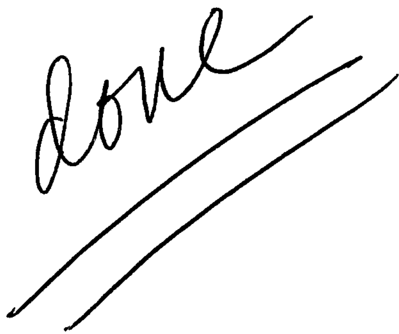

METHANE PRODUCING BACTERIA IN THE PURPLE FROSTING OF THE GREAT SIPPEWISSET SALT MARSH

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An important ecological aspect of all organisms is their relationship with other organisms and with their environment. A particular bacterial species may act differently depending upon whether or not another organism is present, and in what numbers the other organism is present. Seasonality causes many changes to the bacteria's microenvironment such as temperature, salinity, substrate concentration, etc. It is very important to remember the living and non-living components that affect an organism in its natural habitat, rather than simply studying the organism in a controlled laboratory situation using pure cultures.

My work with methane producing bacteria (MPB) was certainly an ecologically-based project. Rusty Johnson and I, working with James Ferry, studied methane producing bacteria found in the Sippewisset Salt Marsh in an area referred to as "purple frosting" (1). The purple frosting is found in sandy areas of the marsh which are submerged twice daily by the ocean tide. The sand used in our experiments is from areas which were segregated from lots of vegetation, being at least a couple feet from the nearest eel grass. The sand, unlike the highly complicated, laminated sand found in other areas of the marsh, is composed simply of a purple surface layer which can be as thick as 5 mm. Below this the sand is white, with seemingly little organic matter.

When the presence of MPB was first noted in the purple frosting, 2 questions arose. First, how are the methanogens, until now defined as strict anaerobes, surviving in an area which must contain O_2? Possibly, the production of gases by other organisms in the layer is driving O_2 out of the sand, but certainly some O_2 must be present. Secondly, with a predominance

relationship exists between the PB and the MPB? The thought was that the PB could be supplying H_2 via photosynthesis to the MPB.

James Ferry's original light-dark prescription bottle experiment (2) seemed to indicate that methanogenesis was light dependent. In this experiment purple sand samples and 5 mls of marine media (3) were added to three different prescription bottles. In one bottle the sand was combined with marine med. (.025% $Na_2S \cdot 9H_2O$) and incubated at 30° in the light. A second bottle was prepared in the same way, but covered with aluminum foil. The third bottle was prepared with modified marine media (no $Na_2S \cdot 9H_2O$ added) and incubated in the light. A significantly higher amount of methane was produced in the first bottle, while very little was found in the absence of light or $S^=$. This would lead one to believe that photosynthetic organisms are supplying the MPB with a substrate. If, as was our idea, purple sulfur bacteria are supplying H_2 to the MPB, they could only be supplying H_2 efficiently if plenty of light and $S^=$ are present. I considered various aspects of this phenomina.

I. Sulfate Reducing Bacteria

In James Ferry's original experiment, a lag in methane production of 2-3 days was seen in all the bottles. Why would the MPB, which are producing methane in the marsh, stop for a short period when brought into the lab? Possibly some sort of shock occurred in transport. Another possibility could be that when the 5 mls of marine media were added to the bottles, the media changed the conditions in the sand such that the MPB were temporarily outcompeted by other organisms. When this 2nd organism runs out

outcompeting the MPB for the H_2 (by the $SO_4^{=} + 4H_2 \longrightarrow S^{=} + 4H_2O$ pathway) until the sulfate present in the sand is depleted. I checked the numbers of Sulfate Reducing Bacteria present in the purple frosting to see if they were present in high enough numbers to be a potential cause of the lag. I also checked the effect of manipulation of the SRB on the lag period.

Materials and Methods

Enumeration of SRB was done using Postgate's medium 3 (4) with 2.5% NaCl and 2% agar, 10 mls of the media was placed in anaerobic roll tubes and purged with 100% N_2. One gram of purple sand was diluted from 10^{-1} to 10^{-9} in tubes of Postgate's media three without agar and shaken well to break up any clumps. From this series, agar roll tubes were prepared in triplicate from 10^{-2} to 10^{-10}. The experiment was done on 2 separate occasions. Tubes were incubated at 30°C. Black colonies were counted when they appeared in 4-5 days.

The effect of manipulation of SRB on the lag period was studied as follows. Prescription bottles were prepared using 80% N_2- 20% CO_2 atmosphere, purple sand and 5 mls of marine media. The sand was obtained from cores with a radius of 3.5 mm. About 2 mm of the purple sand was scraped from these cores. Six bottles were set up with the following media modifications.

1 - 1 mM Na_2S, 15 mM Na_2SO_4

2 - 1 mM Na_2S, - Na_2SO_4

3 - 5 mM Na_2S, 15 mM Na_2SO_4

4 - 5 mM Na_2S, - Na_2SO_4

5 - 10 mM Na_2S, 15 mM Na_2SO_4

Methane production was measured by gas chromatography using a Carle Basic chromatograph equipped with a silica gel column attached to a flame ionization detector.

Results

The 1st sulfate reducing bacteria enumeration experiment lead to the result of 54.0×10^2 sulfate reducing bacteria per gram of sand. The second resulted in 8.67×10^2. Although these 2 results differ widely, this is not surprising. The temporal and spacial differences in the marsh are considerable. Sulfate is brought in with each new tide, but not all areas receive an equal amount.

The addition of sulfate to the prescription bottle experiment was done to determine if an increased amount of sulfate would cause a longer lag period. This would be expected if the sulfate reducers were causing the lag. Graph 1 shows the results, which I interpret as follows. Rusty Johnson (2) showed that methane production was highest in media with 10 mM added Na_2S, lower with 5 mM, and lowest with 1 mM. It appears that added sulfate is immediately reduced by the sulfate reducers, and the evolved $S^=$ changes the final $S^=$ concentration of the bottle. In the case of an initial $S^=$ concentration of 1 $mM^=$, the increase in $S^=$ concentration results in better growth and methane production because the final $S^=$ concentration is closer to the optimum. In the case of an initial $S^=$ concentration of 10 mM, the increase in $S^=$ concentration results in an inhibitory amount of $S^=$, thus growth with added $SO_4^=$ is diminished and there's less methane produced. With an original $S^=$ concentration of 5 mM, the increase in $S^=$ concentration

this is an interesting phenomina, the effect on the lag phase which I was hoping to observe, was not obvious. Therefore, the sulfate reduced bacteria are probably not responsible for the lag in methane production.

II. Effect of Ammonia

Odom and Wall (5) reported that *Rhodospeudomonas capsulata* photo evolves H_2 by the nitrogenase system. Ammonia inhibits the nitrogenase system, and therefore H_2 evolution. If ammonia if left out of the *R. capsulata* growth media, H_2 production drastically increases.

If the purple bacteria in the purple frosting are supplying MPB with H_2, and if they evolve this H_2 from their nitrogenase system, one would expect to see an increase in H_2 production by the purple bacteria if NH_4^+ is left out of the media, and therefore an increase in methanogenesis.

Materials and Methods

Prescription bottles were prepared with purple sand, marine media, and an 80% N_2-20% CO_2 atmosphere as described earlier. Two bottles were prepared, both with 10 mM Na_2S, one with ammonia, one without. Methanogenesis was measured by gas chromatography.

Results

Graph 2 shows the effect of leaving ammonia out of the growth medium. Methane production decreased substantially. The results were inconclusive. Perhaps the nitrogenase activity didn't produce H_2. A possible explanation is as follows. With removal of NH_4^+ from the media the MPB no longer have a nitrogen source and therefore don't grow efficiently. Apparently they are

To accurately test the effect of leaving NH_4 out of the media, one would have to supply the MPB with an alternate N source. Unfortunately, such an alternative is not known, although there's a recent report that MPB can fix N_2 (S. Zinder & L. Daniels, unpublished results).

III. <u>Combination of MPB With Photosynthetic Organisms</u>

A pure culture of MPB, strain H_2E, has combined with enrichments for photosynthetic bacteria to test the possibility of light dependent methanogenesis. In the light-dark prescription bottle experiments repeated by Rusty Johnson (2), the results of J. Ferry's original experiment could not be reproduced. Instead of seeing more methanogenesis in the light, we got results which showed higher amounts of methane production in the dark. A possible reason could be that as the summer progressed, the concentration of fermentative organisms in the purple frosting drastically increased. Therefore, experiments performed later in the summer used sand which differed greatly from sand used in the initial experiment. Organisms which ferment in the dark were producing such high amounts of H_2 that any relationships between photosynthetic bacteria's H_2 production and methane production were masked. To test this possibility, namely to see if light dependent methanogensis is a true phenomina, one must remove all other organisms from the experiment. Therefore, I tried to get pure cultures of purple bacteria and combined these with a pure culture of H_2E.

<u>Materials and Methods</u>

Various medias were used for the enrichment of photosynthetic bacteria. The organisms I used were grown in marine media (pH 7.2) roll tubes. Colonies

green, one purple. Absorption spectras were obtained from both cultures on a Cary 14 Spectrophotometer. Phase contrast microscopy was also used for verification of cell type and to check purity of the enrichments.

A spectral analysis was also performed directly on the sand. Pigments were extracted using methanol and analyzed on the Cary 14 to see all of the photosynthetic pigments present in the frosting.

Two separate combination experiments were set up. In the 1st, 11 tubes were prepared containing 5 mls of marine media with various modifications in an 80% N_2 - 20% CO_2 atmosphere. 1 ml of the H_2E culture and 1 ml of the purple enrichment culture was added to each. Methane production was measured by gas chromotography for a period of 5 days. The identity of the 11 tubes is as follows.

1. $-S^=$
2. 1 mM $S^=$, + hv
3. 1 mM $S^=$, - hv
4. 1 mM $S^=$, 15 mM $SO_4^=$, + hv
5. 1 mM $S^=$, - NH_4^+, + hv
6. 5 mM $S^=$, + hv
7. 5 mM $S^=$, - hv
8. 5 mM $S^=$, 15 mM $SO_4^=$, + hv
9. 5 mM $S^=$, = NH_4^+, + hv
10. 10 mM $S^=$, + hv
11. 10 mM $S^=$, - NH_4^+, + hv

The 2nd experiment used 12 tubes of 10 mls of marine media in a 80% N_2 - 20% CO_2 atmosphere set up as follows.

1. 1 ml H_2E, + hv
2. 1 ml H_2E, + hv
3. 1 ml H_2E, - hv
4. 1 ml H_2E, - hv
5. 1 ml H_2E, 0.5 ml purple enrichment, + hv
6. 1 ml H_2E, 0.5 ml purple enrichment, + hv
7. 1 ml H_2E, 0.5 ml purple enrichment, - hv
8. 1 ml H_2E, 0.5 ml purple enrichment, - hv
9. 1 ml H_2E, 0.5 ml green enrichment, + hv
10. 1 ml H_2E, 0.5 ml green enrichment, + hv
11. 1 ml H_2E, 0.5 ml green enrichment, - hv
12. 1 ml H_2E, 0.5 ml green enrichment, - hv

Results

Due to lack of time pure cultures were never obtained. The purple enrichment showed a single peak at absorbance which indicated the presence of bacteriochlorophyll A. Therefore, the enrichment contains Purple Sulfur Bacteria. Microscopy confirmed this observation. The enrichment was composed of Chromatium spc. Approximately 99% of the cells present were Chromatium.

The green enrichment was far from a pure culture. It did show a peak of absorbance which indicated the presence of Chlorophyll A. Microscopic observation determined that the green culture was an enrichment for Green Bacteria and Cyanobacteria. Various cell types were present including many which appeared to be Anabeania and Volvox. Many other cell types were also present.

and BChl A were present in the purple frosting. Therefore, any light dependent methanogenesis would have to be due to either Purple Sulfur Bacteria, Green Bacteria, or some Cyanobacterium.

The results of the 2 combination experiments are on Table 1 and Table 2. The numbers indicate relative peak height at the highest sensitivity.

No significant amount of methane was produced in any of the tubes of either experiment. Therefore, neither the green nor purple enrichment are sufficient to support the growth of methane producing bacteria in an atmosphere which lacks H_2. Purple Sulfur Bacteria and Cyanobacteria do not appear to be supplying H_2 to the MPB.

Possibly another photosynthetic organisms exist in the purple frosting which does supply MPB with H_2. The purple sulfur bacteria, Thiocapsa was found to predominate in other purple layers of the marsh. Possibly this is the organism which causes light dependent methanogenesis.

IV. Most Probable Numbers of Methane Producing Bacteria and Amount of Combustible Carbon in the Purple Frosting

Materials and Methods

The most probable numbers of MPB in the purple frosting was tested on three separate occasions. In trial I the layer used was a greyish-purple. In trial II and III the layer was highly pigmented and bright pink. In trial I and II the test was run on the purple layer itself (PL) and on the non-purple sand directly below the purple layer (sub-PL). In trial III only the purple sand was tested. It was tested immediately after being retrieved from the marsh and again after being exposed to air for 48 hours. All trials were performed in triplicate.

Anaerobic tubes of mineral media (pH 7.2) were prepared. One gram of sand was added to 9 mls of media to achieve a 10^{-1} dilution. This was further diluted out to 10^{-10}. Tubes were incubated at 30°C in the light. Methane production was checked about 1 month after innoculation by gas chromatography.

The amount of combustible material present in the purple frosting was determined and compared to the amount in beach sand collected from the Stony Beach parking lot. Sand was dried in a 70°C drying oven for 24 hours in a crucible. The crucible was then weighed, placed directly over a bunsen burner for 1 hour, and weighed again. The empty crucible was also weighed. The percentage loss in weight was calculated.

Results

The results of the 3 trials for most probable numbers are given in Tables 3, 4, and 5. The results are given as relative peak heights with the gas chromatograph set at the highest sensativity.

In trial IA&B, IIA&B, and IIIA one could conclude that there are at least 10^3 MPB/gram sand. In trial IIIB one could conclude that there are at least 10^2. Trial IIIB was the one in which the sand core was left out in the lab for 48 hours and therefore, pretty well dried out. Due to the drying and probable decrease in metabolism of many of the organisms in the sand, the core's O_2 content probably increased substantially. Therefore, it's not surprising that the number of MPB has decreased. The fact that there is any CH_4 produced, and actually a substantial amount of MPB present, indicates possible O_2 tolerance of the MPB.

The results of all three trials are sadly erratic. I think this must

amounts of methane that are observed all the way out to 10^{-10} are peculiar. Peaks on the gas chromatograph due to residual methane in the lines were not observed at other times, so would not explain the peaks seen at the high dilutions. Possibly there are in fact many MPB, as many as 10^{10} per gram of sand, but at the high dilutions they are not growing well. This could be due to the fact that there is some component of the purple frosting which is diluted to limiting concentrations and won't allow efficient growth of MPB in the tubes above 10^{-3}.

Another interesting aspect of the most probable number experiment is that the MPB are not found in higher amounts in the purple surface layer than in the sub-surface layer. If purple photosynthetic bacteria promote the growth of MPB by supplying them with H_2, you would expect to find MPB habitating the purple surface layer preferentially. Instead, the MPB don't seem to prefer either layer. This is possible evidence that light dependent methanogenesis doesn't exist.

Finally, the combustible carbon in the purple frosting was calculated at .988% of the total weight of the sample. This compares to .446% for the sample taken from the Stoney Beach parking lot.

Suggestions for Further Work

I. The most probable number of the MPB in the purple frosting must be determined more accurately. I would suggest using media prepared from filter sterilized marsh H_2O to avoid the possibility of not supplying the MPB with a needed substrate. I would also suggest extreme mixing of tubes to assure the dissociation of clumps. Tubes should be placed in the dark

II. The combination experiment should not be given up on. Possibly another purple sulfur bacteria, for example, <u>Thiocapsa</u> exists in the frosting, and it is this organism that causes light dependent methanogenesis.

III. Identification of all the microbial members of the purple frosting should be attempted. Discovery of all the microbial components of the sand could shed some light on the goings on of the purple sand.

1. Happel, A. 1982. Microbial Ecology summer course report.

2. Johnson, R. 1984. Molecular Aspects of Cellular Diversity summer course report.

3. Sowers, K.R., Beron, S.F., and Ferry, J.G. 1984. Appl. Environ. Microbiol. 47:971-978.

4. Postgate, J.R. 1963. Appl. Microbiol. 11:265-267.

5. Odom and Wall. 1983. Appl. Environ. Microbiol. 45: 1300-1305.

Na_2S, 15 mM Na_2SO_4(▲); 1mM Na_2S, no added Na_2SO_4(△); 5mM Na_2S, 15mM Na_2SO_4(●); 5mM Na_2S, no added Na_2SO_4(○); 10mM Na_2S, 15mM Na_2SO_4(■); 10mM Na_2S, no added Na_2SO_4(□).

umoles CH_4/50 ul sampl

10

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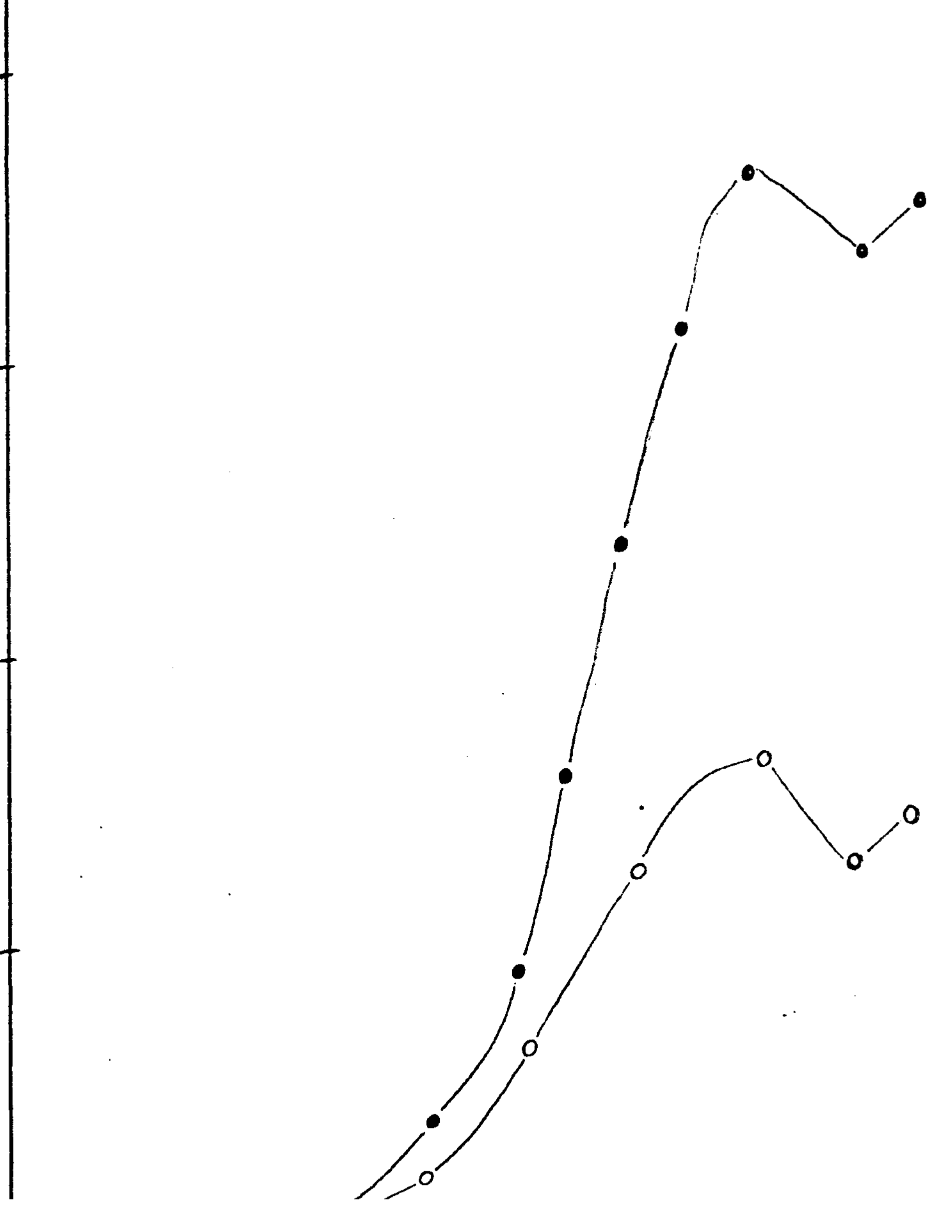
Na_2S(o); 10mM Na_2S, 0.05% NH_4Cl(•).



indicated), 1 ml H_2E culture, and 1 ml purple bacteria enrichment culture..

	Day 1	Day 2	Day 3	Day 4	Day 5
$-S^=$	32	12	36	23	12
1mM $S^=$, +hv	29	23	30	24	12
1mM $S^=$, -hv	24	7	28	16	14
1mM $S^=$, 15mM $SO_4^=$, +hv	34	12	28	14	12
1mM $S^=$, $-NH_4^+$, +hv	23	16	30	18	14
5mM $S^=$, +hv	36	14	18	16	12
5mM $S^=$, -hv	30	16	20	18	8
5mM $S^=$, 15mM $SO_4^=$, +hv	33	10	24	20	10
5mM $S^=$, $-NH_4$, +hv	73	70	98	82	70
10mM $S^=$, $-NH_4^+$, +hv	20	16	50	36	14
10mM $S^=$, +hv	27	14	24	20	14

TABLE 2

Combination Experiment # 2

Anaerobic tubes containing 10 mls marine media, GE(green enrichment), or PE(purple enrichment), and 1 ml H_2E culture.

H_2E, +hv	18	15	12	22	10
H_2E, +hv	18	14	10	16	18
H_2E, -hv	19	10	14	18	10
H_2E, -hv	28	14	16	22	10
H_2E, PE, +hv	27	10	14	28	10
H_2E, PE, +hv	37	12	18	30	14
H_2E, PE, -hv	26	13	18	24	14
H_2E, PE, -hv	34	9	16	20	12
H_2E, GE, +hv	29	14	12	20	20
H_2E, GE, +hv	29	12	12	18	18

Probable Numbers of Methane Producing Bacteria

Sand samples diluted from 10^{-2} to 10^{-10} grams sand per ml of media. All trials d

iplicate(A, B, C).

3-Trial I

Purple Surface Layer

-2	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-}
x20	26	16	11	10.5	8	6	11	10
x20	30	15	20	5	8	3.5	5.5	10
x20	28	19.5	25	6	5	7	12	6

Sub-Purple Surface Layer

x200	37x10	20	9	6.5	4	5	10.5	12
2x200	30	16	13	3	3	3	5	2.5
3x200	30	>100x200	11	7	3.5	3	10.5	3.5

robable Numbers of Methane Producing Bacteria

and samples diluted from 10^{-2} to 10^{-10} grams sand per ml of media. All trials d

plicate(A, B, C).

4-Trial II

urple Surface Layer

-2	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-}
2x10	17	17	24	14	13	15	16	14
4x10	132	11	12	13.5	12	12	8	12
0x10	10	12.5	12	9.5	10	9	10.5	15

ub-Purple Surface Layer

x20	63x20	5	6	7	6	7	9	12
00x200	17x20	13	10	10	12			10
x200	44x20	18.5	12	9				16

'robable Numbers of Methane Producing Bacteria

;and samples diluted from 10^{-2} to 10^{-10} grams sand per ml of media. All trials d
.plicate(A, B, C).

5 -Trial III

e Surface Layer-48 Hour Sand Sample

-2	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-}
		37		21		22		34
)	12	12	20		38	28	72	12
)x200	4	12	32	28		22	35	

e Surface Layer-0 Hour Sand Sample

)0x200	1300	26	40	36	56	40	32	35
x200	55	34	34	36	46	33	36	28
)0x200	450	54	31	42	28			