Direct Plate Isolation of Anaerobes from Environmental Samples

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Abstract

Over the years a number of methods have been developed to grow strict anaerobes and even obtain single colony isolates but none of these methods utilized conventional petri plates. The purpose of this study was to test the possibility of anaerobic direct agar plating of environmental samples for efficacy and to determine if novel isolates could be obtained. Core samples of sediment from School Street Marsh (freshwater) and from Oyster Pond (saltwater) then inoculated on a freshwater (FW) and a saltwater (SW) base agar media with or without added electron acceptors and /or donors (H_2 or trimethylamine). Plate counts and 16S rDNA were done on isolates selected at days 6, 13, and 15. The number of colonies was nearly 100-fold higher from the saltwater sample over time. A wide variety of unique colony morphologies were seen on the plates along with a high number of pigmented colonies. Nearly 50% of the colonies picked for 16S analysis resulted in PCR products that could be sequenced. More than 50% of 16S PCR sequences analyzed with ARB appear to be bacteria not previously cultured. Several monophyletic groups were found with no relatives closer than 90%. This method reduces enrichment bias and provides a new method for obtaining novel organisms from environmental samples in culture. Isolates can then be obtained of these novel organisms to determine more completely their metabolic activities and function in the environment.

Introduction

Over the years a number of methods have been developed to grow strict anaerobes and even obtain single colony isolates including roll tubes and shake tubes (1,7). The development of an anaerobic intrachamber incubator allowed growth of a wider variety of anaerobes from environmental samples on conventional agar-containing Petri plates (5). Previous use of direct plate techniques has primarily focused on aerobic environmental samples including soil and activated sludge (3,6). Anaerobic direct plating has been focused on rumen content analysis and pathogen isolation (2). Other methods developed to retrieve and cultivate "unculturable" organisms include the use of membranes containing solidified agar, but recovered isolates are aerobic (4). The purpose of this study was to test the possibility of anaerobic direct agar plating of environmental samples for efficacy and to determine if novel isolates could be obtained.

Materials and Methods

Media

The media used in this experiment include a freshwater (FW) and a saltwater (SW) base media with or without added electron acceptors and /or donors. Two different electron donors were used: H_2 or trimethylamine (TMA). BES was added to some media to inhibit methanogens . In the freshwater media, Na_2SO_4 was added to media #3 and #6 to provide a sulfur source while in seawater media it was excluded. All media was prepared following anaerobic procedure.

Experimental Protocol

The experiment was designed as described in Table 1. Samples were obtained by taking a core sample of sediment from School Street Marsh (freshwater) and from Oyster Pond (saltwater). Samples were placed in the anaerobic chamber and then homogenized in a sterile beaker. One gram was diluted in a 10-fold dilution series in either FW or SW sterile broth without added BES, Na_2SO_4 or TMA. Two hundred microliters of each dilution was spread on each media indicated in Table 1 with 10^{-3} to 10^{-7} dilutions plated for each sample. All plates were incubated at room temperature in an anaerobic intrachamber incubator with the chamber containing an atmosphere composed of 66% Gas A (80% N_2/20% CO_2/0.1% H_2S) and 33% Gas B (80% H_2/20% CO_2).

Isolate Analysis

Plates were removed from the incubation chamber into the anaerobic chamber for examination at day 6, 13, and 15. Colonies were picked with sterile micropipet tips and cells placed into sterile microfuge tubes. Plate counts were performed at days 6 and 13. Identity of each colony pick was determined by16S rDNA PCR with the resulting sequence analyzed for nearest neighbors with ARB.

Table 1. Experimental Design

Inocula: A- School Street Marsh (freshwater)

B- Oyster Pond (saltwater)

Media:

Freshwater Media	Salt Water Media
1. $H_2 + CO_2$	1. $H_2 + CO_2$
2. $H_2 + CO_2 + BES$	2. $H_2 + CO_2 + BES$
3. $H_2 + CO_2 + Na_2SO_4$	3. $H_2 + CO_2 - Na_2SO_4$
4. TMA	4. TMA
5. TMA + BES	5. TMA + BES
6. TMA + Na_2SO_4	6. TMA - Na_2SO_4

Results

Plate counts revealed that saltwater isolates (Oyster Pond) could grow on all SW and FW media at counts above 1×10^5 while freshwater isolates (School Street Marsh) took considerably longer to come up on SW media (Table 2). The number of isolates was nearly 100-fold higher from the saltwater sample over time.

A wide variety of unique colony morphologies were seen on the plates along with a high number of pigmented colonies (primarily pinks, reds, and browns) (Figures 1-6). Of the colonies picked for 16S analysis, nearly 50% resulted in PCR products that could be sequenced (Table 3). ARB analysis of isolates revealed that the majority were organisms that had not previously been cultured and, for many, had no close relatives except for some clones.

Table 2. Enumeration of Freshwater and Saltwater Samples.

A- School Street Marsh (freshwater)

	Wk 1 (7 days)		Wk 2 (14 days)	
Media Type	FW	SW	FW	SW
1. $H_2 + CO_2$	1.0×10^4	$<1.0 \times 10^3$	1.8×10^4	$<1.0 \times 10^3$
2. $H_2 + CO_2 +$ BES	2.0×10^4	$<1.0 \times 10^3$	3.7×10^4	1.6×10^4
3. $H_2 + CO_2 + Na_2SO_4$	1.0×10^3	$<1.0 \times 10^3$	2.8×10^4	4.2×10^4
4. TMA	4.0×10^4	$<1.0 \times 10^3$	1.5×10^4	1.7×10^4
5. TMA + BES	1.0×10^4	$<1.0 \times 10^3$	6.8×10^4	9.0×10^3
6. TMA + Na_2SO_4	$<1.0 \times 10^3$	$<1.0 \times 10^3$	2.4×10^4	ND

B- Oyster Pond (saltwater)

	Wk 1 (7 days)		Wk 2 (14 days)	
Media Type	FW	SW	FW	SW
1. $H_2 + CO_2$	1.2×10^5	4.5×10^5	4.6×10^6	3.0×10^6
2. $H_2 + CO_2 +$ BES	3.0×10^5	5.0×10^5	4.2×10^6	2.6×10^6
3. $H_2 + CO_2 + Na_2SO_4$	2.5×10^5	5.2×10^5	2.8×10^6	3.5×10^6
4. TMA	3.0×10^5	2.0×10^5	1.9×10^6	1.4×10^6
5. TMA + BES	4.0×10^5	4.0×10^5	1.8×10^6	2.7×10^6
6. TMA + Na_2SO_4	2.6×10^5	3.0×10^5	2.7×10^6	4.0×10^6

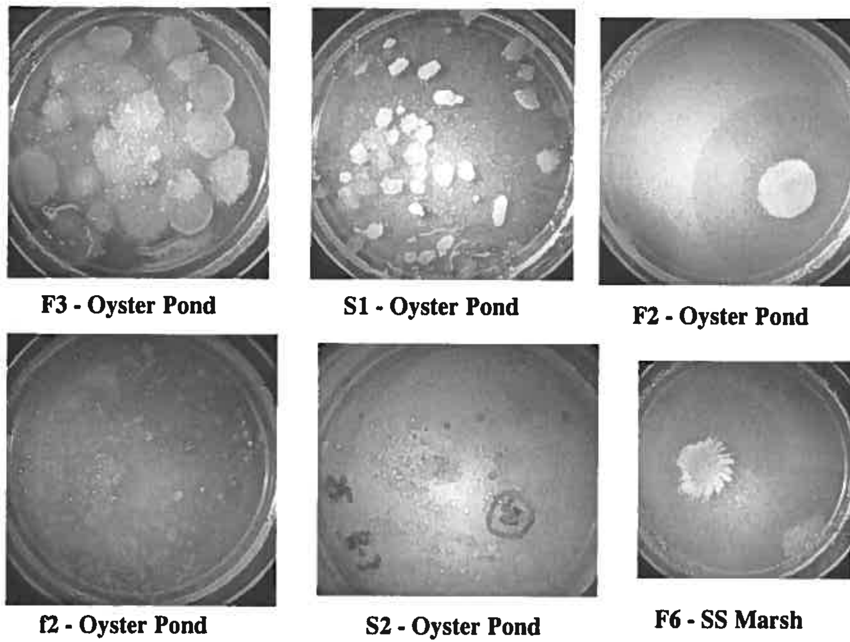


Figure 1. Representative plates showing colony morphologies on various media.

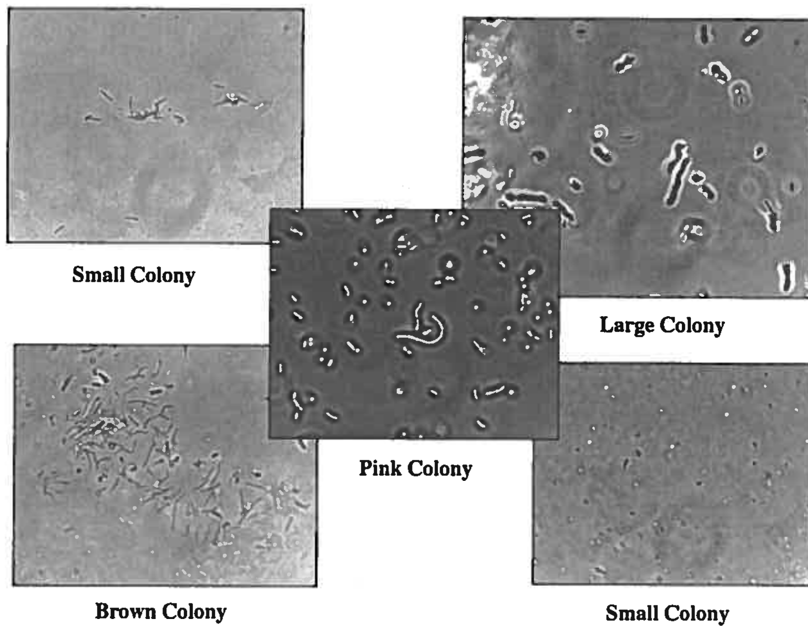


Figure 2. Representative cell morphologies from individual colonies.

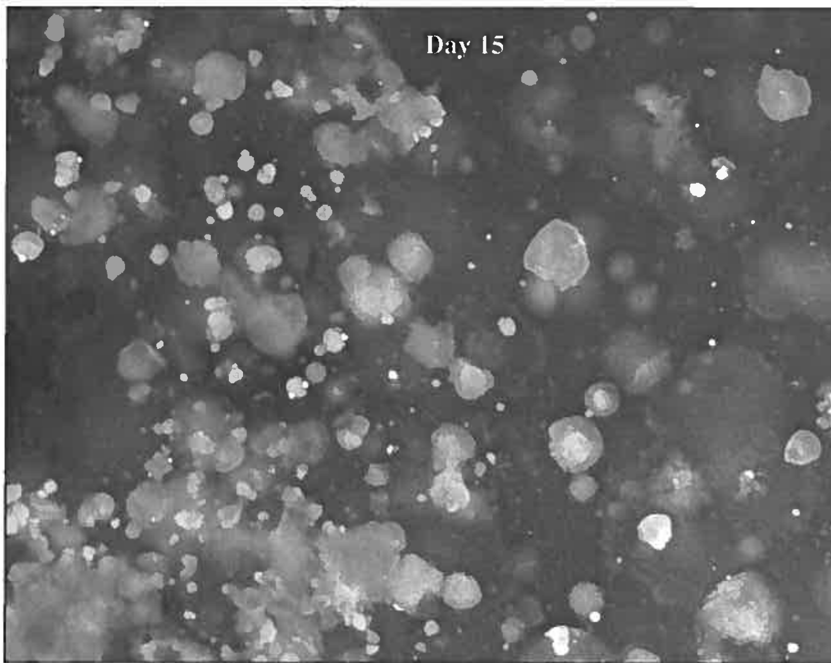


Figure 3. Pigmented colonies from Oyster Pond on SW1 media.

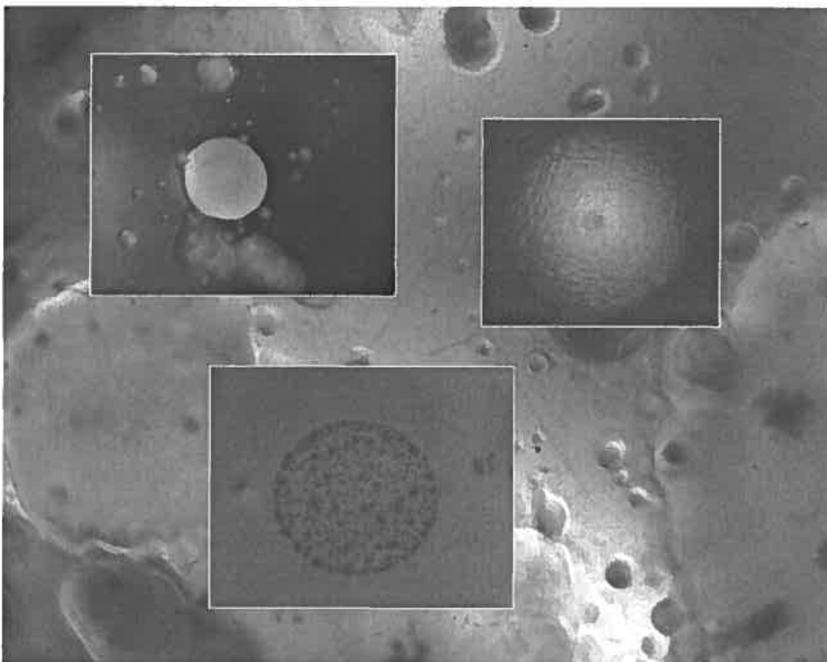


Figure 4. Various colonies from Oyster Pond on SW3 and FW6 media.

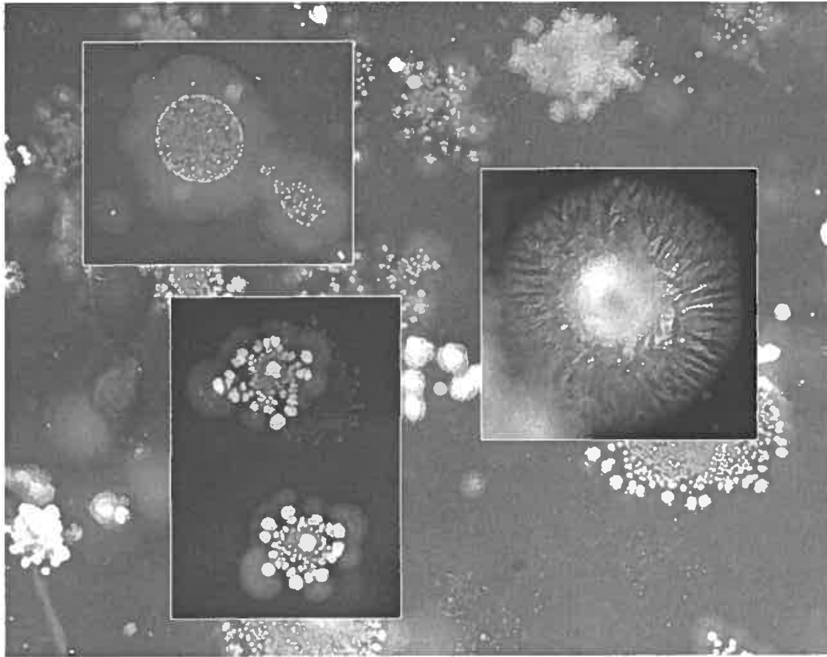


Figure 5. Various colonies from Oyster Pond on SW1 and SW3 media.

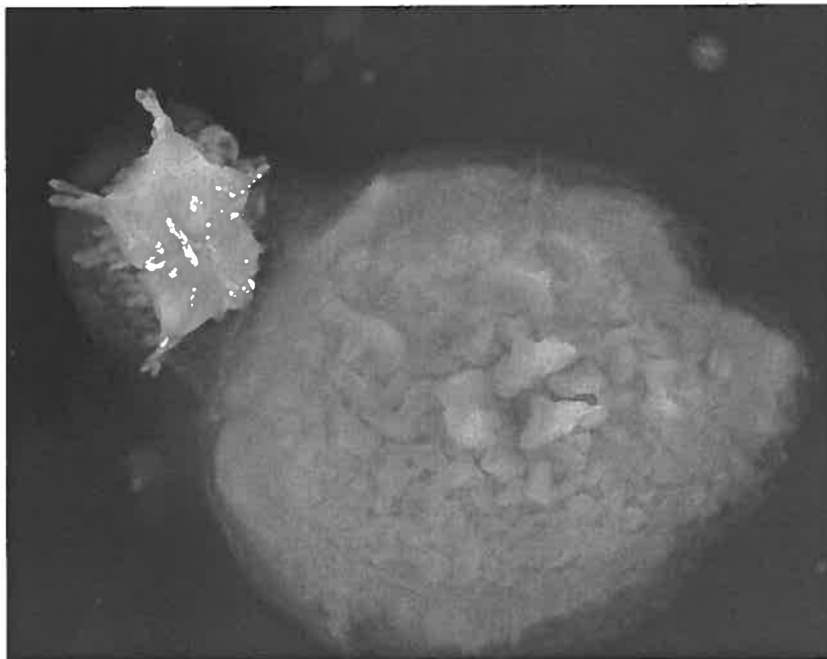
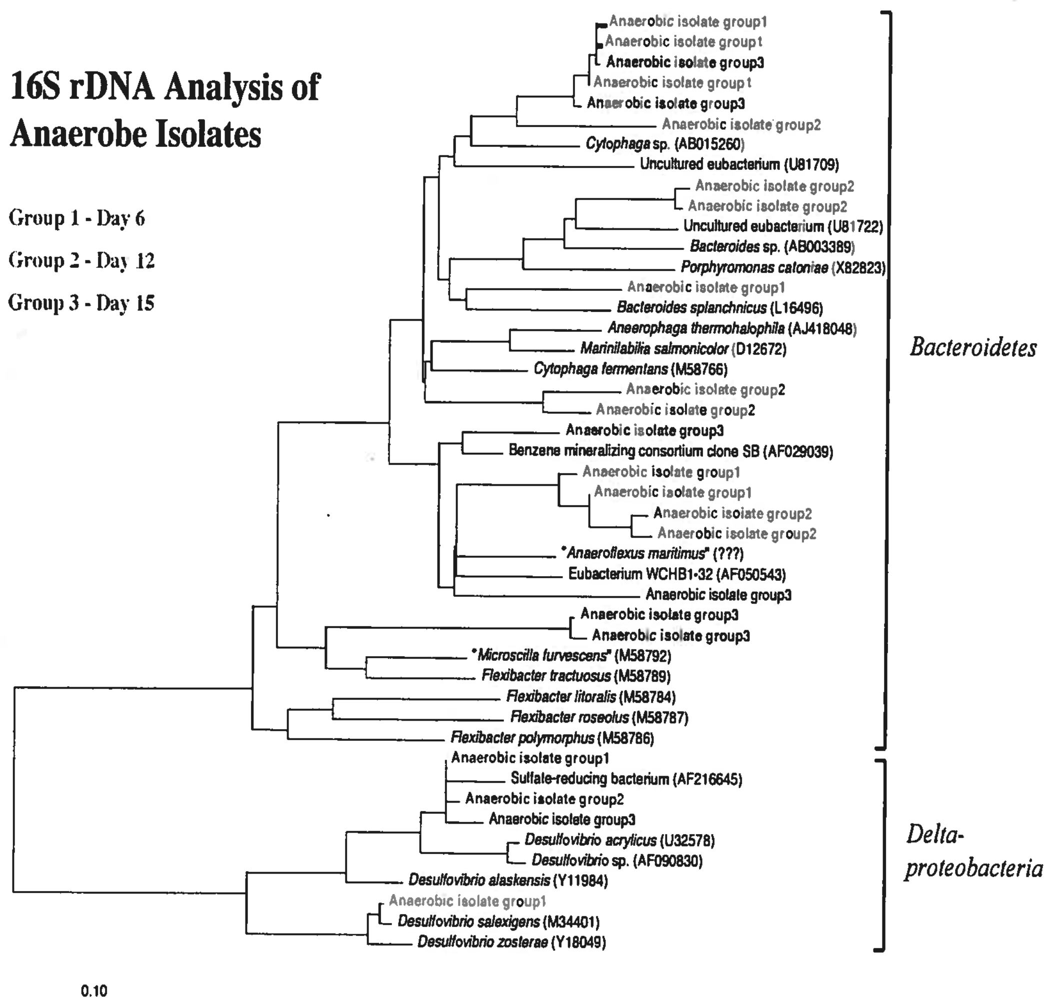


Figure 6. Colonies from Oyster Pond on SW4 media.

Table 3. PCR recovery of Single Colony Isolates

	Colonies Picked	PCR Seq	Percent Recovery
Day 6	14	8	58%
Day 12	16	8	50%
Day 15	26	5	19%

Figure 7. ARB Tree of Anaerobic Direct Plate isolates



Observations and Conclusions

Anaerobic agar-utilizing bacteria appear in less than a week and form large colonies that may obscure more interesting organisms. Use of a dissecting microscope on sacrificed plates revealed that micro-colonies appear after 13 days of incubation and these colonies may be too small to effectively pick in the anaerobic chamber. Closer observation suggests micro-colonies may require other organisms for growth (many of these were growing on the edge of larger colonies). This method allows ready visualization of colony morphology and ease in picking colonies for isolation and 16S rDNA analysis. In addition, plate washes can be done on sacrificed plates to obtain a more accurate picture of diversity since micro-colonies that are difficult to obtain while working in the anaerobic chamber can also be amplified and sequenced.

More than 50% of 16S rDNA PCR sequences analyzed with ARB appear to be bacteria that have not previously been cultured. Several monophyletic groups were found with no relatives closer than 90% and other isolates had relatives at least this far removed. The majority of isolates had the closest ancestor more than a genus removed. Nearly all of these new clades were in the CFB group with some sulfate-reducers also recovered. Archeal primers were attempted without success but could be optimized and used for PCR in the day 15 samples to more completely capture and characterize isolates. This method reduces enrichment bias and provides a new method for obtaining novel organisms from environmental samples in culture. Isolates can then be obtained of these novel organisms to determine more completely their metabolic activities and function in the environment.

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