Phylogeny of Culturable *Marinomonas sp.* Utilizing Reduced Phosphates in the Woods Hole Harbor

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The objective of this study was to isolate and determine the phylogenetic affiliation of culturable sea bacteria capable of assimilating reduced phosphates (e.g. Phosphite, Hypophosphite, and Methylphosphinic Acid) as their sole phosphorous source under laboratory conditions. While the ability to oxidize phosphite (+III) to phosphate (+V) has been identified for the freshwater bacterium, *Pseudomonas stutzeri* WM 88, this may be the first time that salt water isolates have been cultured that possess this metabolic ability. Two isolates in the γ-proteobacteria phylogenetic group (*Marinomonas sp.*) appeared dominant on the hypophosphite and phosphate enriched plate cultures but not on the negative control plates. Taken together, these results may suggest that reduced phosphates play an important role as reducing agents and in introducing phosphorous to the marine system.

Keywords: Phosphite, Hypophosphite, ptxD, Marinomonas

Introduction

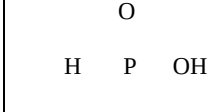
Reduced Phosphate compounds are often overlooked as a biologically available phosphorous source in the environment. And while critical molecules of the cell, including DNA, RNA, and ATP incorporate phosphorous in the oxidized phosphate (+V) form, these may have derived from reduced forms. The challenge in valuing the prevalence of these sources may lie in the inability to accurately measure such compounds reliably using currently available techniques (W Metcalf, *personal communication*). On the other hand, recent studies have elucidated specific operons (e.g. ptxD) which may code for enzymes which oxidize reduced phosphate species, alluding to the importance of this pathway.

Pierrou (1979) has noted that phosphorus is frequently a limiting nutrient in freshwater, coastal, and estuarine systems. Eutrophication, on the other hand, is often caused by excessive inputs of phosphate via domestic, industrial, and agricultural sources, making it relevant to thoroughly understand the phosphorous cycle. Interestingly, it was even proposed by Gulick (1955) that phosphite or hypophosphite served as a major source of phosphorus for the initiation of life on earth.

The major aim of this study is to culture bacteria that are capable of oxidizing reduced phosphate compounds in the environment. While freshwater species such as *Pseudomonas stuzeri* WM88 have been found to possess this function, few salt-water species have been found. In this respect, two salt-water sites were selected from which to culture reduced phosphate oxidizing species.

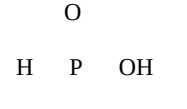
The Phosphate compounds examined in this study

Hypo-phosphite



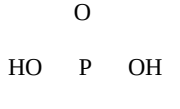
(+I)

Phosphite



(+III)

Phosphate



(+V)

Materials and Methods

Sampling

Two sites were selected from which to enrich for reduced phosphate oxidizers. The first, the Woods Hole Harbor Bay, was reached with the assistance of the Microbial Diversity boat. Approximately 10 liters of sea water were collected approx. 100 cm below the surface of the water. The water was vacuum filtered using a .22 um filter and the waste siphoned off to a waste carboy. Two filters were used as the filter seemed to clog with small debris present in the water sample. Immediately following, the filter was cut into quarter parts and placed inside the enrichment media as described below.

The second site was Trunk River sediment, which might be considered an estuary as the water flow changes in direction with the increasing tide. Qualitatively, this site appears to contain a high level of reduced sulfur and methane gas is readily released from the sandy soil. A 100 mL falcon tube was filled to the 50 mL mark with sediment near the methane bubbling. Approximately 5 grams of sediment was used to inoculate the enrichment media flasks.

Note: as noted in figure X the enrichments were inoculated with either the sea water or the Trunk River sediment, but they were not combined in one enrichment.

Enrichment Cultures

Twenty flasks, each with a different nutrient or sample source regimen, were prepared. The constants shared between each flask were 1M of SW Base, EDTA – Trace Elements, NH4Cl, Na2SO4, MOPS Buffer (at pH of 6.8). Flasks were autoclaved for 45 minutes then each of filtered 12-Vitamin Solution, Vitamin B-12, and either glucose (.20%) or succinate (20 mL per Liter). Prior to inoculation, filtered 1mM reduced phosphite sources were added as follows:

Flask	Sample Site	Carbon Source	Phosphorous Source	Growth
1	Sea	Glucose	Phosphorous Acid	-
2	Trunk	Glucose	Phosphorous Acid	-
3	Sea	Glucose	Na Hypophosphite	+
4	Trunk	Glucose	Na Hypophosphite	+
5	Sea	Glucose	Methylphosphonic Acid	+
6	Trunk	Glucose	Methylphosphonic Acid	+
7	Sea	Glucose	K Phosphate	+
8	Trunk	Glucose	K Phosphate	+
9	Sea	Glucose	-	-
10	Trunk	Glucose	-	-
11	Sea	Succinate	Phosphorous Acid	+
12	Trunk	Succinate	Phosphorous Acid	+
13	Sea	Succinate	Na Hypophosphite	+
14	Trunk	Succinate	Na Hypophosphite	+
15	Sea	Succinate	Methylphosphonic Acid	+
16	Trunk	Succinate	Methylphosphonic Acid	+
17	Sea	Succinate	K Phosphate	+
18	Trunk	Succinate	K Phosphate	+
19	Sea	Succinate	-	-
20	Trunk	Succinate	-	-

Agar Plates

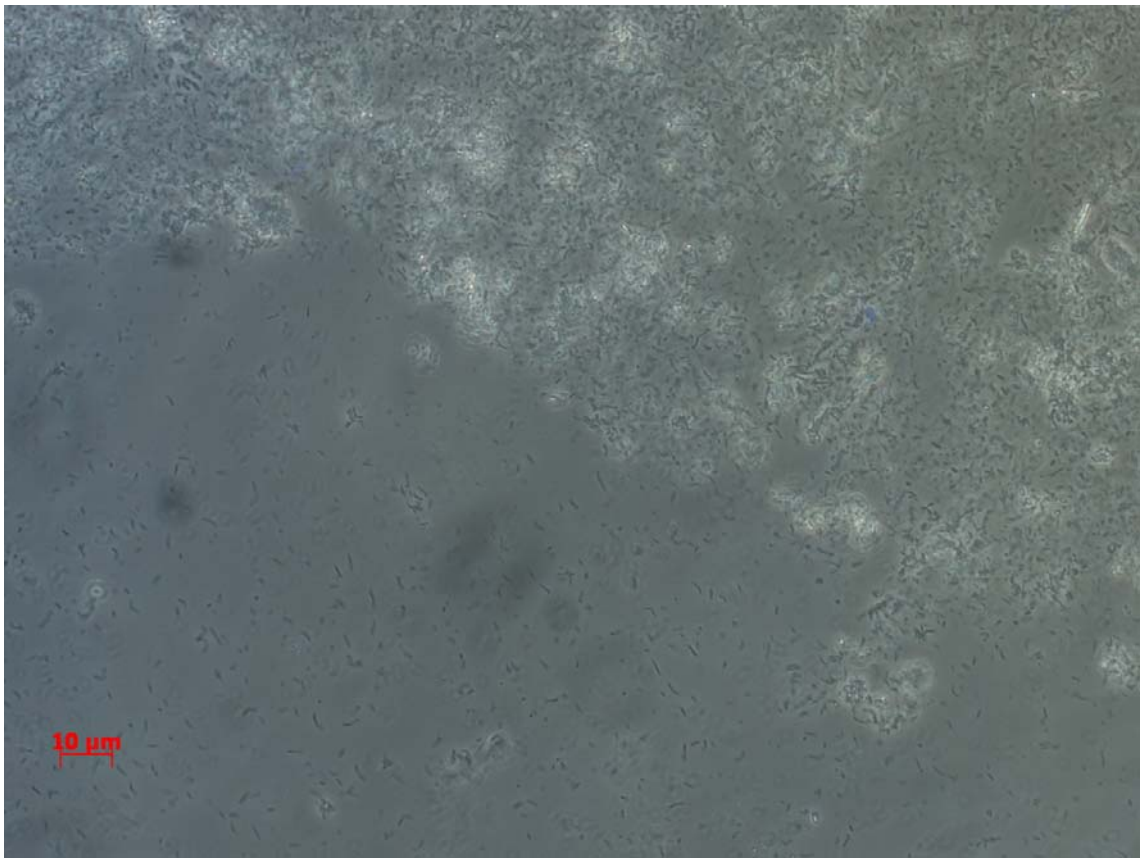
Plates were created using the same Phosphorous-free nutrient broth as outlined above. 1% agar was added to the media following three washes of the agar to better ensure that any phosphates in the agar were washed away. Plates were streaked from the broth flasks with significant growth after five days (see results) using the loop. Growth appeared as early as 24 hours.

Colony PCR and 16 S sequencing

Colonies from the plates were picked using a sterile toothpick and placed into 20uL of 0.05% Tween and placed in the thermocycler for lysing at 100 C for 5 minutes. The 16S rRNA genes was targeted using the universal eubacterial primers 8F and 1492R as follows: 95C for 5 minutes, followed by 20 cycles of 95 C for 30s, 55C for 30s, 72C for 1 min, and one 5 min extension at 72C. Amplicons were exposed to ExoSap (.25 uL) and PCR H20 (3.25 uL) in the thermocycler at 80C for 15 minutes and 9C until taken out of the thermocyler and sequenced by the ABI capillary sequencer in the laboratory of Mitch Sogin at the Marine Biological Laboratory. Resulting sequences were aligned using ARB.

Results

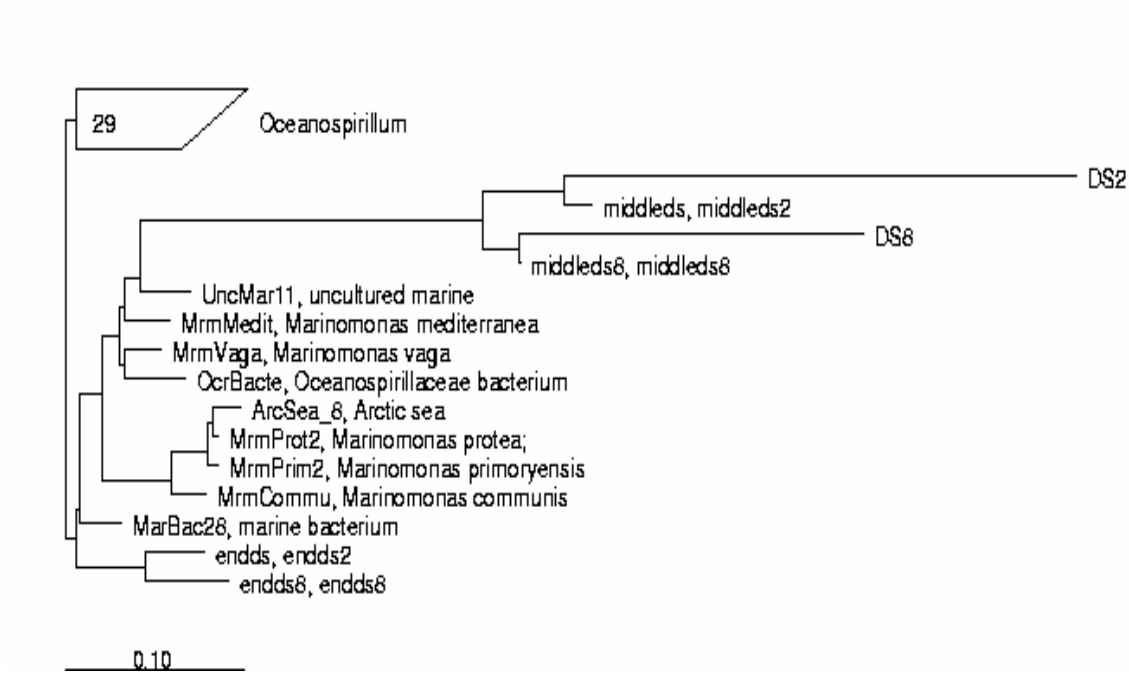
A diverse array of morphotypes appeared under the microscope from the broth flasks, especially for the samples from Trunk River. Trunk River flasks had a great number of eukaryotic flagellates, actively filter feeding on the mostly stationary cells.



Motile Rod morphologies surrounding a mass of stationary rods. From Glucose/Hypophosphite/Sea Plate.

The plates that I took most interest in were from Flask 3 (Glucose, Hypophisphite enriched from the Sea). As seen in Table 1, flask growth was apparent in the hypophosphite flask but not the phosphite flask. This countered my assumption that hypophosphite oxidizes to phosphite before being oxidized to phosphate. Perhaps this represented a novel pathway in which the phosphite step was skipped. To test this idea, I plated the growth from Flask 3 onto glucose plates with both hypophosphite and phosphite. Colonies sequenced from each of these plates were determined to be *Marinomonas sp.*, even though the sequences apparently had a significant amount of unknown bases. It was later found that other samples from the 96-well plate that were seuquenced also presented significant base anomalies (see the work by Ku-San, a fellow student of this course in 2005).

In the end, however, the *Marinomas sp.* appear to be the most dominant isolates that were cultured on the plates. DS2 is from the plate of Glucose/Phosphite/Flask 3 and DS is from the plate of Glucose/Hypophosphite/Flask 3. Please ignore the the "middleds" and "endds" branches.



Marinimonas spp. (gamma proteobacteria)
Phylum *Proteobacteria* Stackebrandt et al., 1986
Class "*Gammaproteobacteria*"
Order "*Oceanospirillales*"
Family "*Oceanospirillaceae*"
Genus *Marinomonas* Van Landschoot & De Ley, 1983

Discussion

Though the biologically-mediated ability to oxidize reduced phosphate species may be a fairly ubiquitous process, its prevalence within the microbial world is still relatively unknown. This study has identified two isolates of *Marinomonas sp.* to have the ability to grow on a reduced hypophosphite and phosphite media. If confirmed, this could represent one of the first of such isolates from the salt-water environment. In future studies, an attempt should be made to further uncover the diversity (richness, eveness) of the prevalence of this oxidation pathway, helping to elucidate the extent to which reduced phosphates play in the marine system.

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