoA + formate → pyruvate + CoA). Although this reaction pathway has been shown to be feasible *in vitro*, it was not clear if this pathway operates *in vivo*. Further physiological studies revealed that anaerobic growth of these bacteria in the presence of formate in defined medium supplemented with Fe(III)-nitrilotriacetic acid as the electron acceptor was attributed to three amino acids that were routinely added to defined *Shewanella* media[71], whereas formate was used as electron donor[72].

These examples clearly illustrate the potential of metabolic modelling as a predictive tool to develop new hypotheses or evaluate experimental data[73]. As our insight into sensing, regulation and metabolism in *S. oneidensis* MR-1 matures, and cell-level models that integrate regulation and metabolism are developed, our understanding at the gene and network level will improve, which is a necessary prelude to a systems-level understanding of natural shewanellae populations.

Understanding the *Shewanella* genus

The *Shewanella* genus includes 48 recognized species (see Further information for a link to a List of Prokaryotic names with Standing in Nomenclature). This ecologically and physiologically diverse group includes not only free-living organisms in redox-stratified environments that vary in nutrient composition, salinity, temperature, redox potential and barometric pressure, but also species that live in association with other organisms as syntrophs, symbionts, epibionts and pathogens (reviewed in (REFS 7,21)). Seventeen *Shewanella* genomes have been fully sequenced to date, six are being sequenced and two have been assembled from the Sargasso Sea metagenomic data[74] (TABLE 2). Sequenced genomes were selected based on their known energy metabolism, bioremediation capabilities, phylogenetic relatedness and the environments they inhabit, providing an opportunity to study genomic diversity from an evolutionary framework and potentially identify functions that enable niche inhabitation or define species-level differentiation. This data set also provides a unique chance to understand the core functionality of a genus and investigate how well a systems-level understanding of a model organism can be extended to other species within a genus.

As a foundation for the comparative analyses of multiple sequenced *Shewanella* spp., we put considerable effort into refining the annotation (gene and function predictions) of the *S. oneidensis* MR-1 genome. Capitalizing on the availability of other genome sequences and the *S. oneidensis* MR-1 AMT-tag proteome database[75], we validated the expression of hypothetical proteins[76,77], identified mistakes in the genome sequence, adjusted start-codon positions[78,79], identified degenerate[29] and missed genes, and eliminated genes that were unlikely to code for proteins. Functional predictions were updated using both traditional approaches (for example, domain analysis[80]) and by exploring the literature for experimental evidence of function (mainly homologous proteins found in other bacteria). Using the Pathways Tools Software[81], these proteins have been linked to metabolic-pathway maps, which revealed erroneous functional predictions and missing functional assignments. We are currently using this highly curated annotation to improve the annotation of proteins that were deduced from other *Shewanella* genomes by identifying protein orthology and metabolic subsystems reconstruction.

To investigate functions that are characteristic of all or only some species, we evaluated protein orthology among the first ten fully sequenced, assembled genomes (TABLE 2). This group was found to share a common core genome of 2,137 proteins, which accounts for approximately half of the predicted proteins in each genome. Compared with core genomes defined in *E. coli* and *Prochlorococcus*, the *Shewanella* spp. core genome encodes a higher number of proteins that import and degrade fermentation end products (carboxylates), amines and amino acids, which reflects the abundance of these materials in the environments that these organisms inhabit[21]. The core terminal-electron acceptor complexes are restricted to those used for aerobic respiration, mainly because of the limited anaerobic respiratory capabilities of *Shewanella denitrificans* (discussed below). Likewise, proteins that are necessary for lactate, formate and hydrogen utilization and are commonly used as electron donors for experimental analyses of *Shewanella* spp. are missing from the core genome owing to their absence in *Shewanella woodyi* and/or *S. denitrificans*. Furthermore, the large number of *S. oneidensis* MR-1 proteins with a role in environmental sensing is not reflected in the *Shewanella* spp. core genome. For example, only 7 of the 27 *S. oneidensis* MR-1 chemoreceptors, none of which has a PAS-sensing domain, are shared among all of the strains. Interestingly, fewer diguanylate cyclases and phosphodiesterases are found in the core set, whereas 50% of the hybrid diguanylate cyclase–phosphodiesterase proteins are conserved across the *Shewanella* group, which could be a unique feature of this genus. This combination of gene and population data helps us to understand the relationships between habitat and genomic content in this genus.

Initial investigations into the diversity of respiratory subsystems in the sequenced *Shewanella* genomes led to several interesting observations. For example, *S. denitrificans* has a limited respiratory capacity compared with other strains[82] (M.F.R., unpublished observations). Subsystems analysis of *S. denitrificans* suggests that this species lacks the ability to synthesize menaquinone and can grow anaerobically only through dissimilatory reduction of nitrate to nitrogen. All other strains encode respiratory systems that are similar to those recognized in other bacterial genera, as well as those that involve cell-surface-localized electron-transfer proteins that provide their hosts with

AMT-tag proteome database
An accurate mass and time (AMT)-tag proteome database that contains identifications for tryptic peptides based on analysis by capillary liquid chromatography followed by tandem mass spectrometry of various cellular lysates of a single organism.

Core genome
In the context of this Review, the core genome refers to the set of proteins that have been found in each *Shewanella* genome analysed to date and are of high sequence similarity and are therefore predicted to encode the same function.

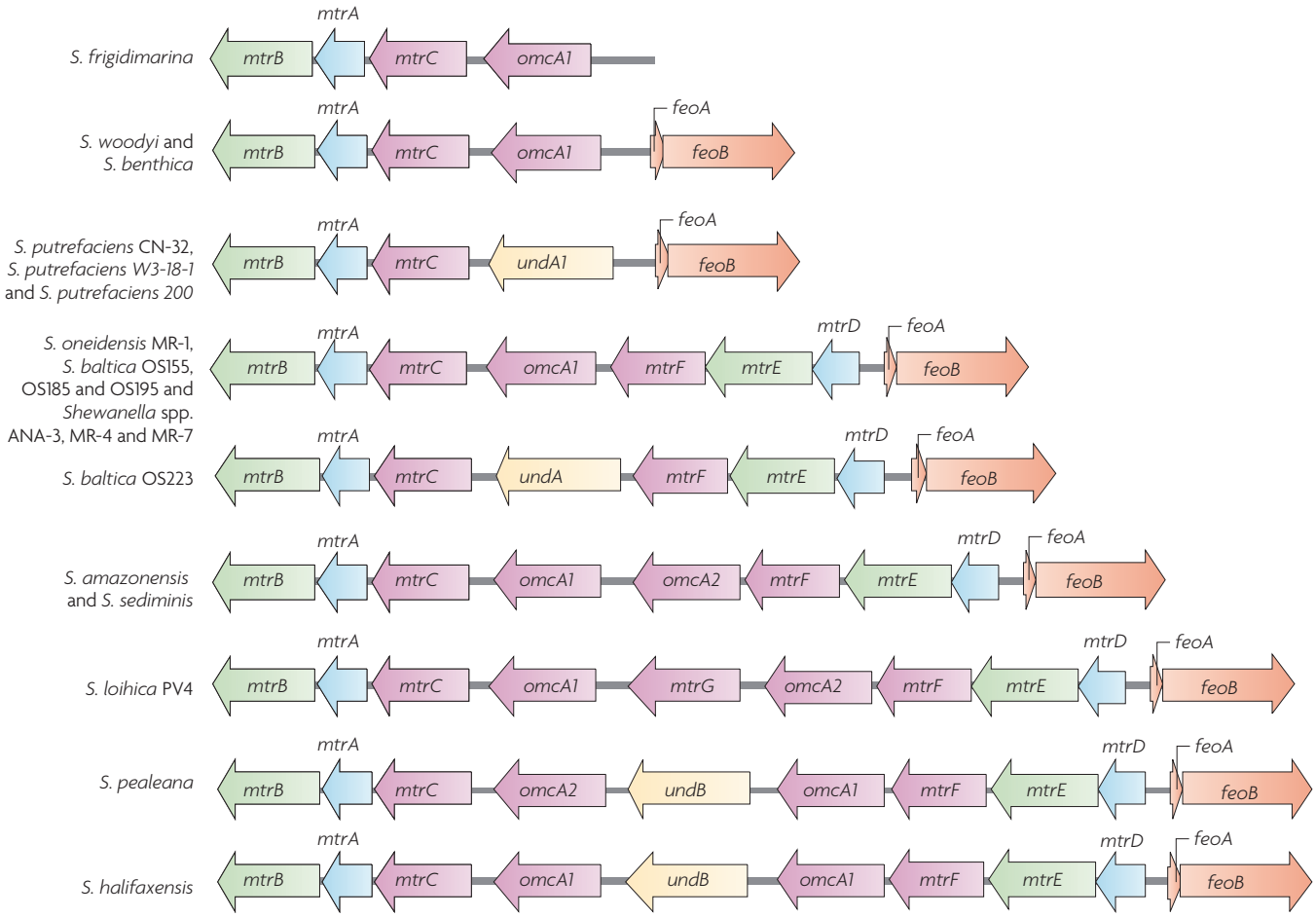


Figure 2 | **Comparison of the metal-reductase-containing locus in *Shewanella* spp.** A single locus that encodes genes which are associated with metal reduction was found in each sequenced genome with the exception of *Shewanella denitrificans*. MtrA and MtrB are essential for reduction of Fe(III) in *Shewanella oneidensis* MR-1, and their sequences are similar to those of MtrE and MtrD, respectively. Although the functions that are encoded by both *mtrC* and *omcA* also contribute to the metal-reducing phenotype, this phenotype has not been observed for *mtrF* mutants, presumably because conditions that promote its expression have not been tested. The genes that encode new 10-haem cytochromes that have been identified in this locus were named *mtrG* and *mtrH*, whereas the new 11-haem cytochromes were designated *undA* and *undB*. With the exception of the periplasmic MtrA and MtrE decahaem cytochromes, all of the *c*-type cytochromes (pink) that are encoded in this locus are predicted to be cell-surface-exposed lipoproteins.

the ability to mediate direct electron transfer to outer-membrane-impermeable electron acceptors, such as iron and manganese oxides. Similar to *S. oneidensis* MR-1, each of these strains has a single locus that encodes a periplasmic (MtrA) and cell-surface (MtrC) decahaem cytochrome *c* and an integral outer-membrane protein (MtrB) that has an unknown function but is essential for the reduction of metal oxides[45] (FIG. 2). A second feature of this locus is the presence of 1–3 10-haem (OmcA, MtrF, MtrG and MtrH) or 11-haem (UndA and UndB) *c*-type cytochromes, all of which are predicted to be localized to the outer membrane. Some strains also encode the closely related, but functionally uncharacterized, *mtrDEF* genes in this locus. However, most or all of these additional genes are controlled by distinct promoters, which indicates that the expression of each of these proteins responds to different signals and might also reflect differences in substrate specificity.

By contrast, variability in the use of DMSO can be provided by the presence of multiple copies of entire DMSO-reductase-containing subsystems in a single organism, differences in the types of electron-transfer components in these subsystems (FIG. 3) and the high degree of variability within the protein sequence of the DMSO-reductase components. Although only proteins that are encoded by the type I *dmsEFABGH* locus in *S. oneidensis* MR-1 have been found to mediate DMSO reduction[58], the similarity in protein sequence, gene order and gene locus suggest that the other related loci are paralogous. Indeed, it has recently been demonstrated through complementation analysis that the *E. coli ynfEFGHI* and *dmsABC* loci are paralogous[83]. Because only one locus was expressed under the tested growth conditions, the defective DMSO-reductase phenotype that was observed after disruption of genes in the *dmsABC* locus mistakenly led to the conclusion that only one locus was responsible for this activity. A condition that

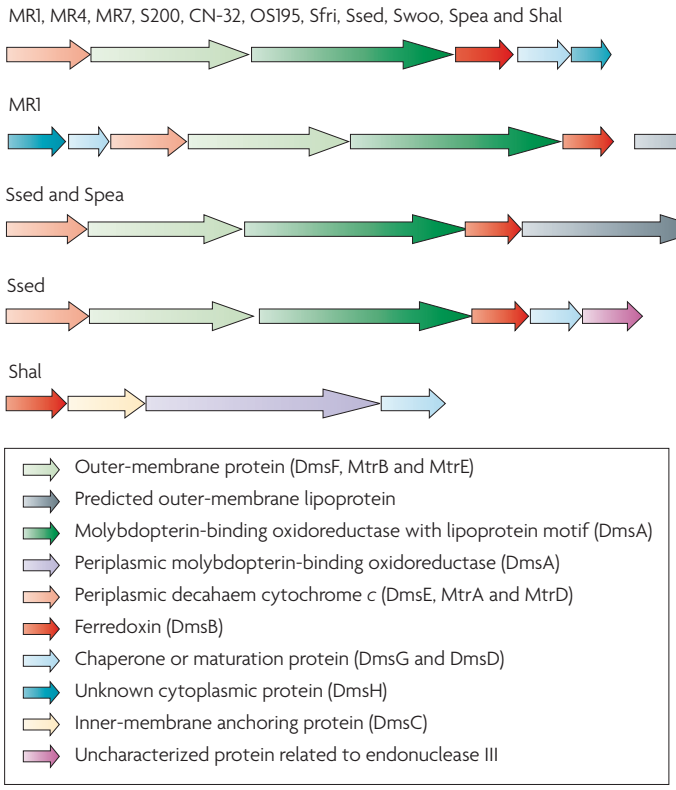


Figure 3 | **Five dimethyl sulphoxide (DMSO) family subsystems.** The subsystem types are distinguished by gene order. Deletion of type I components in *Shewanella oneidensis* MR-1 leads to a severe defect in DMSO reduction. This system is present in 10 of the 20 sequenced *Shewanella* species: two in *Shewanella frigidimarina* and 4 in *Shewanella sediminis*. Compared with the type I subsystem, the type II and III subsystems contain an additional gene that is predicted to encode an outer-membrane lipoprotein, whereas the type IV subsystem has an added gene that encodes an endonuclease-III-related protein. The type IV subsystem is more characteristic of *Escherichia coli*, as it lacks genes that encode the MtrAB-like module (DmsEF), it is predicted to encode a periplasmic localized DmsAB reductase complex and it encodes the inner-membrane anchoring protein DmsC. The occurrence of DmsC in only the type V locus suggests that the remaining reductase complexes are CymA-dependent. CN-32, *S. putrefaciens* CN-32; MR1, *S. oneidensis* MR-1; MR4, *S. oneidensis* MR-4; MR7, *S. oneidensis* MR-7; S200, *S. putrefaciens* S200; OS195, *S. baltica* OS195; Sfri, *S. frigidimarina*; Shal, *S. halifaxensis*; Spea, *S. pealeana*; Ssed, *S. sediminis*; Swoo, *S. woodyi*.

promotes expression of the type II *dmsGHEFAB* locus by *S. oneidensis* MR-1 has not been established, which leaves open the possibility that this locus and other loci depicted in FIG. 3 can mediate the same activity.

All of the DMSO family subsystems, with the exception of the type V DmsC-containing subsystem, seem to share a requirement for the CymA inner-membrane quinol reductase, a periplasmic decahaem cytochrome *c* (MtrA, MtrE or DmsE) and an outer-membrane protein (MtrB, MtrD or DmsF) with the metal reductase subsystems. The use of an MtrAB-like module in surface-mediated electron-transfer systems seems to extend beyond the *Shewanella* genus, as it is required for iron oxidation in *Rhodopseudomonas palustris*[84] and we have observed genes that encode this module adjacent to putative multi-haem cytochromes (also predicted to be lipoproteins) in *Vibrio* spp., *Dechloromonas aromatica*, *Halorhodospira halophila*, *Magnetospirillum magneticum* and *Geobacter uraniumreducens*. This suggests that the MtrAB-like module performs a similar function in different types of electron-transfer pathways. Comparative genomic analysis has provided a more complete understanding of the genes that are likely to provide the characteristic physiology of the *Shewanella* genus and has provided insight into the diversity that is found in some of the respiratory subsystems. Combining more studies of this type with the genetic, biochemical and physiological studies that are needed to validate these predictions will bring us closer to the goal of a systems-level understanding of the *Shewanella* genus.

Towards systems biology of *Shewanella* spp.

A firm foundation for systems-level understanding of members of the *Shewanella* genus is being laid through coupled computational and experimental characterization of *S. oneidensis* MR-1 and detailed comparative genomic analysis. We have provided several examples in which this analysis has already been used successfully to identify previously unknown cellular processes and pathways. Continued use of these strategies to analyse sequenced *Shewanella* species and strains isolated from different environments with varying levels of phylogenetic relatedness should provide further understanding of how speciation mechanisms and the environment influence genotype and phenotype. Ultimately, this will bring us closer to the goal of being able to predict physiological characteristics of new *Shewanella* isolates from their genome sequence.

Developing an integrated model of metabolism and regulation of *S. oneidensis* MR-1 and using information gained from this organism to facilitate a systems-level understanding of other members of the *Shewanella* genus is a realistic objective. As this knowledge matures, it should provide the underpinnings that are needed to move further along the continuum of system complexity towards the study of *Shewanella* cell populations and the role of *Shewanella* species within microbial communities. There has been renewed appreciation for the fact that populations of genetically identical cells can exhibit extensive physiological heterogeneity when grown under homogeneous conditions (reviewed in REF. 85). To understand the importance of this heterogeneity in *Shewanella* species ecophysiology, measurements of individual cells in populations will be important, especially under conditions that reflect the range of their typical habitats.

Environmental populations of *Shewanella* spp. are probably composed of multiple genotypes, such as the *Shewanella baltica* populations in the Baltic Sea[86]. It is possible that these different genotypes reflect different specializations within the population. Alternatively, many of these genomic differences might have little adaptive importance, as has been proposed for *Vibrio splendidus* populations[87]. Combining computational, experimental and field studies to examine mixed populations from well-studied organisms, such as *S. baltica*, might provide some insights into how different *Shewanella* genotypes are likely to position themselves or interact within a community. Continuing to isolate, characterize and sequence wild strains of *Shewanella* from different habitats

is likely to provide us with a better understanding of how environmental factors drive evolution and shape speciation, as is being done for *Prochlorococcus*[88].

Metagenomics is another approach that has been successfully used to probe the genomic diversity of wild populations (for example, REFS 74,89), and its use is expected to grow as sequencing costs decrease and technology improves[90]. As many of the genes that are encoded within a genome are not expressed under relevant environmental conditions, community proteomics and transcriptomics can focus attention on those pathways that are important for ecophysiological function. These approaches have begun to be used successfully to characterize low-diversity microbial communities[91–93]. Using these approaches to characterize the ecophysiology of *Shewanella* species will certainly be more difficult, owing to the highly diverse nature of the members of this genus. However, we think that as our understanding of the physiology of *Shewanella* species from different environments deepens and new computational and experimental approaches are developed, community-based genomics, transcriptomics and proteomics will begin to provide important insights into the role of *Shewanella* species within microbial communities. These developments will facilitate our progression towards a systems-biology understanding of *Shewanella* populations.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
ccmB | *ccmC* | *cheA* | *crp* | *cyaA* | *pdhR* | *zwf*
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Dechloromonas aromatica | *Escherichia coli* | *Geobacter uraniumreducens* | *Halorhodospira halophila* | *Magnetospirillum magneticum* | *Rhodopseudomonas palustris* | *Shewanella baltica* | *Shewanella denitrificans* | *Shewanella oneidensis* MR-1 | *Shewanella woodyi* | *Vibrio splendidus*
Entrez Protein: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=protein
CymA | EtrA | FruR | MtrA | MtrB | MtrC | MtrF | NagC | OmcA

FURTHER INFORMATION

List of Prokaryotic names with Standing in Nomenclature: http://www.bacterio.cict.fr/

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