Final Report

Integrated Genome-Based Studies for *Shewanella* Ecophysiology

Project ID: 0013353

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This report covers the portion (Objective 4.2) of the Shewanella Federation work conducted by Michigan State University (MSU) with a subcontract to Georgia Tech (GT).

Aim: *Shewanella* Population Genomics - Understanding Evolution, Ecophysiology and Speciation

Task 4.2.1 "Genetic and ecophysiological bases defining the core and diversification of *Shewanella* species"

Task 4.2.2 "Determine gene content patterns along redox gradients."

Task 4.2.3. "Investigate the evolutionary processes, patterns and mechanisms of *Shewanella*."

The most notable deliverable of our work under Task 4.2.1 was a paper published in 2009 (***PNAS 106:15909-14***), which summarized the collaborative efforts of several Shewanella Federation labs (PNNL, MSU, UCLA, MBL and ANL), led by Drs Tiedje and Konstantinidis. In this paper we reported on the gene-content and sequence diversity of the *Shewanella* genus and showed that the most rapidly evolving genes of the genus are related to mechanisms that sense and adapt to the environment (Fig. 1). We also determine, by a combination of whole-genome, whole-proteome and physiological data, the metal reduction capabilities and underlying genetic elements for several *Shewanella* strains. Collectively, these findings provide important new information toward identifying the most effective *Shewanella* strain for cleaning up specific contaminants and metals in a given environment, beginning with the extensively contaminated DOE facilities at several National Laboratories and extending to metal contaminated sites worldwide. The results also suggest that similarity in gene regulation and expression should constitute another parameter for describing new microbial species. The species concept represents an unsettled issue for microbiology that has major practical consequences for reliable diagnosis of infectious disease agents, intellectual property rights, bioterrorism agent oversight, and quarantine. Another product of our work under Task 4.2.1 was a paper published in 2010 (***Appl Environ Microbiol. 76(9):2980-8***), which validates the use of oligoarrays using complete genome sequences. This work facilitated gene-expression studies under defined laboratory conditions, which were conducted as MSU and employed strains other than the reference strain (the genome used to build the microarray). It will also assist future experiments that will apply oligoarrays to the study of the gene-content and expression variation within natural bacterial populations, *in-situ*.

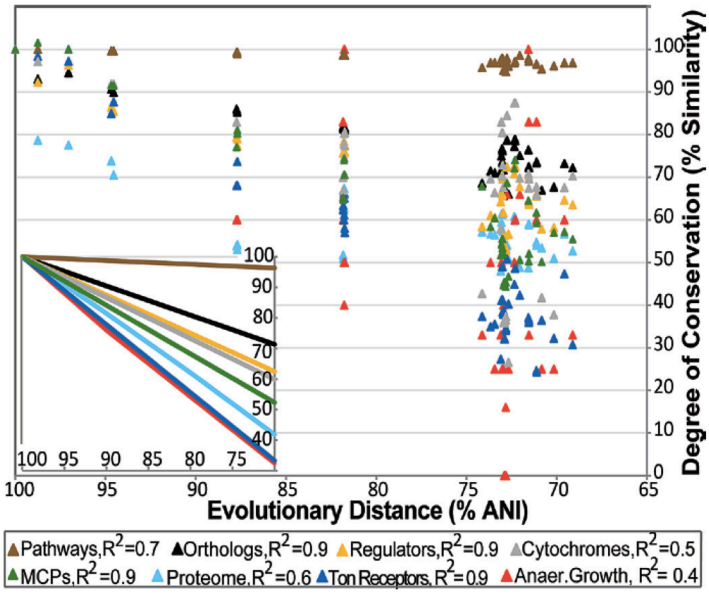

Fig. 1. Modeling bacterial genotypic and phenotypic conservation across an evolutionary gradient. Each of the traits shown (see legend) was compared among 10 *Shewanella* genomes in a pairwise manner (45 comparisons in total). The fraction of shared traits was determined for each pair of strains and plotted against the average nucleotide identity (ANI) of the respective strain pair. Inset 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%. Adapted from PNAS 106:15909-14.

The computational part of our work under Task 4.2.2 & 4.2.3 was primarily performed by the Georgia Tech team). For this task, we focused our efforts on a subset of the *Shewanella* genomes, namely the four genomes of *S. baltica*, for the advantages that this entailed: i) these genomes were recovered from different depths in the Baltic Sea that were characterized by different redox potentials and nutrient availability (i.e., an environmental gradient); ii) the

genomes are closely related, which facilitated bioinformatic sequence comparisons; and iii) they are members of the "same" natural population, thus, the findings based on these genomes reflect the processes that occur *in-situ*. Our findings revealed that the isolates from similar depths had exchanged extensive parts of their auxiliary and core genes in order to adapt successfully to the unique physicochemical conditions of the depth. Importantly, the latter genes were exchanged in very recent past, presumably as an effect of isolates' seasonal migration across the water column. No other available bacterial genomes in the public databases (GenBank) show such extensive and rapid genetic exchange as these co-occurring *S. baltica* genomes (Fig. 2). Therefore, our findings reveal that genetic exchange in response to environmental fluctuations may be surprisingly rapid, which has important broader impacts for understanding bacterial evolution and for modeling bacterial responses to human-induced environmental impacts such as release of contaminants (Task 4.2.3). Our comparisons have also unraveled the genetic determinants that enable each *S. baltica* isolate to adjust to the specific redox potential at its depth of isolation. For instance, the strains isolated from more anoxic waters shared exclusively several genomic islands and operons devoted to anaerobic metabolism and transport such as two copies of the nitrate reduction operon as well as dimethyl sulfoxide reductases (DMSO). Laboratory microarray analysis (work conducted at MSU) revealed that some of these (apparently) ecologically important genes were expressed by the strains in response to anaerobic growth with nitrate and thiosulfate, indicating that they may be functional (Task 4.2.2). This work was published in 2011 (***The ISME J. 5(1):131-40***). In order to expand upon our initial comparative analyses of the *S. baltica* genomes, we submitted a proposal to sequence five additional *S. baltica* strains to the DOE-JGI Community Sequencing Program. These strains were strategically selected, based upon the relationships to other sequenced strains revealed through the MLST and comparative genomic hybridization analyses, to expand our understanding of *S. baltica* strain diversification. This proposal was approved in 2009 and the first genome sequences became

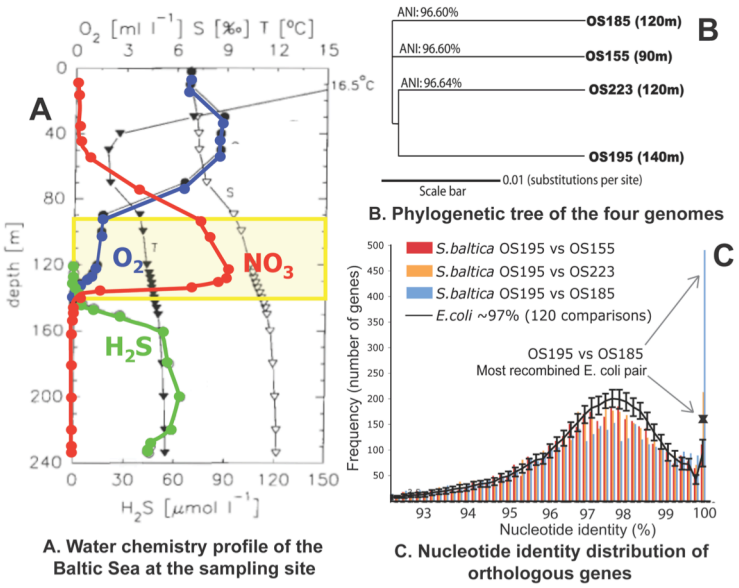

Fig. 2. Rapid and extensive genetic exchange among the *Shewanella baltica* genomes. The figure shows: i) the water chemistry profile at the site of isolation of the four *S. baltica* genomes compared (**A**); ii) the whole-genome phylogeny of the genomes based on the concatenated sequences of all core genes (n= ~2,500) that showed no evidence of recombination (**B**); and iii) the nucleotide identity among the orthologs of selected pairs of genomes (**C**). Note that about 500 orthologs between OS185 and OS195 show ~100% nucleotide identity, contrasting with <150 orthologs for the remaining *S. baltica* genome pairs or any pair of *E. coli* genomes (solid line). Therefore, the former genes, most likely, have been exchanged between OS185 and OS195 in very recent past. Error bars represent one standard deviation from the mean for the *E. coli* comparisons.

available to us in late 2010. Analysis of these genome sequences confirmed and expanded upon our observations with the initial set of four genomes (published in ***Journal of Bacteriology, (5):1236***). Under Task 4.2.2 we have also developed and optimized protocols that employ the next generation sequencers such the Roche 454 Titanium and Illumina GA-II and Hi-Seq for the metagenomic analysis of natural microbial assemblages (one article published: ***Oh et al, Appl Environ Microbiol. (17):6000-11***). These protocols will be instrumental in evaluating the findings mentioned above from the *Shewanella* isolates against complex microbial communities *in-situ*, probably beyond the duration of the project.

The phylogenetic and phenotypic characterization of a selected group of 46 *Shewanella baltica* strains was done. We also inferred their genomic contents through microarray analysis and investigated adaptation of some *S. baltica* strains to conditions in the natural redox gradient.

Phylogenetic diversity. Multilocus Sequence Typing (MLST) is an important means to study phylogenetic diversity and population structure among closely related microorganisms. We determined phylogenetic relatedness of this *S. baltica* population through MLST with a novel strategy in gene selection [Figure 3], and compared it with the traditionally recommended genotyping for *Shewanella* using the house-keeping gene, *gyrB*, enconding the B subunit of the DNA gyrase, and with the whole genome clustering using data from comparative genomic hybridization (CGH). Nine clades were consistently recovered from these three approaches, suggesting genetic homogeneity within these genotypes. Particularly, two clades, MLST-J and K, contain strains from both 1998 and 1986 with very high sequence identity (100% and 98.7%, respectively). This result suggested genetic continuity of strains obtained in 1986 and 1998, and that the later strains are very likely descendants of those from 1986. We also determined habitat association of above identified MLST genotypes through AdaptML based on isolation procedures and sizes of inocula. Isolation procedures were indicative of favored nutrient and redox conditions; whereas sizes of inocula may suggest the *in situ* living states, with small inocula related to higher concentration of free-living cells, and large inocula indicative of particle attachment. A total of five distinct habitats were identified [Figure 3]. In particular, two of the largest clades MLST-A and E each associated with a distinct habitat. MLST-A strains were projected on to habitat H0, characterized by recovery most frequently from high-carbon (a, b, c) or thiosulfate-supplemented medium (e) using moderate size of inocula (0.1 to 1 mL). MLST-E strains were associated with habitat H1, characteristic of isolation almost exclusively using high-carbon medium (a, b, c) and small inocula (0.001-0.01 mL), suggesting a different lifestyle with different nutrient sources. In summary, the phylogenetic analyses combined with habitat determination, together revealed genetic differentiation within the *S. baltica* population most likely resulted from resource partitioning.

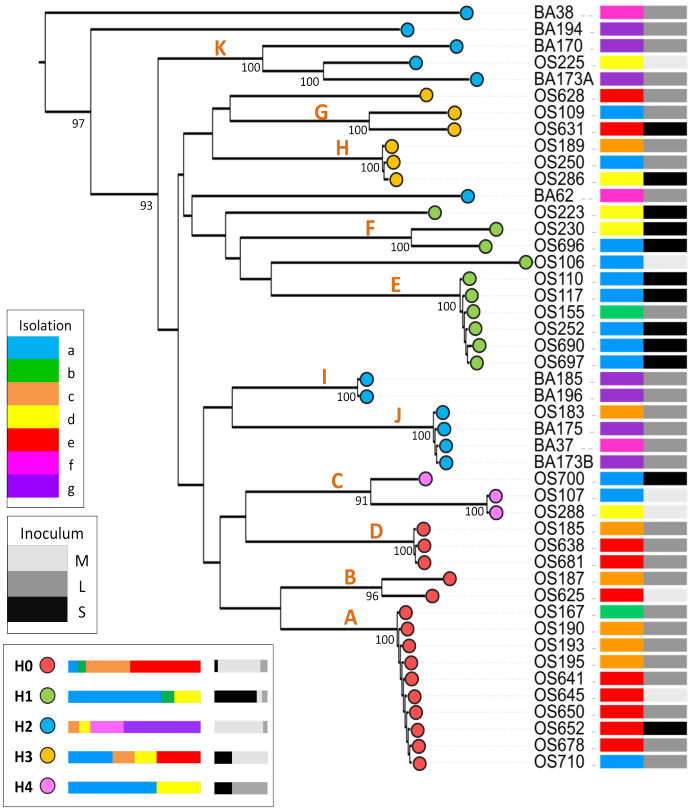

Figure 3. Phylogeny and habitat association of the *S. baltica* population. Tree was constructed with Neighbor Joining algorithm using concatenated sequences from seven MLST genes. Strains starting with 'OS' were isolated from 1986 or 1987, whereas strains starting with 'BA' were from 1998. The strain *S. oneidensis* MR-1 was used as out-group but is not shown. Bootstrap values above 50% are shown on branches based on 1000 bootstrap replicates. Projected habitats are labeled by colored circles that reflect trends in distributions among different isolation procedures or sizes or inoculum. The isolation procedures are as follows: (a) Nutrient Broth medium supplemented with nitrate under anaerobic condition (b) ZoBell agar plate under aerobic condition (c) ZoBell agar plate under anaerobic condition (d) 1:10 diluted Nutrient Broth medium supplemented with nitrate under anaerobic condition (e) Liquid nitrate plus thiosulfate medium under anaerobic condition without added organic carbon source (f) ZoBell (1/5 dilute) agar plate under aerobic condition (g) Nitrate plus thiosulfate agar medium under anaerobic condition without added organic carbon source. In addition, 'S' indicates small inoculum of less than 0.1 mL; 'M' indicates moderate inoculum size between 0.1 mL and 1 mL; 'L' indicates inoculum of 10 mL.

Metabolic diversity of *Shewanella* species. *Shewanella* plays an important role in the cycling of organic and inorganic materials in the environment. In order to further investigate metabolic diversity among *S. baltica* strains, we employed the Biolog-GN system to screen for the utilization of 95 different carbon sources. *S. baltica* strains were able to utilize an average of 36 compounds under aerobic conditions. Among the carbon sources tested, 19 were not used by any *S.baltica* strains, while 11 substrates, including cis-aconitic acid, sucrose, D-gluconic acid, L-glutamic acid, dextrin, maltose, a-D-glucose, L-serine, N-acet.-D-glucosamine, lactic acid, and inosine, could be successfully consumed by all strains studied. In addition, strains from the same genotype were found to share similar metabolic diversity. Correlation analysis suggested significant correlation between carbon source utilization and the genetic relatedness among strains. For instance, strains in the genotype MLST-E shared a distinct metabolic pattern and used a significantly fewer number of carbon sources compared to other clades. In particular, substrates including succinate acid, aspartic acid, and a-keto butyric acid failed to stimulate growth of any strains in this genotype. Our analysis also showed a strong effect of temporal distribution on the metabolic profile. *S. baltica* strains isolated from 1998 managed to grow on 44 compounds, while *S. baltica* strains isolated from the 1980s were able to use only 34 compounds in average. Specifically, capability of using compounds including D-fructose, bromo-succinic acid, α -ketobutyric acid, glucose-1-phosphate, acetic acid, α-hydroxybutyric acid, glucose-6-phospate and L-alanine was found in a significantly larger percentage of strains isolated in 1998 compared to those isolated in the 1980s ($p < 0.001$), possibly corresponding to a

shift in selective pressure in the Baltic Sea environment. In summary, detailed comparison of phenotypic profiles among *S. baltica* strains combined with phylogenetic and ecological characterization yielded further insights into the phenotypic changes that occurred within short evolutionary stretch and shed light on the selective pressure in the natural redox gradient in the Baltic Sea.

Comparative transcriptomics and genomics of *S. baltica* population. Comparative transcriptomics and genomics are important means in investigating specialization of microorganisms into various niche conditions. We designed oligoarrays based on all genes and intergenic regions longer than 200 bp from four initially sequenced *S. baltica* genomes. Through hybridizing cDNA of OS185 and OS195–two strains isolated from microaerophilic and anoxic layer of the Baltic Sea, respectively–from cells grown under aerobic, anaerobic nitrate and thiosulfate conditions, we observed significant regulatory differences on both common genes and strain-specific genes. We also identified a number of genes potentially important for specialization under anoxic conditions, such as the outer membrane cytochromes MtrABC and OmcA (Shew185_1576-1579), the nitrate reductase-like thiosulfate-inducible operon (Shew185_3866-3877), the anaerobic DMSO reductase (Sbal195_2225-2230), etc. Meanwhile, genome plasticity of 42 unsequenced *S. baltica* strains was explored via Comparative Genomic Hybridization (CGH), through which heterogenic gene distribution was revealed relating both individual strains and distinct genotypes. Further analyses identified over 100 genes that are potentially important for specialization into anoxic water environment. On the contrary, specialization on the gene level into microaerophilic condition was not well supported. Loss of over 300 genes in the 1998 strains compared to the 1980s' strains was also found, suggesting possible shift of selective pressure in their niche environments. In summary, these results allowed us to better understand which genes play importance roles in specialization in the redox gradient, and what genetic changes have led to ecological divergence among the *S. baltica* population. .

Examining nitrate specific adaptation of *Shewanella* species through competition assays. Two of the sequenced strains, *S.baltica* OS185 and *S.baltica* OS195, were selected for examining adaptation to niches with distinct nitrate conditions. OS185 was isolated from an oxic-anoxic transition zone where nitrate was found at the highest concentration along the water column, and OS195 was isolated from an anoxic zone characterized by a depletion of nitrate. We found that although the two strains shared similar growth rates in minimal medium with nitrate as the sole electron acceptor while grown individually, OS185 rapidly out-competed OS195 while they were grown in co-culture. This observation suggested OS185 was better adapted to nitrate rich conditions, which was consistent with the natural conditions where they were isolated. We then profiled the transcriptomes of OS185 and OS195 in co-culture and in pure cultures individually through RNAseq by Illumina sequencing. Our results suggested, under conditions both with and without the competitor strain, OS185 was able to alter its gene expression more actively toward the nitrate condition, mostly through up-regulation of redox related genes. These analyses collectively suggested that OS185 is better adapted to

nitrate-rich conditions and provided explanations on the level of gene expression for the competition outcome between OS185 and OS195.

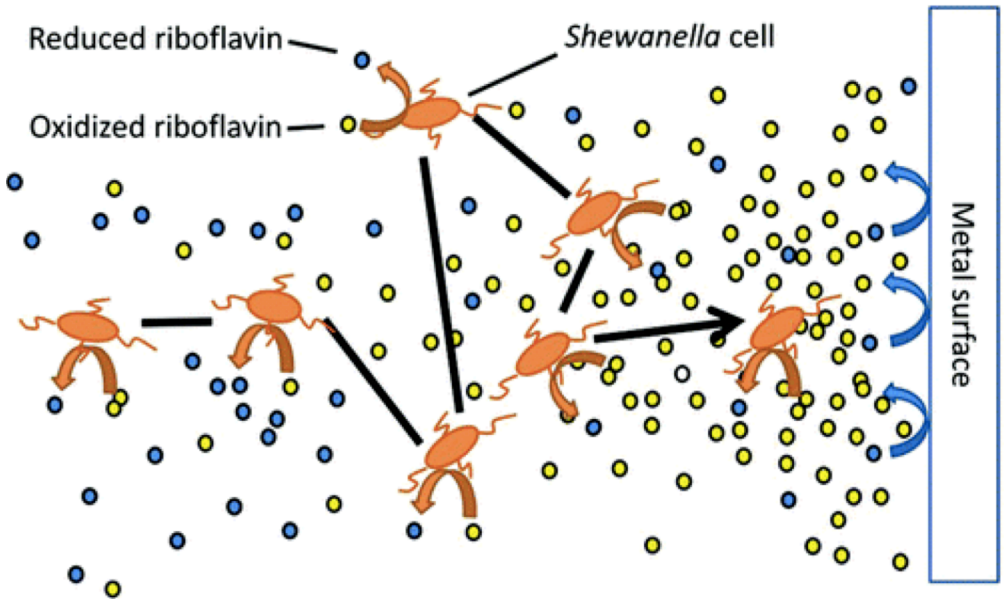

Fig. 4. A schematic diagram of electron shuttle-mediated energy taxis in *Shewanella* metal reduction. We proposed that *Shewanella* use riboflavin as both an electron shuttle and an attractant to direct cell movement toward local sources of insoluble electron acceptors. The cells secrete reduced riboflavin, which diffuses to a nearby particle containing an insoluble electron acceptor and is oxidized. The oxidized riboflavin then diffuses away from the particle, establishing a spatial gradient that draws cells toward the particle. The proposed mechanism was supported by experimental and mathematical modeling results.

Understanding the taxis *Shewanella* in response to its redox environment. *Shewanella* species are famous for their broad range of terminal electron acceptors and the ability to perform taxis towards both soluble and insoluble electron acceptors, which may help explain their nearly ubiquitous presence in widely disparate environmental niches around the world. Studies of *Shewanella*'s tactic properties are important to understand their competitiveness and roles in elemental (e.g. nitrogen, sulfur, iron, manganese and others) cycling and bioremediation (e.g., precipitation of soluble uranium oxides). Population-level microbial taxis involves a complex interplay of cellular process (e.g., growth, metabolism, chemotaxis, and random motility) and molecular processes (e.g., diffusion of electron donors and acceptors that serve as attractants). This study focuses the use of multiple approaches including engineering tools, biological assays and mathematical models to study population-level growth and taxis of *Shewanella* in response to applied and cell-generated gradients of soluble electron acceptors. The model was able to reproduce key trends of the observed cell growth and migration patterns in either diffusion gradient chamber (DGC) or swarm plates, which validate the use of our approaches to measure and simulate *Shewanella*'s taxis in response to electron acceptor gradients.

New hypotheses relevant to *Shewanella*'s taxis were investigated. The ecological niches constructed by various electron acceptor gradients might be responsible for the distribution of *S. baltica* strains with different genotypes and hence different chemotactic behaviors. We studied the impact of opposing gradients of nitrate and fumarate on the chemotactic behaviors of *S. oneidensis* MR-1 fumarate reductase and nitrate reductase mutants, where the mixture of mutant strains could be partially separated into two populations via formation of two separate chemotactic bands, one moving toward each electron acceptor source. We also studied cell behavior in the presence of insoluble electron acceptor manganese dioxide. A novel mechanism, mediated energy taxis, is proposed by which *Shewanella* use self-secreted riboflavin as both an electron shuttle and an attractant to direct cell movement toward local sources of insoluble electron acceptors (Fig. 4.). Mathematical models based on these proposed mechanisms were able to predict experimental trends. This work was published in ***Environ Sci and Technol*** **in 2011 and 2012.**

PUBLICATIONS

Peer-reviewed articles

1) A. Caro-Quintero, J. Deng, J. Auchtung, I. Brettar, M. Höfle, James M. Tiedje, and K. T. Konstantinidis. Genome sequencing of five Shewanella baltica strains recovered from the oxic-anoxic interface of the Baltic Sea. Journal of Bacteriology (2012), (5):1236.

2) S. Oh, A. Caro-Quintero, D. Tsementzi, N. DeLeon-Rodriguez, C. Luo, R. Poretsky, and K. T. Konstantinidis. Metagenomic insights into the evolution, function and complexity of the planktonic microbial community of Lake Lanier, a temperate freshwater ecosystem. Applied and Environmental Microbiology (2011), (17):6000-11.

3) A. Caro-Quintero, J. Deng, J. Auchtung, I. Brettar, M. Höfle, J. Klappenbach, and K. T. Konstantinidis. Unprecedented levels of horizontal gene transfer among spatially co-occurring Shewanella bacteria from the Baltic Sea. The ISME Journal (2011), 5(1):131-40.

4) S. Oh, D. R. Yoder-Himes, J. Tiedje, and K. T. Konstantinidis. Evaluating the performance of oligonucleotide microarrays for strains of increasing genetic divergence to the reference strain. Applied and Environmental Microbiology (2010), 76(9):2980-8.

5) K. T. Konstantinidis, M. H. Serres, M. F. Romine, J. L. M. Rodrigues, J. Auchtung, L.-A. McCue, M. S. Lipton, A. Obraztsova, C. S. Giometti, K. H. Nealson, J. K. Fredrickson, and J. M. Tiedje. Comparative systems biology across an evolutionary gradient within the Shewanella genus. PNAS (2009), 106(37):15909-14.

6) Li, R., Tiedje, J. M., Chiu, C., and Worden, R.M. Soluble electron shuttles can mediate energy taxis toward insoluble electron acceptors. Environ Sci Technol. 2012 Mar 6;46(5):2813-20

7) Li, R., Auchtung, J.M., Tiedje, J. M., and Worden, R.M. Shewanella oneidensis MR-1 chemotaxis in a diffusion gradient chamber. Environ Sci Technol. 2011 Feb 1;45(3):1014-20.

Book chapters

1) K. T. Konstantinidis. Metagenomic insights into bacterial species. In Handbook of Molecular Microbial Ecology II: Metagenomics in Different Habitats. Frans J. de Bruijn editor. John Wiley & Sons, Inc. Hoboken, New Jersey, USA. 2011.

2) J. Cole, K. T. Konstantinidis, R. J. Farris, and J. M. Tiedje. Microbial diversity and phylogeny: extending from rRNAs to genomes. In Environmental Molecular Biology. W-T. Liu and J. Jansson (eds). Horizon Scientific Press. Norwich, UK. 2009.

Publications under review or prepared

1) Deng, J., Brettar, I., Luo, C., Auchtung, J.M., Konstantinidis, K.T., Rodrigues, J.L.M., Höfle, M., and Tiedje, J.M. Stability, genotypic and phenotypic diversity of Shewanella baltica population in the Baltic Sea. Under review.

2) Deng, J., Auchtung, J.M., Caro-Quintero A., Konstantinidis, K.T., Brettar, I., Höfle, M., Tiedje, J.M. Toward understanding the diversity and environmental specialization of a Shewanella baltica population: an integrated comparative transcriptomic and genomic approach. In draft.

3) Deng, J., Chai, B., Auchtung, J.M., Li, R., Cole, T.T., Brettar, I., Höfle M., Konstantinidis, K.T., Tiedje, J.M. Niche Adaptation and Divergent Transcriptional Response by Two Shewanella Baltica Strains during Competition. In draft.