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Analysis of Fatty Acid and Growth Profiles in Ten *Shewanella* spp. to Associate Phylogenetic Relationships

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14. ABSTRACT

Shewanella spp. are from a large family of bacteria (Shewanellaceae) used for studying fundamental stress responses from environmental cues. Therefore, a systematic and controlled alteration of growth conditions could be used to uncover associations between phylogenetically dissimilar microorganisms from the same genus using physiological responses. To understand these changes, a shift in fatty acid length distributions and growth of ten phylogenetically diverse *Shewanella* spp. were monitored when grown in a chemically defined culture medium at pH 6, 7, or 8. Under these different growth conditions, the *Shewanella* spp. systematically shifted fatty acid carbon chain length profiles to adapt to different environments. There was an observed shift to longer fatty acid carbon lengths with increased pH, as well as a change to a predominant type of fatty acid (i.e., terminally branched) in six of the ten species at pH 7. However, these trends were not consistent among all phylogenetically related strains tested, but resulted in new associations between dissimilar *Shewanella* spp. based on physiology.

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Analysis of fatty acid and growth profiles in ten Shewanella sp. to associate phylogenetic relationships

Significance and Impact of the Study: Ten strains from an aquatic bacterial genus (*Shewanella*) can be grouped based on growth and fatty acid profile shifts but the groupings are inconsistent with phylogenetic relationships. Specifically, *S. loihica*, *S. oneidensis,* and *S. amazonensis* are one potential grouping based on their uniform unique responses to a change in acidity but are not closely related phylogenetically. This is the first comparative study of 10 strains from the *Shewanella* genus which has led to novel insight into physiological changes based on fatty acid and growth profiles that are not predicted from phylogenetic relationships.

Abstract

Shewanella spp. are from a large family of bacteria (Shewanellaceae) used for studying fundamental stress responses from environmental cues. Therefore, a systematic and controlled alteration of growth conditions could be used to uncover associations between phylogenetically dissimilar microorganisms from the same genus using physiological responses. To understand these changes, a shift in fatty acid length distributions and growth of ten phylogenetically diverse *Shewanella* spp. were monitored when grown in a chemically defined culture medium at pH 6, 7, or 8. Under these different growth conditions, the *Shewanella* spp. systematically shifted fatty acid carbon chain length profiles to adapt to different environments. There was an observed shift to longer fatty acid carbon lengths with increased pH, as well as a change to a predominant type of fatty acid (i.e. terminally branched) in six of the ten species at pH 7. However, these trends were not consistent among all phylogenetically related strains tested, but resulted in new associations between dissimilar *Shewanella* spp. based on physiology.

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Introduction

The stress response of a bacterium to its surrounding environment can provide valuable insight into its phylogenetic relationship to other strains within a particular family (Lebedinsky, *et al.*, 2014, Leong, *et al.*, 2015). Many of the model organisms used for systematic physiological stress studies are from *Bacillus* or *Escherichia* spp. with only select environmental strains finding similar applications (Yin & Gao, 2011). Members of the *Shewanella* genus are endemic to aquatic environments and have been shown to be metabolically flexible (Venkateswaran, *et al.*, 1999, Kato & Nogi, 2001, Hau & Gralnick, 2007) making them ideal candidates for broad applications in biogeochemical and bioremediation research (Fredrickson, *et al.*, 2008). Greater than 52 strains of *Shewanella* have been identified and sequenced making it one of the top families of aquatic bacteria available for phylogenetic studies.

Investigating changes in phospholipid fatty acid trends is an established analytical method used from clinical epidemiological studies to environmental ecological community surveys (Welch, 1991). Fatty acid profiles can be used to characterize taxonomic differences in environmental samples such as changes in microbial community structure in soil (Frostegard & Baath, 1996, Narendrula & Nkongolo, 2015). Additionally, previous research suggests that the reliable identification of family classifications could be detectible from fatty acid profiles (Zelles, 1999). Depending on the target species, shifts in the distribution of fatty acids are more iconic and useful for characterization specific applications to species and genus. Ultimately, culture growth conditions are influential on cellular fatty acid quantification (Welch, 1991) and therefore responses to defined culture conditions controlling a single environmental variable (i.e., acidity) could lead to potentially new associations within deeply branching phylogenetic lineages like *Shewanella*.

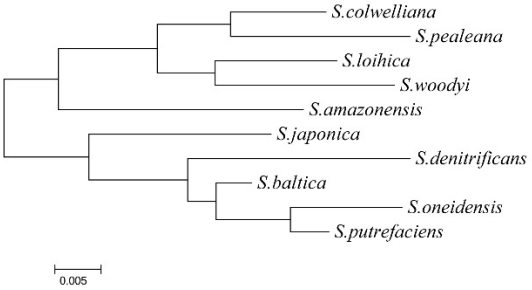
The aim of this study was to determine if systematic shifts to the fatty acid and growth profiles would occur in response to changes in acidity using ten phylogenetically diverse *Shewanella* spp. in the same growth medium. These data will provide a better understanding of how acidity impacts several strains of *Shewanella* and are beneficial for ecological studies or downstream biotechnological applications where phylogenetic similarity cannot predict a particular physiological response. The growth characteristics are reported for each species when grown in native ATCC propagation medium (native media). When these species were

then grown in a defined medium composed of half-strength Marine Broth adjusted to pH 6, 7, or 8 in a 50 mM phosphate buffer, both growth characteristics and fatty acid profiles were able to be compared.

Results and Discussion

Shewanella spp. are part of a large aquatic bacterial family and have developed a wide range of regulatory systems that are both flexible and robust (Fredrickson, *et al.*, 2008). The ten strains of *Shewanella* used for this work had broad phylogenetic diversity (Fig. 1) and were isolated from mostly marine environments. *S. putrefaciens* was the only strain that was not originally isolated from an aquatic environment but from an oil pipeline (Obuekwe & Westlake, 1982). *S. putrefaciens* shares the closest phylogenetic relationship with freshwater *S. oneidensis* (Venkateswaran, 1999) and thus was included in this study to determine if other strains of *Shewanella* share a similar response to acidity and develop a potential connection to strains isolated from within an aquatic environment to other environments.

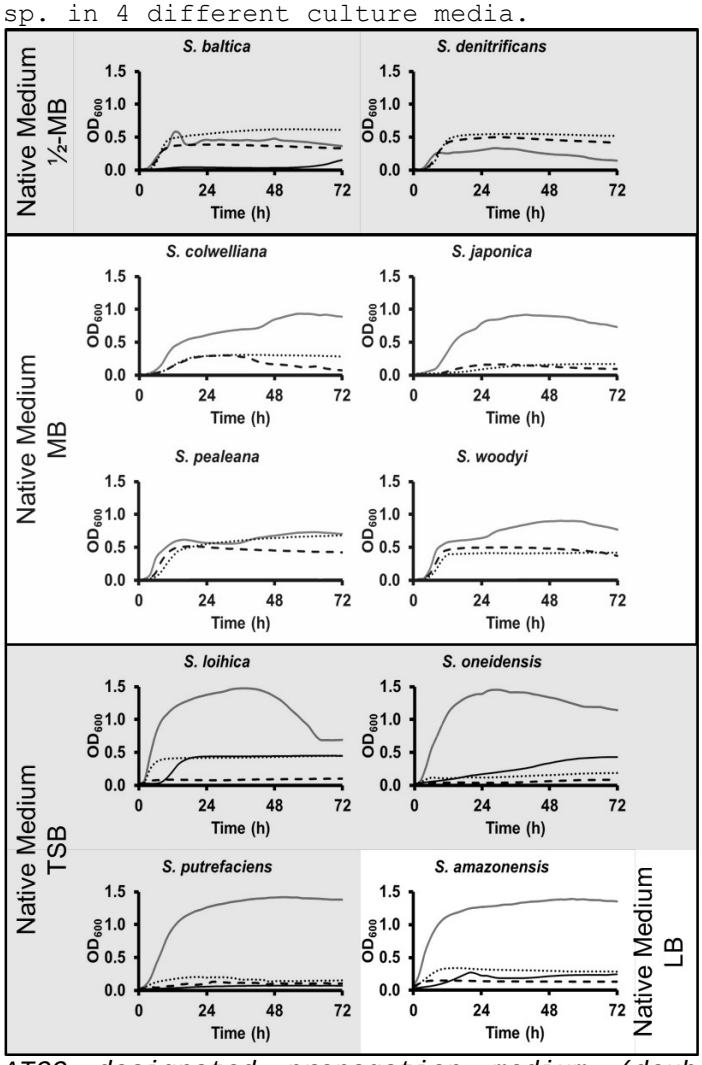
Figure 1. Phylogenetic tree of the ten *Shewanella* spp. used in this study. The scale bar represents the amount of genetic change normalized by the length of the sequence.



Shewanella spp. growth at defined acidity

Physiological changes between phylogenetically diverse strains of *Shewanella* can be correlated in order to group the strains based on their response to identical growth conditions. The uniform growth conditions are in contrast to growth in their native propagation media which is considerably different between the strains. Changes in the growth rate from ten *Shewanella* spp. were compared using optical density measurements at 600 nm (OD_{600}) in native media and in a defined media at pH 6, 7, or 8 (Fig 2). After several iterations, the defined medium that supported growth of most of the strains tested was marine broth diluted to half strength with 50 mM phosphate buffer (½-MB). The 50 mM phosphate buffer mitigated any significant (>0.5 pH units) changes to the acidity of the culture medium during growth experiments. This defined media base was used to test physiological responses to growth in pH 6-8.

Figure 2. Growth curves for each *Shewanella* sp. in 4 different culture media.



ATCC designated propagation medium (double line), ½-MB (diluted with 50 mM phosphate buffer) at either pH 6 (solid line), pH 7 (dotted line), and pH 8 (dashed line).

All strains showed growth (OD_{600} 0.33-1.47) in native medium (Table 1) but the complexity of the cellular responses to the different native media based on nutrient composition and salinity alone makes growth in a defined medium necessary to compare physiological changes across several strains. Only ***S. baltica*** and ***S. denitrificans*** were originally isolated from brackish waters and generated higher cell densities at pH 7 in ½-MB compared to the suggested propagation medium, which was (½-MB). At pH 8, the OD_{600} for ***S. denitrificans*** was ~1.5 times higher than in native media.

Neither *S. baltica* nor *S. denitrificans* grew at pH 6 (>0.2 in ½-MB). Generally, strains whose native media was MB or ½-MB did not grow at pH 6 but were able to grow at pH 7 and 8. This trend was not observed for some of the strains with a native medium of tryptic soy broth (TSB) or Luria-Bertani (LB) when cultured in ½-MB.

TSB was the native propagation medium for *S. loihica, S. oneidensis*, and *S. putrefaciens*. When these strains were grown under all experimental conditions the OD_{600} ranged from 5-31% of the growth in the native propagation medium with *S. oneidensis* growing to the highest turbidity at pH 6. *S. putrefaciens* showed the highest turbidity in ½-MB at pH 7, although there was little change in growth across the acidities tested. Lastly, *S. amazonensis* (native medium LB) showed similar trends to *S. oneidensis, S. putrefaciens*, and *S. loihica* with pH 7 generating the highest cell densities.

There was a strong correlation with growth between the 10 investigated *Shewanella* spp. outside of their phylogenetic relationship (Fig. 1) when comparing the growth results from the native growth medium (Table 1 and Figure 2) and defined medium. Even though these species were phylogenetically diverse, there were clear trends within the ½-MB media experiments. *S. denitrificans* and *S. baltica* are closely related and are both cultured in ½-MB. These strains also grew to a higher OD_{600} at pH 7 (compared to growth in ½-MB) with no appreciable growth at pH 6. *Shewanella* strains such as *S. colwelliana*, *S. pealeana*, and *S. woodyi* could definitely be grouped based on growth responses in ½-MB outside of the phylogenetic lineage. *S. amazonensis* was another interesting outlier as the observed growth characteristics were more similar to a *Shewanella* sp. that was not isolated from a marine environment (i.e., oil pipeline) even though it was isolated from sea water sediment. This unique behavior is also consistent from a phylogenetic standpoint since no other strain tested is closely related to the *S. amazonensis* (Fig. 1).

Table 1. Comparison of growth characteristics and origin of 10 *Shewanella* sp.

Strain	Origin [strain]	Reference	Native medium	Growth Native media [OD]	Growth: ½-MB (pH 6) [OD]	Growth ½-MB (pH 7) [OD]	Growth ½-MB (pH 8) [OD]
S. baltica	Brackish water [OS155]	(Brettar, *et al.*, 2001)	½-MB	0.57	0.16	0.62	0.39
S. denitrificans	Brackish water [OS217]	(Brettar, *et al.*, 2002)	½-MB	0.33	0.01	0.56	0.50
S. colwelliana	Estuarine containing juvenile oysters [LST]	(Labare & Weiner, 1990)	MB	0.93	0.01	0.31	0.31
S. japonica	Sea Water (Troitza Bay) [KMM3299]	(Ivanova, *et al.*, 2001)	MB	0.92	0.01	0.17	0.16
S. amazonensis	Sea water sediment [SB2B]	(Venkateswaran, *et al.*, 1998)	LB	1.39	0.27	0.34	0.15
S. woodyi	Seawater waste [MS32]	(Makemson, *et al.*, 1997)	MB	0.91	0.02	0.42	0.50
S. loihica	Thermal Vent (Sea Water) [PV-4]	(Gao, *et al.*, 2006)	TSB	1.47	0.45	0.45	0.11
S. pealeana	Gland of female squid [ANG-SQ1]	(Leonardo, *et al.*, 1999)	MB	0.73	0.01	0.68	0.52
S. oneidensis	Freshwater sediment [MR-1]	(Venkateswaran, *et al.*, 1999)	TSB	1.45	0.43	0.19	0.09
S. putrefaciens	Oil pipeline [200]	(Picardal, *et al.*, 1995)	TSB	1.42	0.08	0.21	0.13

OD: maximum optical density at 600nm from 4 replicates (standard deviation of all replicates was 8%); Descriptions of origin and strain from www.ATCC.org

Effect of pH on Fatty Acid Profiles

General growth trends are a crude (yet effective) indicator of general physiological responses to environmental stressors and are typically supported quantitatively by shifts in fatty acid profiles (Suutari & Laakso, 1994, Sajbidor, 1997, Quivey Jr, *et*

al., 2000). A complete compilation of the fatty acid profiles from ten ***Shewanella*** spp. grown in three acidities (pH 6, 7, and 8) is presented in Table 2. Certain ***Shewanella*** strains could not be used for a complete comparison between all of the acidities tested based on their lack of growth in ½-MB. However, four species showed growth under all three conditions (***S. oneidensis, S. baltica, S. amazonensis,*** and ***S. loihica***) and nine species were able to grow in ½-MB at pH 7 or pH 8.

Table XXX. Fatty acid composition of ***Shewanella*** spp. when cultured at pH 6, 7, or 8 in half-strength Marine Broth.

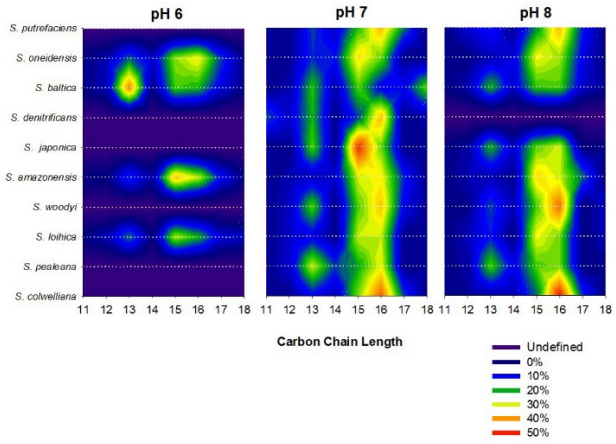
		S. amazonensis			***S. baltica***			***S. colwelliana***			***S. denitrificans***			***S. japonica***		
		pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8
Straight chain saturated fatty acids	11:0											14%				
	12:0		3%	3%		3%	5%					3%				
	13:0	7%	2%	5%			22%		4%						23%	
	14:0		5%	1%			5%									
	15:0	16%	6%	8%			7%		15%							
	16:0	8%	10%	9%		8%	5%		12%	19%		6%			8%	14%
	17:0			4%												
	18:0					24%										
Monounsaturated fatty acids	16:1ω9	20%	21%	22%	20%		20%		32%	35%		30%			16%	17%
	17:1ω8		11%		7%				13%	11%						
	17:1ω9	10%				9%										
	17:1ω10			13%			7%									
	18:1ω9															
	18:1ω10						2%									
	18:1ω11															
Terminally branched saturated fatty acids	10:0 *iso*															
	12:0 *iso*				4%					2%		3%				3%
	13:0 *iso*	5%	3%		41%	21%			5%	4%		20%				22%
	14:0 *iso*	3%				3%	2%		3%	4%						7%
	15:0 *iso*	20%	23%	28%	22%	23%	17%		10%	17%		13%			43%	26%
	15:0 *anteiso*														5%	
	17:0 *iso*									3%						
Hydroxy fatty acids	12:0 3-OH			1%												
	14:0 3-OH															
	16:0 3-OH															
	18:0 3-OH											3%				
TOTAL*		**89%**	**84%**	**94%**	**94%**	**91%**	**92%**		**94%**	**95%**		**92%**			**95%**	**89%**

		S. loihica			***S. oneidensis***			***S. pealeana***			***S. putrefaciens***			***S. woodyi***		
		pH 6	pH 7	pH 8	pH 6	pH 7	pH 8	pH6	pH 7	pH 8	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8
Straight chain saturated fatty acids	11:0															
	12:0		5%						6%				8%			
	13:0				11%				4%			2%	2%			
	14:0								10%			2%				
	15:0								8%							
	16:0	5%	9%	7%	8%	6%	10%		11%	13%		9%	9%		5%	15%
	17:0															
	18:0															
Monounsaturated fatty acids	16:1ω9	15%	19%	20%	23%	18%	19%		19%	14%		27%	30%		30%	31%
	17:1ω8	7%			13%								13%		2%	
	17:1ω9						9%					16%			6%	
	17:1ω10															
	18:1ω9															
	18:1ω10															
	18:1ω11															
Terminally branched saturated fatty acids	10:0 *iso*															
	12:0 *iso*	4%		5%	3%	1%				3%					5%	1%
	13:0 *iso*	16%	8%	12%	4%	12%	5%		24%	25%		11%	11%		23%	15%
	14:0 *iso*				5%	10%	8%		3%	9%		3%	2%			5%
	15:0 *iso*	25%	28%	33%	26%	33%	33%		11%	25%		21%	19%		23%	25%
	15:0 *anteiso*	2%														
	17:0 *iso*															
Hydroxy fatty acids	12:0 3-OH															
	14:0 3-OH															
	16:0 3-OH															
	18:0 3-OH	5%		5%					1%						1%	6%
TOTAL*		**79%**	**69%**	**82%**	**93%**	**80%**	**84%**		**97%**	**89%**		**91%**	**94%**		**95%**	**98%**

Culture conditions that did not reach a threshold turbidity of OD_{600} >0.1 were not included in this study and therefore are grayed out

The strains were compared using fatty acid methyl ester (FAMEs) analysis of extracted cellular fatty acids under the conditions in which there was growth (>0.1 OD_{600}). The results of the fatty acid chain length shifts from all strains at pH 6, 7, and 8 are shown on the intensity plots in Fig. 3. The graphical representation of these data shows clearly how fatty acid profiles changed with acidity. The prevalent fatty acid carbon chain length for ***S. baltica*** at pH 6 was C_{13} (41%), with a total mass percentage of 45% from carbon chain lengths of C_{11-14}; compared to 27% at pH 7 and 34% at pH 8 with ~50% of the fatty acids at carbon chain lengths of 15-16. Two strains that were phylogenetically similar and showed similar fatty acid profile changes with regards to carbon chain length and acidity were ***S. colwelliana*** and ***S. woodyi***. These similarities were more prominent at pH 8. These two strains were also isolated from sea water containing biological waste as opposed to being isolated from within another organism unlike ***S. paleana*** which exhibited a different fatty acid length profile from the rest of the ***Shewanella*** spp. tested. ***S. japonica*** was also unique as it exhibited changes to its fatty acid profile unlike other marine ***Shewanella*** spp. or similar members of its phylogenetic branch.

Figure 3. Intensity maps of fatty acid carbon chain lengths when grown in ½-MB medium adjusted to pH 6, 7, or 8.

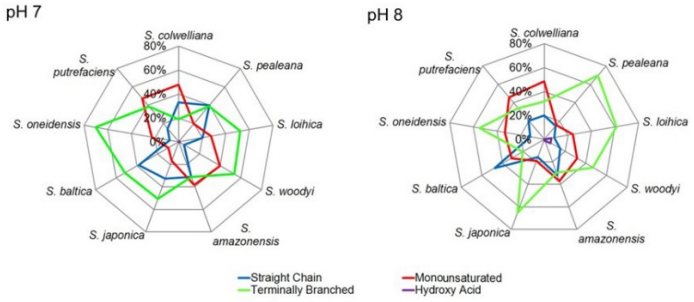


All of the changes in fatty acid branching and composition induced by acidity were evaluated between pH 7 and pH 8 for nine of the ***Shewanella*** spp. tested. Radial maps of the fluctuations in the types of fatty acids at pH 7 or pH 8 are shown in Fig. 4. In all but three species (***S. putrefaciens, S. colwelliana,*** and ***S. amazonensis***), terminally branched fatty acids are the most prominent type at pH 7. ***S. woodyi*** also generated 40% monosaturated fatty acids but unlike ***S. putrefaciens, S. colwelliana,*** and ***S. amazonensis, S. woodyi*** also generated a high percentage of terminally branched fatty acids. The hydroxy acid type was only detected in ***S. loihica*** and ***S. woodyi*** at pH 8 which is consistent with their phylogenetic relationship. The largest difference in the terminally branched fatty acids at pH 8 is observed with ***S. pealeana*** and ***S. japonica.***

When ***S. baltica*** was cultured at pH 8, there was significantly less terminally branched fatty acids but a larger percent of straight chain and monounsaturated fatty acids. There was a clear connection between hydroxyl acid fatty acids and phylogenetic similarity but no general correlation between branching and phylogenetic similarity observed from the radial maps in relation to straight chain, monounsaturated or terminally branched fatty acids.

Four strains (***S. amazonesis, S. loihica, S. oneidensis***, and ***S. baltica***) were able to grow at all three acidities which allowed for fatty acid profiles to be compared across all acidities. The largest variance in the fatty acid profiles with decreasing acidity was observed with ***S. baltica.*** The straight chain fatty acids shifted from undetected at pH 6 to 38% at pH 7 and 48% at pH 8. The percentage of terminally branched fatty acids also decreased as the pH increased. When ***S. baltica*** was grown at pH 6, 71% of the fatty acids were terminally branched; this decreased to 21% when the pH increased to pH 8. As was discussed previously, branching of the fatty acid chains was a poor indicator of similarity but clearly from the results shown in Fig 5a confirms that the changes in chain length can be used for these physiological comparisons. These data clearly show that even though ***S. baltica*** is phylogenetically similar to ***S. oneidensis*** it is different based on carbon chain length distribution induced by acidity which would not have been predicted using phylogenetic analysis. The intensity graph in Fig. 5b indicates that even though all 4 strains are different from a phylogenetic and origin standpoint, ***S. baltica*** was dissimilar to the other three strains at pH 6, there were similar trends evident with ***S. amazonensis, S. loihica,*** and ***S. oneidensis.***

Figure 4. Radial maps of the different types of fatty acids when the pH of the ½-MB medium was shifted from pH 7 to pH 8.



These data show that the response to acidity observed in these experiments cannot be correlated to phylogenetic similarity. Instead, this analysis showed that the fatty acid and growth profiles from different species from the same genus could be altered if grown in the same medium with a defined pH. Based on growth, fatty acid, and native growth media it was clear from these results that ***S. loihica***, ***S. oneidensis,*** and ***S. amazonensis*** are

potentially unique members of the *Shewanella* genus based on the physiological effects to changes in acidity but are not closely related based on the phylogenetic tree. These systematic changes in carbon chain length and branching of fatty acids during microbial growth at pH 6, 7 and 8 from the ten *Shewanella* spp. represents an association of how shifts in the fatty acid distribution provides insight into grouping of strains from the same genus. These experiments provide a straightforward method for fully evaluating the importance of environmental conditions and their connection to a physiological response which would be important for ecological or clinical studies. This work has also identified three strains of *Shewanella* that are unique based on their physiological response to acidity that were not evident from phylogeny.

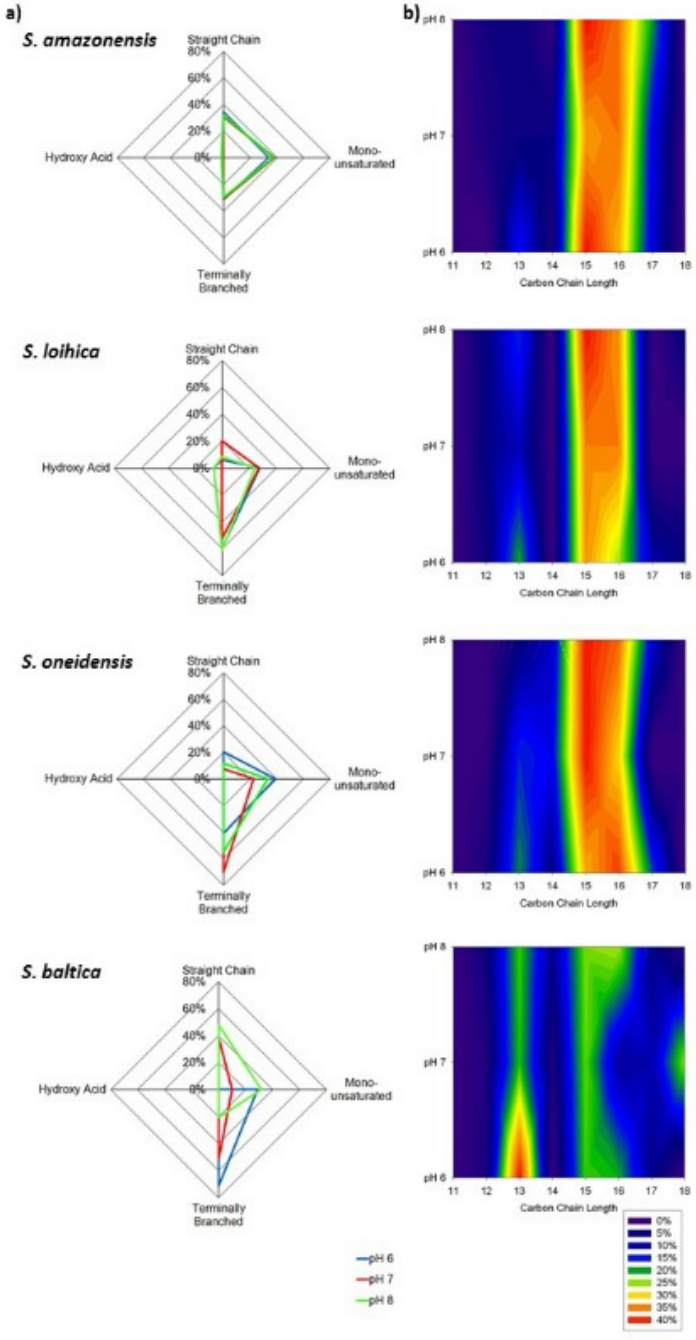


Figure 5. Analysis of the 4 *Shewanella* spp. which were able to grow at pH 6, 7, and 8 in ½-MB. a) Radial and b) intensity maps of fatty acids composition between pH 6-8.

Materials and methods

Bacterial strains and culture conditions

Ten *Shewanella* spp. were purchased from American Type Culture Collection (ATCC) and used in this study (*S. amazonensis* (ATCC Number: BAA-1098), *S. baltica* (ATCC Number: BAA-1091), *S. colwelliana* (ATCC Number: 39565), *S. denitrificans* (ATCC Number: BAA-1090), *S. japonica* (ATCC Number: BAA-316), *S. loihica* (ATCC Number: BAA-1088), *S. oneidensis* (ATCC Number: 700550), *S. pealeana* (ATCC Number: 700345), *S. putrefaciens* (ATCC Number: 51753), and *S. woodyi* (ATCC Number: 51908)). For fatty acid analysis, 50 mL starter cultures were grown in a 125 mL flask from a -80°C glycerol stock, in their designated ATCC growth medium and temperature. Once the culture was at stationary phase a 1:100 transfer was made into a 250 mL flask containing 102 mL of half-strength marine broth (½-MB) (Difco 2216) in 50 mM phosphate buffer at pH 6, 7 or 8 or in the native medium. The cultures grew at 25°C with agitation at 100 rpm until reaching stationary phase. At this point, the cultures were harvested for fatty acid extractions. Culture conditions that did not reach a threshold turbidity of OD_{600} >0.1 were not included in this study.

Phylogenetic analysis

A phylogenetic tree was generated using the 16S rRNA National Center for Biotechnology Information (NCBI) nucleotide sequences from the following *Shewanella* spp.: *S. amazonensis* (Accession: AF005248), *S. baltica* (Accession: AJ000215), *S. colwelliana* (Accession: NR_043074), *S. denitrificans* (Accession: NR_027556), *S. japonica* (Accession: NR_025012), *S. loihica* (Accession: NR_043689), *S. oneidensis* (Accession: NR_036917), *S. pealeana* (Accession: AF011335), *S. putrefaciens* (Accession: X81623), and *S. woodyi* (Accession: AF003549). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-3454.8062) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows: when the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1306 positions in the final dataset.

Evolutionary analyses were conducted in MEGA5 (Tamura, *et al.*, 2011).

Growth curve analyses

From -80°C glycerol stocks, all ten *Shewanella* strains were grown for 48 hr according to the propagation procedures provided by ATCC. Using the 48 hr cultures, 1:100 transfers (200 µL total volume) were made into the 100-well plates fitted for Bioscreen C™, which is an automated multi-well turbidity recording system. Each of the ten species were transferred into four types of growth media in quadruplicates, in addition to a negative control (blank). The four types of media used for each species were ½-MB in 50 mM phosphate buffer at pH 6, 7 or 8, in addition to the native medium designated per species by ATCC (Luria Bertani (LB) for *S. amazonensis;* MB for *S. colwelliana, S. japonica, S. pealeana,* and *S. woodyi;* ½-MB for *S. baltica* and *S. denitrificans;* and trypticase soy broth (TSB) for *S. loihica, S. oneidensis,* and *S. putrefaciens*). OD_{600} measurements were taken every two hours for three days with gentle shaking (25°C). The quadruplicate data curves were averaged to establish growth curves of all 10 *Shewanella* spp. in the four defined growth media.

Fatty acid methyl ester (FAMEs) extractions

After reaching early stationary phase, 50 mL of each culture were harvested and pelleted by centrifuge for 15 min at 3,000 rcf. The pellet was analyzed using published protocols for fatty acid methyl esters (FAMEs) (Eder, 1995). Briefly, the FAMEs extraction procedure involved harvesting the cells, saponifaction with rapid mixing and heat, methylation at 80°C for 10 min, extracting the FAMEs using a 1:1 ratio of hexane and methyl-tert-butyl ether, then washing with an aqueous base solution. A single additional wash with 18MΩ MilliQ™ water was performed to remove trace impurities. Samples were stored in crimp-top vials at -20°C before analysis.

Fatty acid analyses by gas chromatography-mass spectrometry

Products in the FAMEs extracts were identified by gas chromatography-mass spectrometry (GC-MS). Data were acquired with an Agilent 7890A GC equipped with a standard multimode inlet and a 5975C mass selective detector. An Agilent autoinjector with a 10 µL syringe was used to introduce 1.0 µL of neat extract into the inlet which was split at a 60:1 ratio. A DB-1MS (Agilent, 60 m x 0.25 mm x 0.25 µm film) column was used with an oven temperature

program that began at 40˚C, held for 1.5 min, ramped at 10˚C/min to 290˚C and held for 10 min. The MS was scanned from 40 to 350 m/z, resulting in a scan rate of 5.19 Hz.

GC-MS data were analyzed using an in-house program that identified all detected constituents by matching the mass spectra with archived library data through the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (version 2.0g, 2011) (Scientific Instrument Services, Inc.). Peak identification proceeded, using a previously-published technique (Stein, 1999), over the retention time range of 17 to 27 min, the timeframe within which the desired FAMEs products eluted. Once peak definitions were established, product quantification was performed by calculating the peak areas found in the total ion chromatograms (TICs) summed from the original GC-MS data, using trapezoidal representations of the defined ranges. Both the blank and target TICs were corrected for baseline drift and the differences in areas between each defined TIC peak and the same retention time range in the blank TIC were calculated. Peak areas are reported as percentages of the sum of all peak areas.

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