Isolation of a *Shewanella* species that grows anaerobically on nitrate and acetate and aerobically on LB

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Introduction

My initial interest was in isolating and studying sulfur-oxidizing denitrifying autotrophs in the environment and comparing their metabolic versatility to that of *Sulfuromonas denitrificans* [1]. However, we instead enriched for nitrate- and acetate-dependent organisms that are almost certainly heterotrophs.

Methods and Data

Isolation media: Isolation media was concocted as follows: First, "brackish base" (BB) was made by combining a number of salts (Table 1) per liter final volume and autoclaving to sterilize. Then, sodium bicarbonate and potassium phosphate (5g $NaHCO_3$ and 0.5g KH_2PO_4) were dissolved in 50ml H_2O, sterile filtered, and added, along with 1ml sterile filtered trace metal solution (JRL 2016 special sauce).

To this base BB media, one or more of sterile-filtered 10 mM thiosulfate ("S"), 20 mM potassum nitrate ("N"), and/or 10 mM sodium acetate ("A") was added as described below.

To make plates, 1 gram-percent agar was added to the basic solution.

16s Colony PCR: Each colony was picked and touched to 20ul of alkaline PEG200 ("ALP"). This was then boiled for 5 minutes to lyse the cells. 1 ul of

Amount	Chemical
10.5g	NaCl
1.7g	$MgCl_2.6H_2O$
0.125g	$CaCl_2.2H_2O$
0.5g	KCl

Table 1: BB base media salts

Isolate	Stock identifier
1	16-4-MP-CTB-32
2	16-4-MP-CTB-33
3	16-4-MP-CTB-34
4	16-4-MP-CTB-35
5	16-4-MP-CTB-36
6	16-4-MP-CTB-37
8	16-4-MP-CTB-38

Table 2: Strain and stock identifiers for isolates described below.

cell lysate was then added to a 25 ul Promega GoTaq G2-based PCR reaction with the bacterial 16s primers 8F and 1391R. 16 rounds of PCR was performed with a 1.5 minute extension time. After PCR, bands were checked on a gel; then, Sanger sequencing was performed directly from the PCR reactions with the primer 515F.

Data availability: All data is available at https://github.com/ctb/2016-micdiv-report/.

Strain availability: The 7 isolates that transferred successfully from BB to LB to BB are available from the Microbial Diversity course strain collection at the Marine Biological Lab; see Table 2.

Results and Discussion

An enrichment from Trunk River grew with the addition of acetate

I initially designed media to enrich for sulfur oxidizing denitrifiers ("BB+SN"). I inoculated two Pfennig bottles (approx. 40ml) with approximately 2-3 cc of material. The material for the first enrichment, culture 4, was taken directly from the sediment layer on top of the sand in the main channel of Trunk River, approximately 8m from the start of the narrow outflow channel. The material for the second enrichment, culture 8, was taken from the underwater surface of a sea table enrichment that originated from a microbial mat, also taken from Trunk River. Both enrichments were incubated at 30 deg in a foil-lined box to prevent phototroph growth.

After 18 hours, significant turbidity was observed in both enrichments, along with substantial amounts of supersaturated gas, indicating growth. I therefore transferred 1 ml from each enrichment to another Pfennig bottle containing BB+SN. These transfer enrichments, however, failed to grow.

Based on scent, Dr. Leadbetter suspected that the original transfer of sediment contained acetic acid, indicating the presence of significant amounts of

acetate. I therefore added 400ul of 1M sodium acetate to both enrichment cultures, to a final concentration of 10 mM.

After the addition of acetate, both transfers grew to white opacity within about 16 hours at 30 deg. Two subsequent transfers of each culture (1ml into 40 ml "BB+SNA", BB with thiosulfate, nitrate, and acetate) also exhibited similar growth.

Enrichments exhibited nitrate and acetate dependent growth

To further analyze growth conditions, I employed a simple "differential diagnosis" approach and transferred each enrichment to four culture conditions: BB, BB with thiosulfate and acetate (BB+SA), BB with nitrate and acetate (BB+NA), and BB with acetate (BB+A). After incubation for 16 hours, only the BB+NA cultures grew, indicating that the enrichments required both nitrate and acetate but did not require thiosulfate (Figure 1).

Figure 1: Both enrichments 4 and 8 (first and third row) exhibit nitrate and acetate-dependent growth (third column) in anaerobic BB medium. No growth was observed in base BB media, BB+thiosulfate and acetate, or BB+acetate alone.

Enrichments yielded colonies when grown on solid media

I plated 1:500 and 1:5000 dilutions of enrichments 4 and 8 on BB+SNA solid media, and incubated the plates both anaerobically and aerobically at 30 deg. I

also plated the same dilutions on LB and grew aerobically at 30 deg. All plates showed density-dependent growth, although the colonies on the LB plates grew much faster (2-4 times) than either the aerobic or anaerobic BB plates.

Isolate colonies from aerobic LB plates grew successfully in anaerobic culture

I picked 4 colonies grown aerobically on LB from each enrichment (for a total of 8), and inoculated anaerobic BB+SNA cultures with them. 7/8 of the cultures grew within 48 hours, with three (culture 3 from enrichment 8, and cultures 5 and 8 from enrichment 4) growing overnight to opacity.

I then transferred these three isolates (3, 5, and 8) from BB+SNA anaerobic liquid culture back to LB plates, where they again grew (see Figure 2).

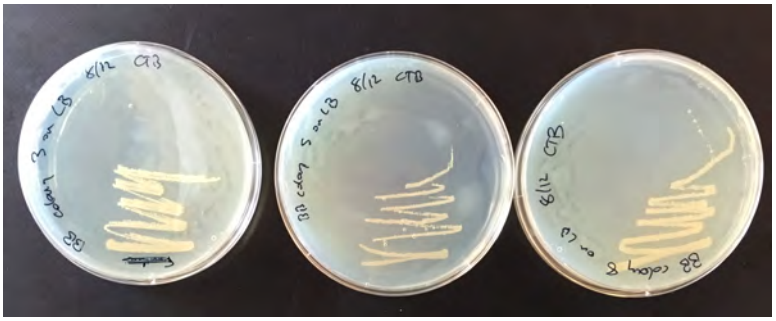

Figure 2: All three isolate colonies grew after transfer from LB through BB+SNA anaerobic culture followed by streaking on LB.

Isolate colonies were identified by 16s colony PCR as *Shewanella* spp.

I picked colonies and performed 16s PCR on the eight LB isolate colonies as described in the Methods. All 8 yielded bands, which were then Sanger sequenced. BLAST of all 8 colonies against NCBI's "nt" database primarily recovered 16s sequences from *Shewanella*; the best hits showed strong similarity (99%) to two species of *Shewanella* (Table 3).

Isolate cultures contain Spirilla-shaped microbes

I examined all three cultures with light microscopy and saw Spirilla-like bacteria (Figure 3). These bacteria appeared to move in a corkscrew-like fashion. This morphology is at odds with their molecular identification as *Shewanella* spp, which are typically rod-shaped bacilli.

Enrichment	Colony	Match percentage	BLAST match	Accession
1	8	99%	*Shewanella* algae strain MAS2736	GQ372874.1
2	8	99%	*Shewanella* algae strain MAS2736	GQ372874.1
3	8	99%	*Shewanella* algae strain MAS2736	GQ372874.1
4	8	99%	*Shewanella* algae strain MAS2736	GQ372874.1
5	4	99%	*Shewanella* sp. Chr-15	JQ863373.1
6	4	99%	*Shewanella* sp. Chr-15	JQ863373.1
7	4	99%	*Shewanella* sp. Chr-15	JQ863373.1
8	4	99%	*Shewanella* sp. Chr-15	JQ863373.1

Table 3: BLAST-based characterization of Sanger-sequenced 16s regions from colony PCRs. All colonies are clearly identified as *Shewanella* at 99% identity.

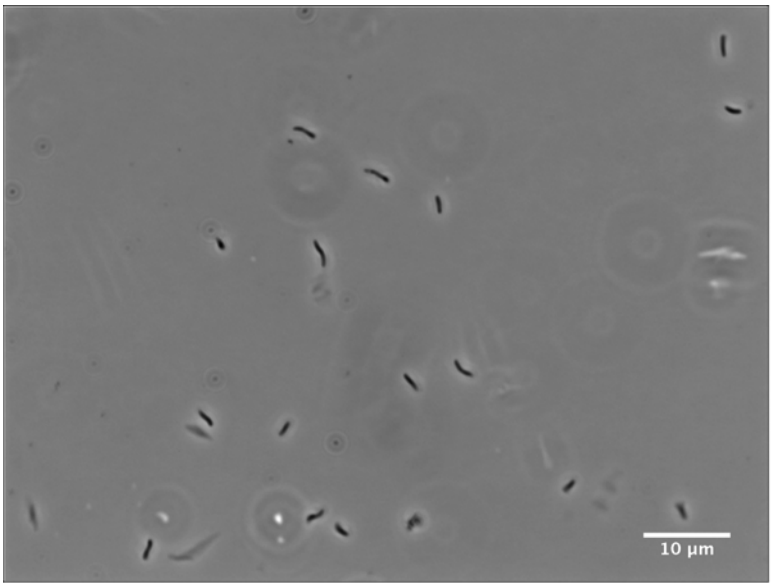

Figure 3: Light microscopy image of isolate colony 8, showing a bent-rod shape characteristic of spirillae; all three isolate colonies (3, 5, and 8) exhibited identical morphology.

Isolate cultures produce some N_2O and little to no methane.

Gas was sampled from headspace in the Pfennig bottles, and analyzed with both mass spectrometry (for N_2O) and gas chromatography (for methane). 10-20 nanomoles of nitrous oxide were seen in 2cc of headspace. Only trace amounts of methane (below 1%) were observed for the three isolates.

Discussion

We robustly recovered two species of *Shewanella* from two enrichments sourced in Trunk River. *Shewanella* species are often facultative anaerobes and can utilize a diverse array of electron acceptors and carbon sources [2]; our results agree. Here we find that our strains grow quickly and robustly both aerobically on LB plates and anaerobically on artisanal brackish media intended to select for sulfide oxidizers, nitrate reducers and acetate consumers.

As there is no genome yet available for these strains, my next proposed step would be to sequence and assemble the genomes of both strains and compare gene complements. Genes present in both would presumably be important for acetoclastic denitrification and should be readily identifiable.

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References

[1] Sievert SM, Scott KM, Klotz MG, Chain PS, Hauser LJ, et al. (2008) Genome of the epsilonproteobacterial chemolithoautotroph sulfurimonas denitrificans. Applied and environmental microbiology 74: 1145–1156.

[2] Venkateswaran K, Moser DP, Dollhopf ME, Lies DP, Saffarini DA, et al. (1999) Polyphasic taxonomy of the genus shewanella and description of shewanella oneidensis sp. nov. International Journal of Systematic and Evolutionary Microbiology 49: 705–724.