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Symbiosis of *Shewanella* spp. on the Surface of Pacific Chub Mackerel, *Scomber japonicus*, Gills.

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Marine Biology

by

Ryan Kich

Committee in charge:

Professor Eric E. Allen, Chair
Professor Doug Bartlett
Professor Jack Gilbert

2022

The Thesis of Ryan Kich is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

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ACKNOWLEDGEMENTS

This thesis, in full, is currently being prepared for submission for publication of the material. Kich, Ryan; Eric E. Allen. The thesis author was the primary investigator and author of this material.

ABSTRACT OF THE THESIS

Symbiosis of *Shewanella* spp. on the Surface of Pacific Chub Mackerel, *Scomber japonicus*, Gills.

by

Ryan Kich

Master of Science in Marine Biology

University of California San Diego, 2022

Eric E. Allen, Chair

The fish gill is a vital organ for fish health involved with gas exchange, nitrogen excretion and salt balance. The fish gill is also a chemically diverse environment which hosts a distinct microbiome, yet little is known about its constituents. Here, we describe the nature of the symbiosis between Pacific chub mackerel *Scomber japonicus* gills and a putative novel species, *Shewanella* sp., using a combination of whole genome sequencing (WGS) of isolated strains and a broad meta-analysis of 16S rRNA gene amplicon data derived from other marine fish species. It was found that three closely related amplicon sequence variants (ASVs) constituted a median of 30% of the gill

microbiome. The successful cultivation of representative isolates matching these environmental ASVs were recovered from mackerel gill samples and whole genomes for these strains were assembled and analyzed. The taxonomy of isolated *Shewanella* spp. strains was established using a phylogenomic approach. Comparative genomics of diverse *Shewanella* species highlighted an enrichment of virulence factors in the gill associated *Shewanella* strains as well as notable loss of function in polyunsaturated fatty acid production, a hallmark characteristic conserved in most members of the genus *Shewanella*. Finally, meta-analysis of fish gill amplicon data suggests that *Shewanella* spp. may associate with the gills of forage fishes and provides evidence of trophic transmission to predators of forage fishes.

INTRODUCTION

In the field of host-associated microbial ecology, we study the interplay between microbial diversity and their multicellular hosts, such as animals, algae, and plants. Multicellular eukaryotes can have diverse communities of symbiotic microorganisms which are distinct from their surrounding environments. These symbionts, whose relations to their hosts range from mutualistic to parasitic, modulate the biology of their hosts in ways that cannot be done by the host itself, extending the host's metabolic and defensive capabilities. For example, there are around 150 times more genes in the average human gut microbiome than in a human host [1]. There is now an effort to characterize diverse microbiomes using metagenomic, metatranscriptomic, and metabolomic data to discover what roles microbes play in each system, as well as how they potentially contribute to the healthy and dysbiotic states of their hosts and environments.

In the past two decades, the advent of Next Generation and Third Generation sequencing technologies such as Illumina (NGS) and Oxford Nanopore Technologies (ONT) have revolutionized the way we study the microbes in the world. In 2022, we can generate large sequencing datasets 6 orders of magnitude cheaper than in the year 2000, taking days rather than years to generate [2]. These data allow us to study microbes which cannot yet be cultured, while elucidating microbial community composition, functional potential, and genetic expression. In recent years, we have applied these technologies to diverse environments such as animal hosts, sediment, and water microbiomes [3].

The fish microbiome is a relatively understudied aspect of fish health. Fish constitute roughly half of vertebrate diversity with over 30,000 described species. Fish are also an important global resource as fisheries support over 200 million lives and

provide a protein source relied on by over 20% of the world's population [4]. There have been recent efforts to characterize the structure and constituents of natural microbiomes of fish (intestinal, gill, mucosal skin, etc.), as well as the physical and biological drivers that constitute them [5], [6], [7]. This knowledge can promote fish health in aquaculture systems, which face productivity limitations and environmental concerns due to diseases. This becomes even more important in the context of a global aquaculture boom which now yields nearly half of ocean subsidies [8].

A relatively understudied aspect of fish health is that of the fish gill microbiome. Fish gills are an extremely versatile organ responsible for gas exchange of adult fish, salt excretion, and nitrogen excretion in the form of urea and ammonia [9]. For filter feeding fish such as anchovies or mackerel, gill rakers are responsible for filtering out food from the water column. In addition to gas exchange and feeding, fish gills are a chemically diverse environment for microbes to live. The gill microbiome has the potential to be a significant driver in fish health because the fish gill's cellular epithelium, a two-cell layer between the internal and external environment, is a potential entry point for pathogens. Probiotic constituents of the gill may also benefit fish health by competitively excluding antagonistic bacteria by competing for nutrients, producing inhibitory compounds, and quorum sensing [10].

To date, constituents of the fish microbiome have been poorly characterized. Previous culture-based studies debated whether there were specific gill adapted bacteria, or only transient bacteria present on fish gills [11]. However, the recent application of new sequencing technologies has allowed us to study a greater fish diversity, with more samples, and with greater resolution. Recent studies using these technologies suggest that

the gill microbiome is not only unique compared to the surrounding environment, but is perhaps the most unique microbial community within the fish microbiome [5]. One recent study on carp showed the presence of intracellular ammonia-oxidizing bacterial symbionts. This presumably assists in denitrifying gill tissues, to the benefit of the fish as excess nitrogen is damaging to tissues [12]. Another study has shown that the trout gill microbiome has the ability to inhibit growth of fungal pathogens of fish [13]. Fish microbiome datasets which cover large spatial and temporal scales and/or fish diversity can be used to identify potential symbiotic relationships of microbes and fish.

In a recent study conducted by Minich *et al.*, the microbiome of the Pacific Chub Mackerel, *Scomber japonicus*, was tracked over a one-year period at monthly intervals [14]. These data focused on the microbiome of the gut, digesta, pyloric caeca, mucosal skin, and gills and show that among fish mucosal microbiota, the gill environment is the most unique environment by beta diversity metrics. A notable point of interest shows that in the gills of *S. japonicus* there is an ever present enrichment of bacteria in the *Shewanella* genus. Enrichment of *Shewanella* on fish gills has been previously reported in Mo'orean reef fishes [6]. However, the nature of the symbiosis between *S. japonicus* and *Shewanella* remains unknown.

The Shewanellaceae are a remarkable family of Gram-negative, facultative anaerobic bacteria containing a single genus, *Shewanella*. The hallmark trait of *Shewanella* is their ability to utilize a diverse array of alternative terminal electron acceptors in the absence of oxygen. To date, there are over 20 known terminal electron acceptors utilized such as nitrate, sulfate, solid metals such as iron and manganese, toxic metals such as chromium (IV) and uranium (VI), and even organics such as

trimethylamine-n-oxide (TMAO) [15], [16]. This makes them the most diverse respiratory organisms yet discovered and allows them to thrive in redox stratified environments. *Shewanella putrefaciens*, the first *Shewanella* to be characterized, is known as a major player in fish spoilage where it utilizes TMAO as a terminal electron acceptor, reducing it to trimethylamine. *S. putrefaciens* has also been shown to have probiotic effects when used in aquaculture settings [17], [18]. *Shewanella* are also known for their psychrotolerance and piezotolerance nature, which may be related to their ability to produce long chain omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA). PUFA synthesis is a strategy for maintaining membrane fluidity in environments of low temperature and high pressure, which is important considering the cold and depth of the ocean's biosphere. It is thought that *Shewanella* may be an important source of EPA in marine animals, as EPA is an essential nutrient for higher trophic level animals that must acquire EPA from diet [19]. *Shewanella* are found in planktonic form in the water column as well as both biotic and abiotic surface-associated environments and have been isolated from deep sea sediments, animal hosts, and seawater [20]. *Shewanella* have previously been noted to be enriched in fish gills, however the role they play in this environment is unknown [6].

In this study, we set out to describe the nature of the symbiosis between these *Shewanella* and the gills of *Scomber japonicus*. The goals of this study are twofold: To isolate representatives of these *Shewanella* for whole genomic analysis, and to explore the extent of the *Shewanella* and gill association in greater fish diversity through an amplicon-based fish gill meta-analysis.

MATERIALS AND METHODS

Isolation

Pacific chub mackerel samples were caught via rod and reel on the Ellen Browning Scripps pier between August 2020 and July 2021. All specimens were immediately euthanized according to the standards of AVMA. Following euthanasia, a sterile cotton swab was used to collect microbial mass on the surface of each gill arch on the left gill. That swab was then washed in an isotonic solution of sterile, artificial sea water contained in an Eppendorf tube. The tube was placed on water ice and immediately transported back to the laboratory. 25 µl aliquots of this solution were spread onto Difco 2216 marine agar (75% concentration), and Sea Water Complete agar. Plates were enriched for 7 days at room temperature. Colonies were picked based on diverse colony morphology and were restreaked onto a plate of the same type of media as picked from.

Colony ID

Well isolated colonies were picked for colony identification via Sanger sequencing of the full length 16S bacterial ribosomal RNA gene. DNA extraction was accomplished using a thermocycler boil step. These extractions were added to a PCR mix containing NEB Taq 2x Master Mix and 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers. Following the PCR reaction, gel electrophoresis was performed to ensure an amplification fragment of 1.5kb, the approximate length of the 16S rRNA gene. Successful reactions were cleaned up using the NEB Shrimp Alkaline Phosphatase. Cleaned PCR products were sent to Eton Bio (San Diego, CA) for Sanger sequencing. For each PCR product, there were two Sanger

sequencing reactions, one using the forward primer, and the other using the reverse primer. A *de novo* assembly of the full length 16S rRNA gene was made with each PCR's respective forward and reverse sequences in Geneious. Following full-length 16S rRNA gene assembly, an NCBI BLAST analysis was performed to determine the closest species match to each isolate. 16S genes were also aligned with the Shewanella amplicon sequence variants (ASVs) from the mackerel time series dataset generated previously to identify isolates which are enriched in the gills [14]. This dataset will henceforth be referred as the 1-year mackerel dataset.

Isolates chosen for sequencing were grown in 100% 2216 marine broth at 20c in a shaking incubator. Culture cell counts were enumerated with a BD Acurri C6 Plus flow cytometer to inform an appropriate culture volume for DNA extraction. DNA extraction was performed using the MoBio Ultraclean Microbial DNA Isolation kit. DNA quantification was measured via fluorometry using the Qubit 2.0. DNA purity was approximated using spectrophotometry via Nanodrop. The DNA fragment size structure of extracted DNA was determined by gel electrophoresis. Purified genomic DNA from each strain was sent to Novogene (San Diego, CA) for library prep and Illumina sequencing. The library prep kit used was NEBNext® DNA Library Prep Kit. Sequencing was done on an Illumina MiSeq using a 2 x 150 paired end method. A total of 4003372 and 6182916 reads were produced for isolate strains P8 and P11, respectively. Another set of aliquots were sent to the Salk Institute for Biological Studies (La Jolla, CA) for library preparation and Oxford Nanopore (ONT) sequencing. At the Salk Institute, a second DNA extraction of P11 was performed using the Circulomics CBB HMW kit. Library prep was done using the SQK-LSK110 kit. Multiplex sequencing was performed

on an ONT = Promethion flowcell R000-133. Library preparation for P11 was done using the SQK-RAD004 kit and sequenced on an ONT Promethion flowcell PR0002 high accuracy base calling R000-132. ONT sequencing yielded 64,568 and 546,092 reads for P8 and P11, respectively.

Genome Assembly

Quality of Illumina and ONT libraries were assessed in FastQC (Andrews and Others 2010). Low quality reads and read ends were omitted using Trimmomatic [21]. Chromosomal assemblies were made using the long read ONT data in FlyE [22]. A subset of the longest reads covering the chromosome an average of 100 times were used in each genome assembly and the minimum read lengths used in assembly were 9800 and 11905 base pairs for P8 and P11, respectively. The minimum overlap of reads was set at 1000 bp. Illumina reads were aligned to the ONT chromosome assemblies using Bowtie2 [23]. Using this alignment, each chromosome was polished using the Illumina data in Pilon [24]. Finally, the polished chromosomes were reordered, making the origin of replication at the start of the sequence. Quality assessment of assemblies were done using QUAST [25]. The completeness of the assemblies was assessed using 312 single copy Gammaproteobacteria marker genes via CheckM [26].

Genomic Analysis

Genome annotation was completed using both Prokka and Rast pipelines [27], [28]. Synteny between the two genomes was visualized using MUMmer2.0 and the Artemis Comparison Tool (ACT) [29], [30]. For phylogenomic analysis, publicly

available genomes of 24 Shewanella species were downloaded from NCBI. An average amino acid matrix of the 24 species with the 2 strains was generated using the Kostas Lab genome-based distance matrix calculator [31]. Next, a multilocus concatenated alignment of 400 universal marker genes was made with Phylophlan 3.0[32]. Using this alignment, a phylogenomic tree was built in GGTree [33]. Five diverse species of Shewanella were chosen for a comparative genomic analysis. A list of orthologs was generated using OrthoMCL [34].

Table 1. Shewanella assemblies used in comparative genomic analyses. A curated list of species was chosen to represent diversity across all clades in the genus. The accession numbers are listed along with the corresponding genome size, GC content, and sample location.

Species	GenBank Assembly Accession	%GC	Genome Size MBP	Sample Location
S. sp. P8	N/A	38.8	4.02	Pacific chub mackerel gill
S. sp. P11	N/A	38.9	4.02	Pacific chub mackerel gill
S. marina	GCA_000614975.1	40.4	4.42	N/A
S. frigidmarina	GCA_003605145.1	40.7	4.83	Chazhma Bay seawater
S. japonica	GCA_002075795.1	40.8	4.98	Russian Sea water
S. livingstonensis	GCA_003855395.1	41.1	4.84	Antarctic sewater
S. piezotolerans	GCA_000014885.1	43.3	5.4	Deep sea sediment
S. halifaxensis	GCA_000019185.1	43.7	5.23	Sediment of a munitions dump off Nova Scotia
S. woodyi	GCA_000019525.1	43.7	5.94	Alborean seawater
S. putrefaciens	GCA_000615005.1	44.3	4.34	N/A
S. psychrophila	GCA_002005305.1	44.3	6.35	Western Pacific Sediment
S. pealeana	GCA_000018285.1	44.7	5.17	Accessory nidamental gland of the squid Loligo pealei
S. denitrificans	GCA_000013765.1	45.1	4.55	Anoxic seawater from Baltic Sea
S. waksmanii	GCA_000518805.1	45.3	4.97	Marine Invertebrate
S. colwelliana	GCA_000518705	45.4	4.58	Oyster containing aquaculture unit
S. atlantica	GCA_003966265.1	45.8	5.39	Atlantic sediment
S. oneidensis	GCA_000146165.2	45.9	5.13	Lake Oneida
S. benthica	GCA_015069875.1	46	4.35	Deep sea
S. xiamenensis	GCA_001723195.1	46.2	5.37	Hospital effleunts
S. baltica	GCA_900456975.1	46.3	5.3	Environmental
S. algidipsicola	GCA_003605125.1	46.6	4.2	Baltic seawater
S. mangrovi	GCA_000753795.1	48.1	4.22	Mangrove sediment
S. chilikensis	GCA_003217175.1	52.4	4.44	N/A
S. algae	GCA_000615045.1	53	4.83	N/A
S. amazonensis	GCA_000015245.1	53.6	4.31	Amazon river delta sediment
S. loihica	GCA_000016065.1	53.7	4.6	Hawaiian Hydrothermal Vent

Meta Analysis of Amplicon Data

Gill microbiome Short Read Archives (SRA) were downloaded from NCBI using a Perl script and were uploaded to Qiita [35]. An independent, closed reference meta

analysis was performed in Qiita, generating an unrarified feature table using the V4-V5 16S rRNA gene amplicon data. The data were left unrarified in order to analyze the largest range of diversity. Subsequent analyses were performed in R.

RESULTS

Shewanella Isolates From S. japonicus Gills

A total of 12 Shewanella isolates were identified from the mackerel gill culture enrichments. Two isolates were chosen for sequencing, namely P8 and P11. P8 was chosen for whole genome analysis as it has a 100% match to the ASV which was most enriched in the 1-year mackerel dataset [14]. A second isolate, P11, was chosen for whole genome analysis as it aligned with an ASV which is present in the 1-year mackerel dataset, but not enriched relative to P8.

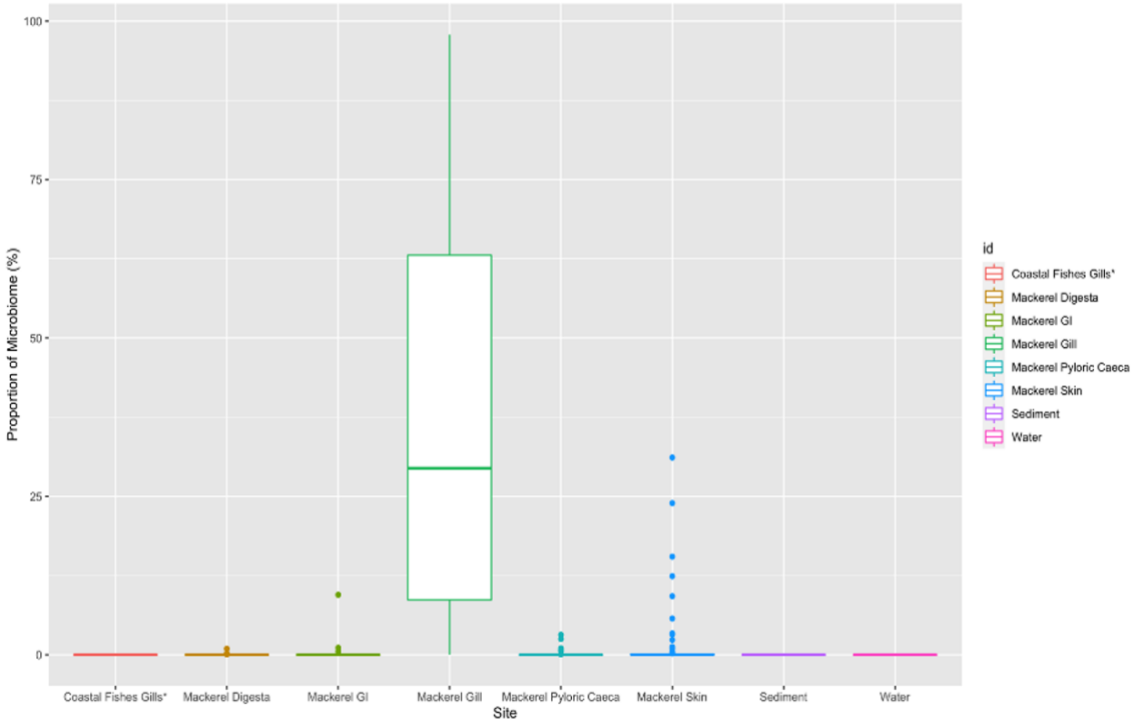

Figure 1. Enrichment of *Shewanella* spp. On the Gills of Pacific Chub Mackerel, *Scomber japonicus*. The relative proportion of *Shewanella* spp. ASVs in mackerel body sites (gills, skin, GI, digesta, pyloric caeca), sediment, water, and coastal fishes gill microbiomes. These data, collected by Minich *et al.*, come from one year of mackerel samples (N=121) collected at monthly intervals. Mackerel gill associated Shewanella ASVs are a concatenated group of 3 ASVs (namely Shew1, 2, and 3) which are present in the majority of mackerel gill samples. *Coastal Fishes Gills (N=7) is a list of multiple species of fish gill samples consisting of opaleye, barred sand bass, California salema, rock wrasse, seniorita, and sargo.

Diversity of Shewanlla From 1-Year Mackerel Data

A dataset collected by Minich *et. al.* comprising one year of monthly mackerel samples collected from multiple mackerel body sites (gills, skin, GI, pyloric caeca, and digesta), environmental samples (water and sediment), and fish gill samples from seven additional San Diego coastal fishes (opaleye, barred sand bass, California salema, rock wrasse, seniorita, and sargo) was used to analyze the diversity of Shewanella on mackerel gills [14]. In this dataset, there were 77 unique ASVs mapping to the Shewanella genus. Of those 77, 50 ASVs were found in mackerel gills. However, three of the ASVs were more enriched and prevalent than the others, as plotted in Figure 1. The three mackerel-associated (MA) *Shewanella* ASVs, namely Shew1, Shew2, and Shew3, were present in 85.12%, 86.77%, 61.98%, of mackerel gill samples, respectively. At least one of the three ASVs were found in 98.34% of mackerel gill samples. These ASVs accounted for 94.24% of all Shewanella hits in the dataset (31.39%, 54.43%, and 8.42%, respectively). In mackerel gill samples, the median enrichment of mackerel *Shewanella* was 30.10% of the total microbiota sampled. The median enrichment of these 3 ASVs on the other mackerel body sites, coastal fishes gills, water and sediment environments is 0%. However, these ASVs are present in some samples for each of the other mackerel body sites, but not in any of the coastal fishes gills, water, or sediment samples. A few samples, particularly in the skin samples, showed high enrichment of mackerel-associated *Shewanella*.

Table 2. Genome assembly statistics of *Shewanella* spp. isolates P8 and P11.

Assembly Statistics		
Strain ID	P8	P11
Chromosome Circularity	Yes	Yes
# Contigs	6	41
N50	4015455	4018545
Total Length (bp)	4271605	4195466
%GC	38.82%	38.85%
CheckM Completeness	100%	100%
CheckM Contamination	0.54%	0.54%
# Predicted Genes	1642	1619

Genome Assemblies of S. sp. strains P8 and P11

Two isolates were chosen for whole-genome shotgun sequencing. The 16S rRNA gene V4 region of isolate P8 was 100% identical to the most enriched ASV, Shew2. Isolate P11 was chosen as it was closely related, yet not enriched in the mackerel amplicon dataset. Genome assemblies of P8 and P11 yielded circular chromosomes of 4.015 and 4.018 Mbp, respectively. The percent GC is 38.82 and 38.85, respectively. The largest contigs from both assemblies yielded 100% complete chromosomes based on the presence of 312 universal Gammaproteobacteria marker genes. Contamination for both strains is very low at 0.54%. P8 and P11 have 1642 and 1619 genes predicted by QUAST.

Table 3. BLAST analysis of marker genes from the enriched strain *Shewanella* sp. P8. Sequences of the 16S rRNA and *rpoB* genes were taken from annotations generated by Prokka.

Isolate	gene	Match	%ID	%Query Coverage	Max Score	Total Score	Acc. Len
P8	16s	S. carasii	95.01	100	2416	19308	4454556
P8	16s	S. algae	94.89	100	2405	19204	4924764
P8	16s	S. woodyi	94.88	100	2405	23797	5935403
P8	rpoB	S. putrefaciens	99	84	3851	3851	4659617
P8	rpoB	S. baltica	99	83.68	3779	3779	4999751
P8	rpoB	S. decolorationis	99	83.4	3718	3718	4719362

The closest species match to *Shewanella* sp. P8 based on full-length 16S rRNA gene alignment was *S. carasii* which was 95.01% identical with 100% query coverage. However, the closest species match based on *rpoB* alignment was *S. putrefaciens*, which was 84% similar with 99% query coverage.

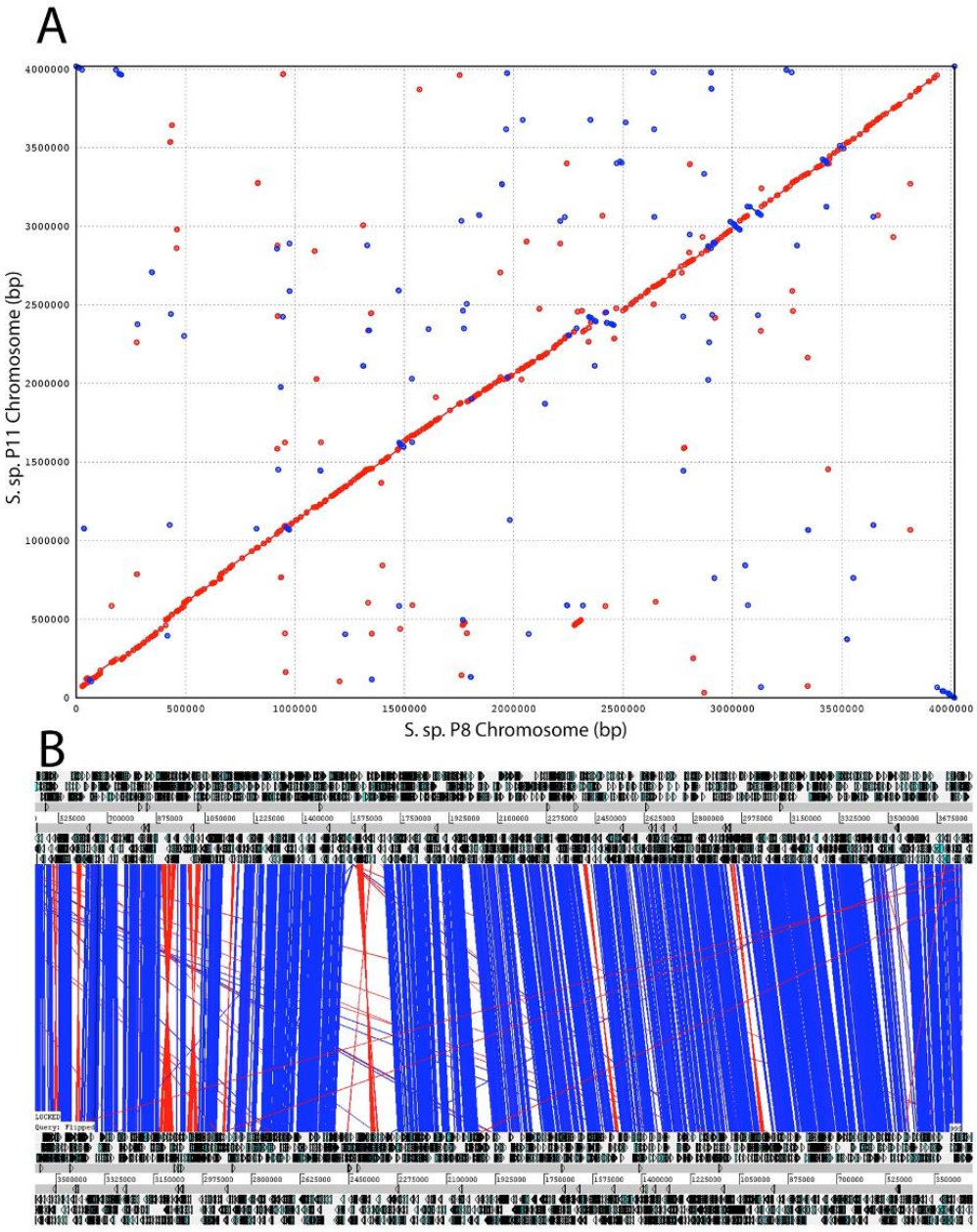

Figure 2. Synteny plots comparing conserved gene order between the genomes of P11 and P8. Mummer and Artemis Comparison Tool (ACT) analyses show the two isolate genomes are highly syntenic. Figure 2A shows synteny plotted by mummer. The chromosome of P11 is on the y axis and the chromosome of P8 is on the x-axis. The red dots and lines show regions which are syntenic between the P8 and P11 chromosomes, where the blue shows areas of discontinuity. Figure 2B shows the synteny visualized by ACT. The chromosome of P8 is on the top, and the chromosome of P11, on the bottom. The blue regions show synteny between the two chromosomes and the red shows regions which are inverted between the two.

Genetic synteny was used to compare chromosomal organization between P8 and P11. Isolate strains P8 and P11 are highly syntenic as shown by the one-to-one slope in the Mummer Plot (See Figure 2A). Short regions of genetic inversions can be seen at 2.4mbp, 2.8mbp, 2.9mbp, 3mbp, and 3.1mbp.

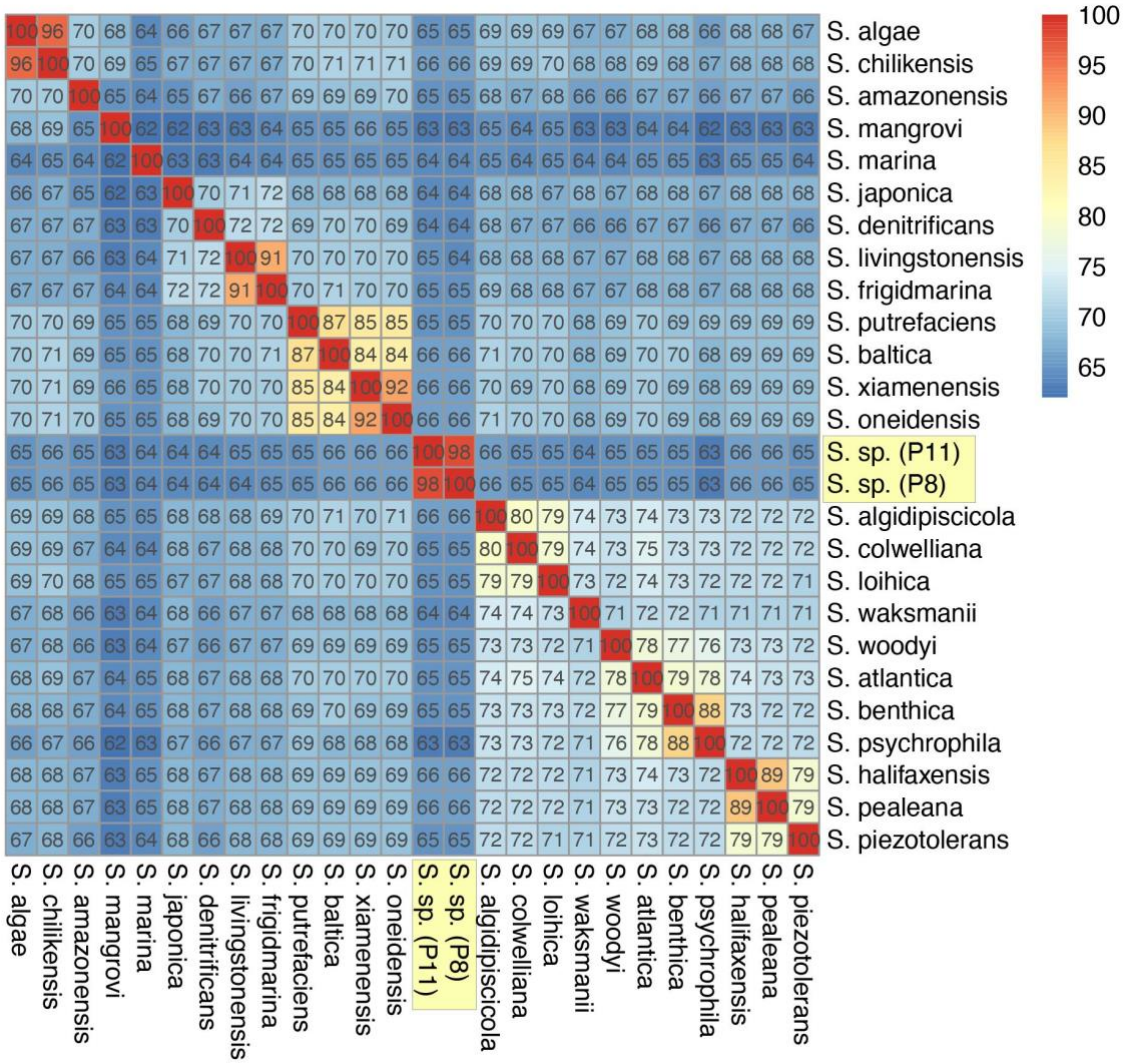

Figure 3. Average amino acid identity (AAI) matrix shows distinction of *Shewanella* sp. P8 and P11 compared to 24 other species of Shewanella.

Average Amino Acid Identity of Shewanella Diversity

An AAI matrix analysis elucidates clades within the *Shewanella* genus. One clade can be seen between *S. algidipiscicola* and *S. piezotolerans* with AAI values greater than 71. AAI suggests another clade between *S. marina* and *S. oneidensis*, with values greater than 68. AAI shows similarity between *S. algae*, *S. chilikensis*, *S. amazonensis*, and *S. mangrovi*. Strains. P8 and P11 show AAI values between 63 and 66 for all comparison species.

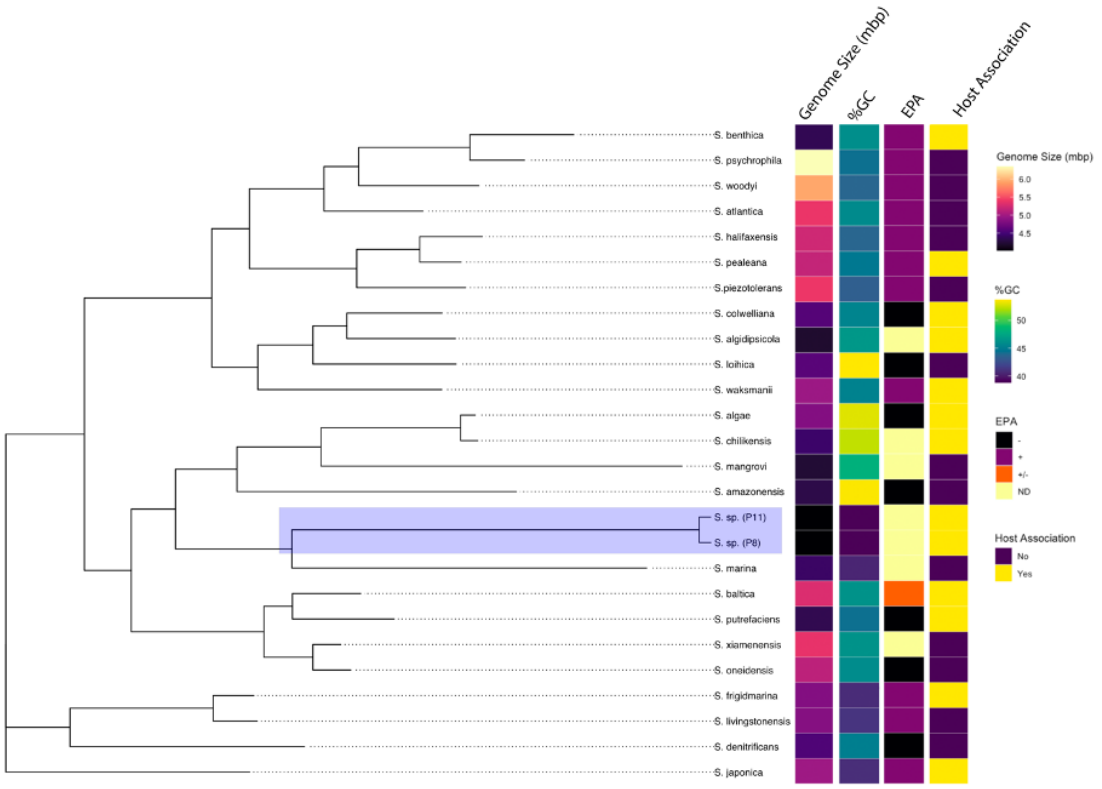

Figure 4. Phylogenomic tree of select *Shewanella* species elucidates placement of strains P8 and P11 in the context of *Shewanella* diversity. This tree was built from a concatenated alignment of 400 universal markers from Phylophlan 3.0. The first and second annotation columns show genome size (in Mbp) and %GC for each genome. The third column annotation shows if there is experimental evidence of omega-3 polyunsaturated fatty acid EPA production of isolates in culture. Experiments of EPA production in *Shewanella baltica* are variable as some experiments have shown production, and others not. The fourth column annotation shows if there is evidence of host association. Evidence of host association is assigned if there are representative isolates deriving from a multicellular organism body site.

Taxonomy of Shewanella based on Phylogenomic Analysis

The multi-locus concatenated phylogenomic tree shows three clades within the *Shewanella* genus. The clade shown on the top, which includes species between *S. benthica* and *S. waksmanii*, seems to be more tightly clustered than the other groups. This clade appears to generally have larger genome sizes, with medium to high %GC (43.3-53.7%). This group seems to be more consistent in production of EPA with the exception of *S. algidipisicola*. Evidence of host association in this clade is highly variable. The second clade includes *S. algae*, *S. oneidensis*, as well as strains P8 and P11. This clade appears to generally have smaller genome sizes and highly variable GC (38.8-53.6%). Most species in this clade have no experimentation of EPA production to date. Those which have been tested have not shown production of EPA with the exception of *S. baltica* which has been shown to produce EPA in some studies, and has been shown to not produce EPA in other studies [36], [37]. Many species within this clade have representative isolates deriving from a multicellular host. The last clade on the bottom, with species such as *S. frigidmarina* and *S. japonica*, appears to have medium to small genome sizes and variable %GC (40.7-45.1%). This group also has variability in evidence of host association and EPA production.

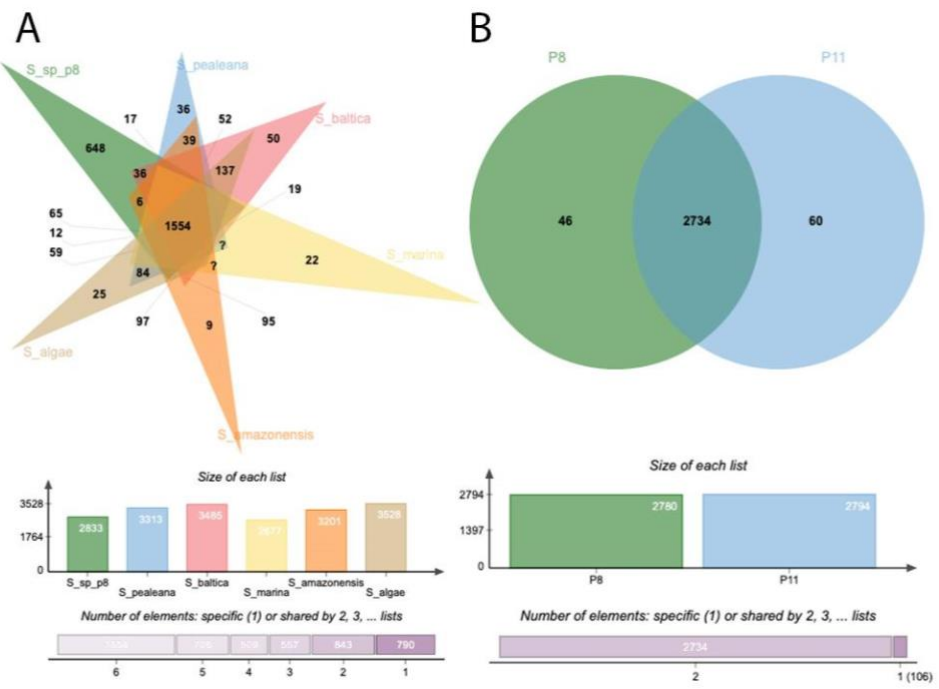

Figure 5. (A) Unique and shared genetic elements (orthologs) of *S.* sp. P8 and five other *Shewanella* species. The numbers inside the outermost shapes represent the number of elements specific to that species. The number in the center shape shows the number of elements shared by all six comparison species. (B) Unique and shared genetic elements (orthologs) of *S.* sp. P8 versus P11.

Comparative Genomics

OrthoVenn2 analysis shows 77 genetic elements (orthologs) which are specific to *S. sp.* P8. The number of specific elements for the other species ranges from 8 in *S. amazonensis* and 45 in *S. baltica*. *S. marina* has the highest number of shared elements with *S. sp.* P8 at 63, and the lowest number of shared elements with *S. sp.* P8 is *S. amazonesis* with 6. There are 1556 elements shared amongst all six species. When analyzing P8 vs P11 using OrthoVenn2, 46 and 60 unique elements were highlighted in

both strains, respectively (See Figure 5B). Most of these differences were either annotated as hypothetical genes or transposons.

Table 4. Relative abundance of mackerel-associated (MA) *Shewanella* on diverse fish microbiomes. MA *Shewanella* were detected in 22 species of fishes beyond Pacific chub mackerel. Quantity of MA *Shewanella* and MA *Shewanella* are reported in relative abundance from unrarified data. Column 'N' shows the proportion of samples of a given species and sample type which MA *Shewanella* were detected.

Host Species	Host Common Name	Sample Type	N	Abundance Total Shewanella	Abundance MA Shewanella
Scomber japonicus	Pacific Chub Mackerel	fish gill	120/121	69.171	63.030
Abudefduf sexfasciatus	Scissortail Sergeant	fish gill	1 of 5	3.830	0.004
Acanthurus nigricans	White Cheek Surgeonfish	fish gill	1 of 5	0.935	0.180
Anisotremus davidsonii	Sargo	fish digesta	1 of 1	0.110	0.110
Cephalopholis argus	Peacock Grouper	fish intestine	1 of 5	11.402	1.044
Chaetodon tichrous	Tahiti Butterfly fish	fish gill	1 of 1	0.665	0.003
Ctenochaetus striatus	Lined Bristletooth	fish gill	1 of 1	0.711	0.091
Cynoscion parvipinnis	Shortfin Weakfish	fish hindgut digesta/	1 of 1	29.381	13.586
Cynoscion parvipinnis	Shortfin Weakfish	midgut digesta	1 of 1	0.427	0.251
Cynoscion parvipinnis	Shortfin Weakfish	fish skin	1 of 1	0.976	0.258
Gibbonsia elegans	Spotted Kelpfish	fish gill	1 of 1	0.004	0.004
Gymnothorax mordax	California Moray	fish hindgut digesta	1 of 1	0.055	0.012
Hyperprosopon argenteum	Walleye Surfperch	fish digesta	1 of 1	0.014	0.014
Lutjanus fulvus	Blacktailed Snapper	fish gill	1 of 4	4.680	0.003
Lycodes cortezianus	Bigfin Eelpout	fish skin	1 of 1	0.303	0.006
Mustelus californicus	Grey Smooth-hound	fish gill	1 of 1	0.004	0.004
Paralabrax clathratus	Calico Bass	fish gill	1 of 1	0.014	0.003
Paralabrax maculatofasciatus	Spotted Sand Bass	fish hindgut digesta	1 of 1	0.093	0.008
Physiculus rastrelliger	Hundred Fathom Mora	fish gill	1 of 1	0.003	0.003
Porichthys myriaster	Midshipman	fish skin	1 of 1	0.004	0.004
Sardinops sagax	Pacific Sardine	fish gill	1 of 1	0.301	0.155
Sardinops sagax	Pacific Sardine	fish hindgut digesta	1 of 1	0.227	0.184
Sardinops sagax caerulea	Pacific Sardine	fish skin	2 of 4	0.859	0.781
Sardinops sagax caerulea	Pacific Sardine	fish skin	2 of 4	33.696	2.446
Sargocentron microstoma	Smallmouth Squirrel fish	fish intestine	1 of 3	10.160	0.275
Seriola dorsalis	Yellowtail	fish hindgut digesta	1 of 1	0.323	0.211
Seriola dorsalis	Yellowtail	fish skin	1 of 1	0.074	0.074
Trachurus symmetricus	Pacific Jack Mackerel	fish hindgut digesta	1 of 1	9.302	3.618
Trachurus symmetricus	Pacific Jack Mackerel	fish gill	1 of 2	0.199	0.106
Paracirrhites arcatus	Arc-eye Hawkfish	gill	1 of 10	17.991	2.888

16s rRNA Meta Analysis

In an amplicon meta analysis of 180 species of fishes, ASVs matching mackerel-associated (MA) *Shewanella* were found in 22 non-Pacific chub mackerel fishes in sample types ranging from gills, guts, mucosal skin, and digesta and in a variety of habitats and geographical locations. Notably, MA *Shewanella* were detected in Moorean reef fishes, deep sea fishes, and a shark gill sample. In most samples in which MA *Shewanella* were detected, they represent less than 1% of relative abundance. However,

MA *Shewanella* were found in >1% abundance of peacock grouper intestine, shortfin weakfish hindgut, Pacific sardine skin, jack mackerel digesta, and arc-eye hawkfish gills. Notably, MA *Shewanella* were detectable in two of three non-Pacific Chub mackerel forage fish gill samples analyzed.

DISCUSSION

The goal of this research was to describe the relationship between environmental Shewanella species and their specific association with the gills of Pacific chub mackerel. This work was inspired by a 16S rRNA dataset which showed a robust enrichment of *Shewanella* which was specific to Pacific chub mackerel gill samples. Isolated strains *Shewanella* sp. P8 and P11 were analyzed via whole genome sequencing to describe the symbiosis between these novel strains and the gills of Pacific chub mackerel. First, the phenomenon of *Shewanella* and mackerel gills is described using amplicon data. Next, isolated strains P8 and P11 are described as a putative novel species, and we report some of their genomic features which appear to be unique within *Shewanella* diversity. Finally, we discuss evidence from the P8 and P11 genomes which suggest evidence of parasitism.

An analysis of 121 mackerel gill samples collected over one year shows that there is a strong enrichment of related *Shewanella* ASVs, constituting a median proportional abundance of 30.1% (see Figure 1). This statistic does not seem to be conflated by a high number of *rrn* operons, as strains. P8 and P11 each maintain five copies, which is consistent with the 4.2 average copy number of *rrn* operons in other Shewanella species [38]. The one-year of monthly mackerel samples shows these *Shewanella* are present in the other body sites in low prevalence and abundance, but are absent in water, sediment, and the gills of other fish species. These data suggest that there is a robust enrichment of MA *Shewanella* specific to mackerel gills. A meta analysis of 180 fish species shows that these *Shewanella* are not exclusive to mackerel, nor Southern California coastal waters, as they are found present in Moorean fish samples in low proportional abundance. There doesn't seem to be a phylogenetic driver of this phenomenon, as the *Shewanella* were not

found in the other thuniid samples. Rather, the driver of this phenomenon may be specific to the life history or physiology of mackerel. The true extent of this phenomenon will not be known until more fish microbiome data becomes available, as this dataset represents less than 0.5% of fish diversity.

Shewanella isolate strains P8 and P11 may belong to a new species. BLAST analysis of the full length 16S rRNA gene shows that the closest relatives to P8 and P11 have a full length 16S rRNA gene sequence that is approximately 95% identical to its closest species-defined relative in NCBI GenBank. This is below 97%, which is the commonly accepted threshold of species delineation [39]. Whole genome AAI data further supports the notion that this may be a new species as the highest AAI values are 66 (see Figure 3), suggesting nearly a genus level difference to other *Shewanella* species [40]. Finally, the analysis of orthologous genes from OrthoMCL and OrthoVenn2, highlight some of the unique metabolic features of these novel Shewanella strains.

One of the major differences of P8 and P11 versus other *Shewanella*, as highlighted by analysis of orthologous genes, is in fatty acid biosynthesis. Notably, the genes responsible for synthesis of EPA are missing in the genomes of P8 and P11 as well as *S. marina*, which is the closest species relative to *S. sp*. by multi locus concatenated alignment (see Figure 4). Previously, the genes for EPA synthesis were thought to be ubiquitous within all *Shewanella* genomes [41]. Conversely, the genomes of P8 and P11 and *S. marina* uniquely have genes responsible for cyclopropane fatty acid synthesis. Cyclopropane fatty acids have shown to have antifungal properties, potentially acting in disrupting fungal membranes or inhibiting synthesis of fungal fatty acids [42]. Fungal pathogenesis of marine fishes remains an understudied field. However, fungi such as

microsporidia are known to infect the gills of marine fishes [43]. Fatty acid metabolism analysis is necessary to determine which fatty acids are actively synthesized by P8 and P11.

Another feature that seems to be unique to P8 and P11 is purine breakdown. Analysis of orthologous genes shows that P8 and P11 are capable of purine metabolism as well as the complete breakdown of uric acid into ammonia, due to the presence of a urea transporter. Breakdown of purines into ammonia could be utilized as a virulence factor for *S. sp*. For example, human pathogens such as *Cryptococcus neoformans* produce ammonia from urea resulting in alkalization and thus damage to tissue (Toplis et al. 2020). Mackerel is known to be high in purine content in its tissues, which suggests a possible virulence pathway for *Shewenella* in mackerel. It is possible that in the gills, breakdown of urea into ammonia could benefit the fish host by denitrifying gill tissues. However, *S. japonicus* is an ammonotelic species, meaning most of its nitrogen is already excreted as ammonia. Only about 10% of its nitrogen is excreted in the form of urea. It is not apparent how purine metabolism and uric acid degradation may benefit *S. sp*. in the gill environment, and metatranscriptomic data from mackerel gills would be necessary to determine if these genes are actively being transcribed in the gill.

The draft genome assemblies of *S. sp*. P8 and P11 show evidence of host-associated symbiosis. The chromosome size of these isolates of 4.02 Mbp is reduced in size compared to the median genome size of a *Shewanella* genome of 4.8-4.9 Mbp [44]. This is consistent with the minimal genome concept in the context of a symbiotic relationship, where the microbe can afford a smaller genome due to the host providing an environmental incentive to the symbiont [45]. This could also help explain why the

mackerel derived *Shewanella* grew poorly on 2216 marine agar, taking five days to form colonies, which is significantly slower compared to other species such as *S. pealeana* which show considerable visible growth overnight. It is also worth noting that the GC content of *Shewanella sp.* P8 and P11 (38%) is exceptionally low, the lowest of any *Shewanella* sequence to date (see Figure 4) [44]. It has been observed that reduced genomes tend to have AT biased genomes. For example, highly reduced genomes of symbionts such as *Canidatus* Zinderia insecticola, which has a 209 kbp chromosome and a GC content of 13.3% [46]. Comparative genomics highlight a number of apparently unique gene clusters which suggest the potential pathogenicity of P8 and P11.

Genomic analysis suggests evidence of parasitism. First, comparative genomic analysis of P8 and P11 show enrichment of type III, IV, and VI secretion systems (T3S, T4S, and T6S) as well as multifunctional-autoprocessing repeats-in-toxin (MARTX) genes, compared to other *Shewanella* genomes. T3/4/6S and MARTX toxins are virulence factors used by pathogens to translocate cytotoxic effector proteins to host cells [47]. P8 and P11 also have several of the genes involved in the formation of toxin coregulated pilus (TCP), a *Vibrio cholerae* pathogenicity signature which allows *V. cholerae* to colonize the human intestine. TCP are a type IV pilus which are often an essential virulence factor of Gram negative pathogens, responsible for aggregating cells together to form microcolonies, aiding in biofilm formation and evasion of the host immune system [48]. Type IV pili are often involved in bacterial adhesion to epithelial cells, which may explain how *S.* sp. is able to effectively colonize the surface of fish gills. Type IV pili are present in all *Shewanella*, however this cluster of virulence-associated pili biosynthesis genes seem to be unique to *S.* sp.

It is important to note that despite the enrichment of virulence factors in the genomes, evidence of disease in the *Shewanella* enriched *S. japonicus* gill samples was not observed. There could be a couple explanations for this. First, *S. sp.* may be an opportunistic pathogen, remaining commensal with the fish host until it becomes less healthy due to age, injury, or disease. *S. sp.* could also be a trophically transmitted pathogen. This would be possible as mackerel are a low trophic level forage fish, which are fed upon by a variety of higher trophic level predators, making mackerel an efficient primary host. This is supported by the fish microbiome meta analysis, which shows the presence of *S.* sp. in gut microbiome samples of predators of mackerel such as yellowtail (*Seriola dorsalis*), shortfin corvina (*Cynoscion parvipinnis*), and the California moray (*Gymnothorax mordax*) (See Table 4). Interestingly, the non-mackerel sample with the highest enrichment of MA *Shewanella* was the hindgut sample of shortfin corvina (13.5%), a pelagic predator of mackerel (See Table 5). This idea is further supported by the presence of *S.* sp. in other low trophic level forage fishes such as the Pacific sardine (*Sardinops sagax*) and the Pacific jack mackerel (*Thrachurus symmetricus*). However, greater sample sizes of both forage fishes and predators of forage fishes would be necessary to draw a strong connection.

Major questions remain unanswered regarding the symbiotic relationship between Pacific chub mackerel gills and *S.* sp. The data analyzed in this study suggest that these novel *Shewanella* are opportunistic pathogens existing on mackerel gills as parasites. However, it does not explain an enrichment that seems to be specific to gills, and specific to Pacific chub mackerel. One major limitation of the genomic analysis of isolate strains *S.* sp. P8 and P11 are the lack of annotation of unique gene clusters. Analysis from

OrthoVenn2 and OrthoMCL show that *S.* sp. has hundreds of gene clusters which seem to be unique to this species. Most of these clusters are annotated as hypothetical proteins which severely limits our ability to understand why they might be associated with fish gills, and specifically with mackerel.

In the context of climate change and global warming, it is important to describe and track potential pathogens to understand the potential impact they may have on fisheries and local ecosystems. Future work is planned to help answer some of these questions. First, monthly samples of *S. japonicus* have continued to be collected since May 2017. Since *S. japonicus* is distributed throughout the Pacific, it will also be important to collect mackerel samples from the North, South, and West Pacific to determine any spatial drivers in this symbiosis. Lastly, a more comprehensive dataset of marine animal microbiomes could help us find this bacterium associated with other ecosystems, organisms, and body sites.

Continued research on the fish microbiome is important in the context of global warming. We must describe the natural state of the fish microbiome, and putative symbionts such as *Shewanella* sp. in order to understand how they are influenced by climate change. Further work in monitoring these changes will help further our understanding of *Shewanella* on ecosystems and fisheries.

This thesis, in full, is currently being prepared for submission for publication of the material. Kich, Ryan; Eric E. Allen. The thesis author was the primary investigator and author of this material.

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