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## INTRODUCTION

Methane-producing bacteria are described as strictly anaerobic chemotrophs that utilize $H_2$ or simple organic compounds such as formate, acetate, methanol, or methylamines as electron donors for which their terminal electron acceptor is $CO_2$ (Mah, 1981), or possibly $S^o$ (Stetter and Gaag,1983). Generally, methanogenesis occurs in habitats that are devoid of $O_2$ and receive large inputs of organic matter. The methane-producing bacteria are often associated with other bacteria capable of metabolizing complex carbon sources to provide the substrates for methanogenesis.

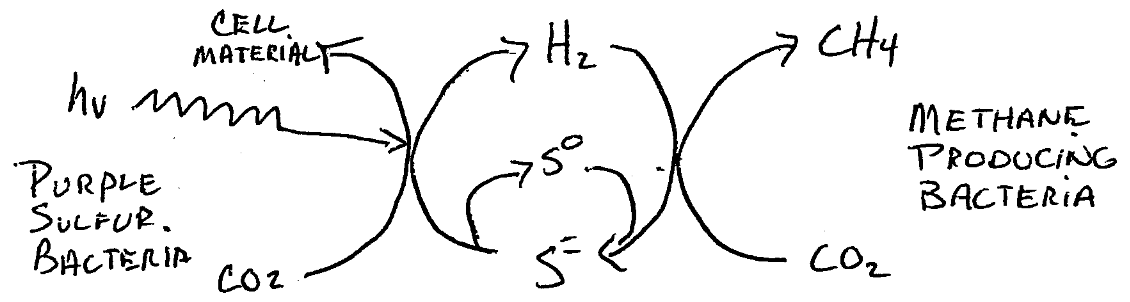
This project examined an unusual methanogenic consortia involving methanogens and purple sulfur bacteria in a superficial habitat of the Great Sippewissett Marsh, that is known as the Purple Sand. Located on the sandy surface of tidal creek beds, the Purple Sand is a 3-4 mm thick layer that contains a population of purple sulfur bacteria which is responsible for the color of the sand. The sand is constantly exposed to atmospheric $O_2$ and oxygenated seawater with the fluctuations of the tides and contains relatively little organic matter. Under these conditions, the discovery of methanogenesis in this habitat was unexpected.

We investigated the possibility of a direct methanogenic consortium where the phototrophic purple sulfur bacteria contribute substrate via $H_2$-transfer to the methanegens during anaerobic photosynthesis, by testing for methane production when samples were exposed to the light compared with methane production in the dark. In addition, we sought to isolate and

methanogens and our isolates to tolerate $O_2$ when exposed to the atmosphere in an effort to explain the presence of these methanogens in this habitat.

## I. Investigation of Light-dependent Methanogenesis

We measured methane production in the Purple Sand layer of the Great Sippewissett and hypothesized that the purple sulfur bacteria contribute $H_2$ to the methanogens by the following mechanism:




In this model, $H_2$ produced by purple sulfur bacteria during photosynthesis is transferred to the methane-producing bacteria to reduce $CO_2$ to $CH_4$. Because the purple sulfur bacteria normally oxidize sulfide to $S^o$, an excess of sulfide and a high $CO_2$ concentration would provide the mechanism to drive the interspecies $H_2$-transfer. The evidence that some methanogens can reduce $S^o$, forming large quantities of $H_2S$ (Stetter and Gaaf, 1983), would provide for the excess sulfide, but would reduce $CH_4$ production, so we decided to incorporate $CH_4$ production measurements in the presence and absence of sulfide in addition to our light/dark experiment. Purple sulfur bacteria produce small quantities of $H_2S$ during fermentative reactions in the dark (Ward, 1984), but oxidize $H_2S$ in photosynthesis which would contribute to driving the light-dependent

Methane was measured using a basic gas chromatograph (Carle 9500). In situ measurements were made of purple sand sites by inserting cut-off glass bottles with butyl rubber stoppers into the sand, covering one of two bottles with aluminum foil to simulate darkness, and using 1cc syringes to extract $CH_4$ samples over time. In vitro $CH_4$ production measurements were done on purple sand slurries containing about 5 grams of sample which were placed in 120 ml serum bottles containing 10 ml of Marine Mineral Media (Sowers, et al,1984);

| | |
|---|---|
| NaCl 2.4% | $Na_2HPO_4$ 0.06% |
| KCl 0.08% | Cysteine HCl 0.025% |
| Yeast Extract 0.1% | $Na_2CO_3$ 0.2% |
| $NH_4Cl$ 0.05% | $Na_2S$ 0.025% |
| $MgCl_2 \cdot 6H_2O$ 1.0% | Mineral MIx 1.0% |
| $CaCl_2 \cdot 2H_2O$ 0.014% | Resazurin 1.0% |

The serum bottles were sparged with $N_2:CO_2$ (80:20) to remove residual $CH_4$, and incubated at 30° under the following:

(1) +hv, $+S^=$

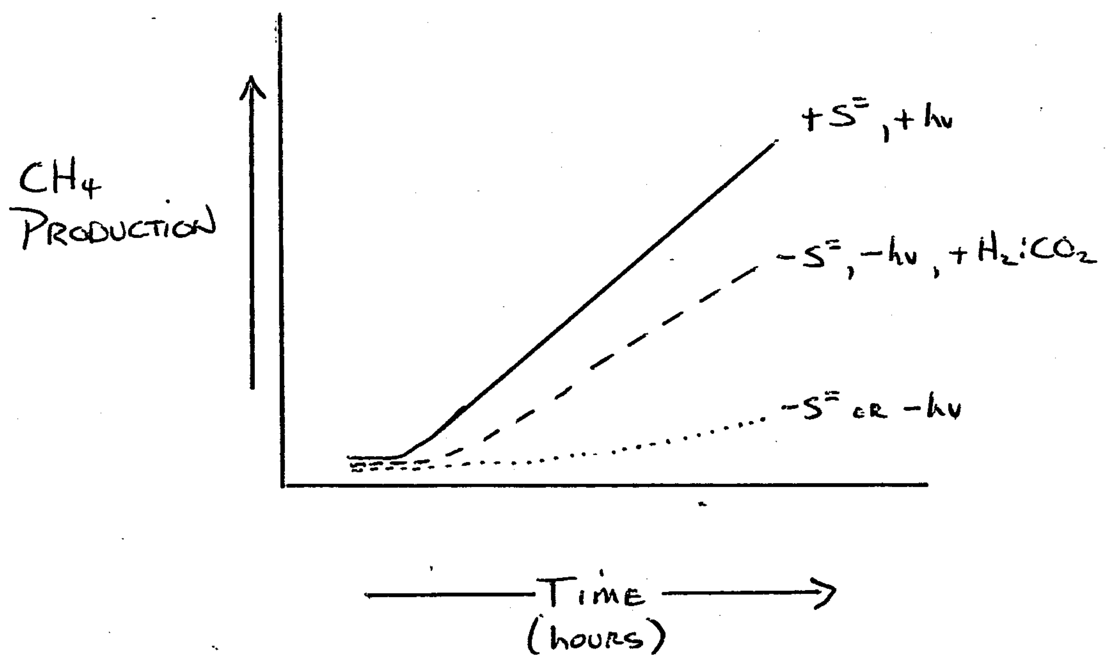
(2) +hv, $-S^=$

(3) -hv, $+S^=$

(4) -hv, $-S^=$, $+H_2:CO_2$ (80:20)

## RESULTS

of $H_2:CO_2$, and the least observed without light or without $S^=$:




To explain the observed lag period, we postulated that sulfate-reducing bacteria may be present in the Purple Sand layer and initially might outcompete the methanogens for $H_2$. To test this, we repeated the experiment with and without added $SO_4^=$ and also did most probable numbers of the sulfate-reducing bacteria and purple sulfur bacteria, but these results were incomplete at the end of the project. Our efforts to repeat the results of the light-dependent methanogenesis experiment failed to duplicate the above results, but this could be related to the changing marsh environment over the summer.

II. Isolation of methane-producing bacteria from the Purple Sand

with a coculture of both purple sulfur and pure isolates of the unknown methanogens present. Due to the slow growth of the methanogens, coculturing was not possible in the given length of time, but three methanogen types were isolated in distinct colonies using variations of the Hungate roll tube technique (Hungate, 1969). Microscopic examination revealed that each of the three unidentified types of colonies had a distinct cell morphology: one was a large, irregular rod that occurred in chains, a second was vibroid, and the third was a highly motile, slender rod. Under epifluorescent microscopy, all three cell types fluoresced at 420 nm UV light, characteristic of the $F_{420}$ molecule unique to the methane-producing bacteria. In colonies located near the roll tube stoppers, cells of two of the methanogens contained uniform refractile bodies, one per cell, which we tentatively identified as endospores, a property not previously observed in methanogens. Endosporulation appeared to be a response to $O_2$ diffusion into the culture, as slides of the methanogens developed many refractile bodies after 2-3 hours exposure to the air while entrapped in the agar between the cover slip and the slide.

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