Studies of *Cytophaga* sp. *puffball*, a facultatively anaerobic marine *Cytophaga*

Jean Huang
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Abstract

Cytophaga group bacteria are known to degrade polysaccharides in the environment and are believed to play a large role in their turnover. Although *Cytophaga* are abundant in soils, freshwater and marine environments in shores and in sediments, relatively few facultatively anaerobic species have been isolated and characterized. The focus of this work is a study of a *Cytophaga* sp. from Oyster Pond sediment that made spherical colonies up to 2 cm in diameter in 1% agar anaerobic shake tubes. The microbe was named *Cytophaga sp. puffball* to commemorate its striking colony morphology. *C. puffball* is an interesting marine *Cytophaga* because it is facultatively anaerobic and can use a large variety of simple sugars as well as polymers agar and gellan gum (Gelrtie) as carbon and energy sources. Its metabolic capabilities in the lab suggest that it has the capacity to participate in a variety of degradation reactions in nature both aerobically and anaerobically. The 16SrDNA of *sp. puffball* is 96% identical to that of *Cytophaga fermentans* (Bachmann, 1955), but unlike this facultative anaerobe, is able to degrade agar and does not carry out fermentation of the propionic acid type. Similar to *Cytophaga xylanolytica, sp. puffball* can degrade xylan, an abundant biopolymer in the environment, anaerobically.

Introduction

Cytophaga are gram-negative bacteria of the phylum *Bacteriodetes* that have gliding motility and are abundant in soil, fresh and marine water and environments. Bacteria of this group are known to degrade complex polysaccharides such as agar, cellulose and chitin. Most strains are known to be strict aerobes, though facultatively anaerobic strains have been isolated and studied. *Cytophaga fermentans* was the first facultatively anaerobic strain characterized (Bachmann, 1955). Several anaerobic strains have since been studied. *Cytophaga xylanolytica,* a freshwater isolate, was found to degrade the biopolymer xylan anaerobically (Haack and Breznak, 1993). These studies show that *Cytophaga* sp. with the ability to metabolize abundant polymers anaerobically exist, and could play a large role in biopolymer turnover. Other anaerobic *Cytophaga* studied have been obligately anaerobic species isolated from human gingival dental plaques such as *Capnocytophaga* (Leadbetter et al., 1979), which needed high CO2 for growth and was sensitive to regular air due to low CO2.

The following study is an initial characterization of a facultatively anaerobic marine *Cytophaga sp. puffball.*

Materials and Methods

Cytophaga sp. puffball origin and isolation

Shallow (5 inches) of sediment was collected from Oyster Pond a marine environment with pH 5.8 on June 15 2004. The sediment had been inoculated into SW#6 medium

(appendix) that contained 20mM TMA, 2.8mM Cysteine and agar as the sole carbon sources with N2/CO2 in the headspace of the Balch tube. The original purpose of the enrichment had been to track methane production by methanogens from this site, and those experiments were carried out for 1 week with the primary enrichment and 10 days with a secondary enrichment before samples were inoculated into anaerobic shake tubes of 1% agar of SW#6 medium. 2 days later, white, spherical colonies of *C. puffball* appeared and were present in shake tubes down to 1 colony in a 10-8 dilution. A single colony grew to 1.5 cm in diameter and had a zone of clearing that extended beyond the colony. Anaerobic media without TMA and cysteine and shake tubes using agarose, washed agar or Gelrite (Sigma) were was made to investigate the metabolic dependence of the bacterium on these compounds. Aerobic HS and 1X SW media (appendix) with either NaHCO3 or Mops as buffer were used to determine if cells could grow aerobically. Different carbon sources were added to aerobic HS medium to test the bacterium's ability to metabolize those compounds.

Microscopy

Cells were examined using light microscopy with a Ziess Axioskop II microscope under 100X oil. Cells were prepared and examined using transmission electron microscopy. Cells were prepared for TEM by putting a drop of cells that were growing exponentially on glucose (OD600 = .550) onto an ionized carbon grid and staining the cells with 2% uranyl acetate.

Microelectrode studies of a single *C. puffball* colony

Sulfide and pH measurements of a 1.5cm diameter puffball colony were made with microelectrodes (Unisense) over a 4cm depth through the colony.

Phylogeny

Cells from a puffball colony in an agar shake tube were resuspended in 20ul of a .05% triton solution and heated at 100°C for 5 minutes in a thermocycler. The supernatant was centrifuged for 10 minutes at 13,000 rpm and .5 ul of a 1X, 1:40 and 1:80 dilution of the lysate was used for 16srDNA amplification. 8F and 1493R primers were used and the resulting products were sequenced with 8F, 1492R, and 519R primers. The contigs were assembled with Sequencher and the phylogenetic position of the 1389bp sequence was calculated in Arb using DNA parsimony with 100 bootstraps.

Analysis of fermentation products

Supernatant from cultures growing aerobically or anaerobically on 20mM glucose were taken and analyzed just when the cultures reached stationary phase. 900ul of centrifuged cell culture was combined with 100ul of malate standard and the sample was filtered through a .2uM filter. A Shimadzu HPLC with an Aminex HPX-87H column (Biorad) was used to analyze the sample with acidified water mobile phase. Initial and final concentrations of glucose from cultures were assayed with a Glucose HK assay kit (Sigma) at initial and final time points. Standards were also run to identify and quantify products.

Results and discussion

Isolation of *C. puffball*

The primary shake tubes from the Oyster Pond samples yielded puff ball colonies after 2 days and other small dark colonies appeared after 4 days. Bacteria from the dark colonies were short motile spirilla and microscopic examination of the lowest dilution (10-6) mixed culture shake tubes showed that there were also spirochetes. It was unclear whether any of these other bacteria were associated with the puffball colonies. The motile spirilla and some spirochetes seemed to grow in the +TMA liquid cultures, but attempts to purify these cultures further shake tubes did not work. No methane was measured in these tubes after 2 weeks though the culture that the innoculum came from had evolved over 3% methane (Figure 1a).

Pure cultures of *C. puffball* were obtained from secondary shake tubes from Oyster Pond sediment in SW#6 medium (Figure 1b). Light microscopy of cells from these white puffball colonies were slender rods 4-7 um in length and .6um wide that had gliding motility (Figure 2a). Cells also flexed and reversed direction. TEM of negatively stained cells showed that cells produced extracellular material (Figure 3). No flagella or pilli were present. There were also curious rod shaped objects that were about 50-100nm in size in the medium and some of these rods were close to the cells (Figure 3). They were about as abundant as the cells themselves and it is unclear whether they are organic or inorganic or even phage. More experiments could be done to determine if the rod shaped objects in the TEM were inorganic compounds in the medium or of biotic origin and associated with *C. puffball*. Negative staining and TEM of the cells in different media could determine whether the rods were inorganic compounds in the medium, which would not be present when the media was changed, or whether they were truly associated with the *Cytophaga*.

Arb analysis of the 1389bp sequence of the16rDNA of a single puffball colony showed that the bacteria are a *Cytophaga* closely related to *Cytophaga fermentans* (Figure 2b). *C. puffball* was 96% similar to *C. fermentans* and 85% similar *C. xylanolytica.*

Carbon sources utilized

At first it wasn't clear what the bacteria were using as a carbon source. The only carbon sources present in the primary anaerobic shake tubes were 20mM TMA, 2.8mM cysteine and agar under N2/CO2 headspace. Though there was clearing in the agar, the agar was not liquefied even by older cultures. Serial dilutions of a portion of a puffball colony were made into anaerobic medium that contained no carbon sources followed by transfer into shake tubes containing different permutations of the possible carbon sources (different gelling agents: agar, washed agar, agarose or Gelrite (Sigma) with cysteine and TMA or missing either component) were made to study the dependency of *C. puffball* on any of the carbon compounds. There was growth in all tubes with the first colonies appearing after 2 days, but colonies were largest and came up first in shake tubes made with regular agar followed by washed agar. This indicated that *C. puffball* was using the solidifying agent as a carbon source because the colonies were similar in size in +TMA +Cysteine shake tubes and shake tubes containing only a gelling agent as the sole carbon source. Colonies were

slowest to appear in agarose tubes. Cells grew well in the presence of Gelrite, though it was difficult to make shake tubes with this compound and growth on it was determined from making a Gelrite plug with anaerobic medium and examining culture microscopically.

Since it was determined that *C. puffball* was using the agar gelling agent as a source of carbon and energy in the shake tubes, it is interesting that the bacterium was present at such high density (10-8 dilution) in innoculum from the secondary transfer of Oyster Pond sediment. This culture contained 20mM TMA and 2.8mM cysteine, which are carbon sources that *C. puffball* does not use. *C. puffball* does not grow on LB, but can grow in .05% yeast extract suggesting that it could be accustomed to less, rather than more, nutrients and so may have been able to survive on trace organics in the secondary enrichment.

A variety of carbon compounds were provided to *C. puffball* to determine if the bacterium could metabolize them (Table 1) at 10mM or .2-1% concentrations. Growth in cultures was monitored with OD600 as well as microscopic examination for culture purity and examination for cell growth on particles of insoluble compounds. *C. puffball* could grow in aerobic HS medium (appendix) with glucose. Similar to other *Cytophaga C. fermentans* and *C. xylanolytica, C. puffball* shared the ability to degrade a variety of simple sugars but was unique from *C. fermentans* in its ability to degrade agar and the component of agar, galactose. Whether *C. puffball* can chemotax to any of the carbon compounds it utilizes might be an interesting avenue for future study.

Fermentation products

It was determined via HPLC analysis of the culture fluids from cultures growing on anaerobically on glucose that *C. puffball* produces predominantly acetate with relatively small amounts of formate, succinate and propionate as fermentation products. The ratio of the products show that the bacterium does not carry out fermentation of the propionic acid type performed by *C. fermentans.* Small amounts of these products were also produced in cultures growing aerobically on glucose, and this may have been due to incomplete aeration of the culture or insufficient amounts of oxygen in the headspace to fully support oxygenic growth. Comparisons of the products from glucose metabolism under anaerobic and aerobic conditions normalized per OD600 yield (Figure 4C) indicated that the bacterium produced more acetate, propionate, formate and succinate under anaerobic conditions. Given that the bacterium grew to a higher yield under aerobic conditions, the growth yield was most likely due to oxidative phosphorylation because the same amount of glucose was available for the cells to use (Figure 4c). There was also a mystery peak at ~9.3 present in both anaerobic and aerobic glucose grown cultures. For future study it would be interesting to determine what this compound is. The identity of the compound was not a usual fermentation product. LC/MS of the culture fluids could potentially be used to further investigate this compound. Though the products of glucose metabolism under anaerobic and aerobic conditions could be determined using the HPLC, fermentation balances could not be completed because there were problems with the glucose assay kit. The amount of glucose calculated used by the microbes anaerobically did not make sense for the amount of products produced. The error could be due to pipetting errors and the

assay could be repeated to complete the fermentation balance. The HPLC products from formation of glucose were reproducible and consistent from culture to culture.

Attempts at plating
Cultures grew well in shake tubes and in liquid culture, but resisted growing on plates except on SW#6 medium (HS anaerobic medium + TMA) in the anaerobic incubator. These cultures grew spreading colonies that were white and pitted the agar. Cultures would not grow under aerobic conditions under aerobic HS medium, LB agar, or 1% YE agar with CO2 headspace conditions tried.

Microelectrode studies of puffball
Sulfide and pH measurements were made of a single puffball colony 1.5cm in diameter using microelectrodes (Unisense). There was no change in sulfide as the electrode traveled through the colony, but there was a.5 unit pH decrease within the colony (Figure 5). This showed that puffball was decreasing pH as a result of fermentation. *C. puffball* may have fermented impurities in the agar since cells grew best in unwashed agar.

Since *Cytophaga sp. puffball* metabolizes a variety of simple sugars and polymers including xylan, agar and Gelrite both anaerobically and aerobically, a hypothesis is that *C. puffball* could be an abundant species in the Oyster Pond environment because of its versatile degradation ability. Primers could be made and FISH could be used to probe the abundance of this *Cytophaga* in the Oyster Pond environment. Also, from his anaerobic plating experiments from Oyster Pond, Craig Oberg from the class recovered many *Cytophaga* sp. and it would be interesting to compare using FISH whether Oyster Pond may have more *Cytophagas* than other marine sites in Woods Hole, MA.

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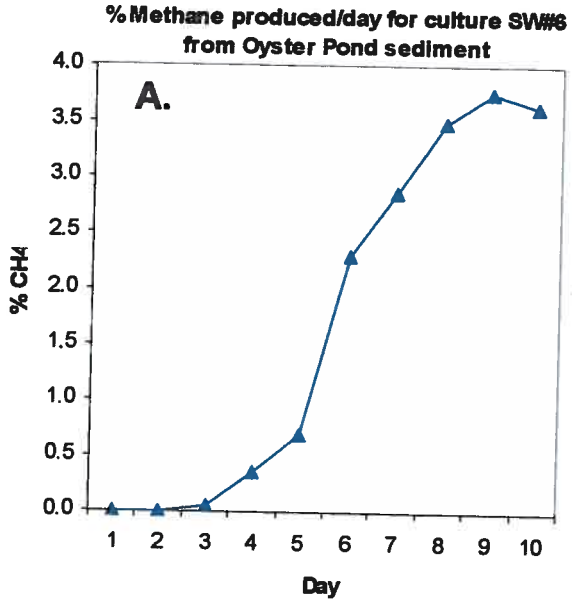
Leadbetter, E.R., S.C. Holt and S.S Socransky. 1979. *Capnocytophaga*: Mew Genus of Gram-Negative gliding bacteria. Arch. Microbiol, 122, 9-16.

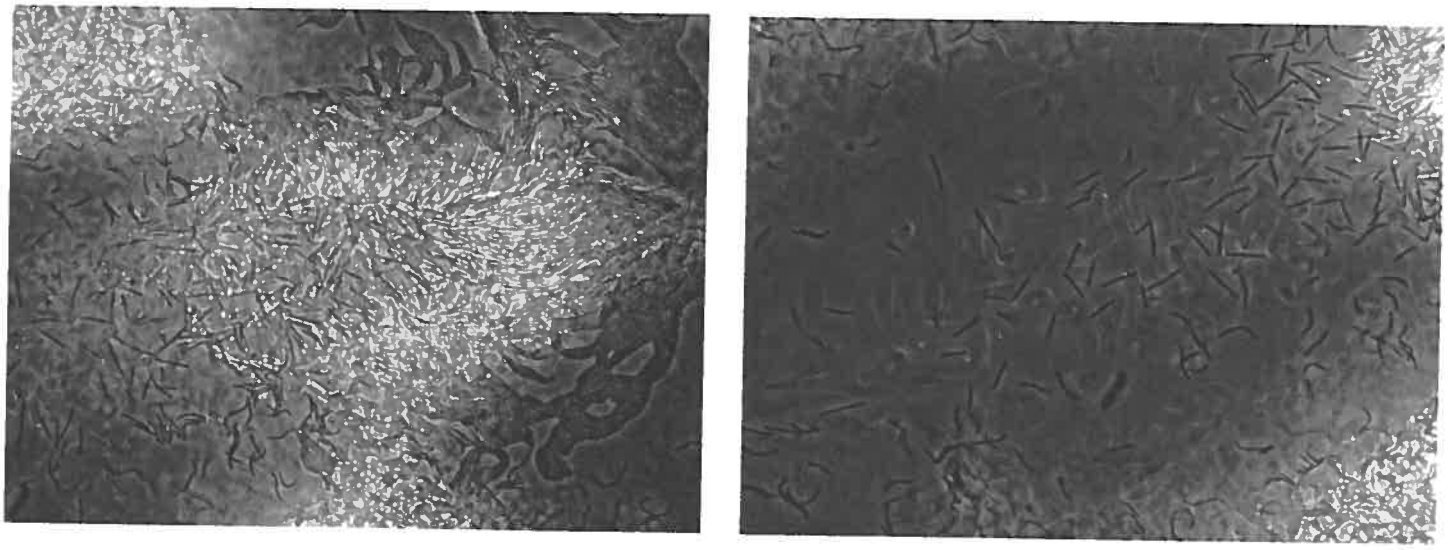


B.

Figure 1. Environmental origin of *Cytophaga sp. puffball* isolate. A. A secondary transfer of the Oyster Pond sediment culture from which *C. puffball* was obtained produced up to 3.7% methane over 10 days in SW#6 medium. *C. puffball* came from shake tubes from this enrichment. B. Seconday shake tube transfers obtain pure cultures of *C. puffball.*

Figure 2. Morphology and phylogenetic position of *Cytophaga sp. puffball*. **A.** 100X light microscopy of *C. puffball* cells. **B.** Phylogenyof *C. puffball*. 1383 bp of the 16srDNA of *Cytophaga sp. puffball* was aligned in ARB and a tree was calculated using Phylip DNA parsimony tree with 100 bootstraps.

A.



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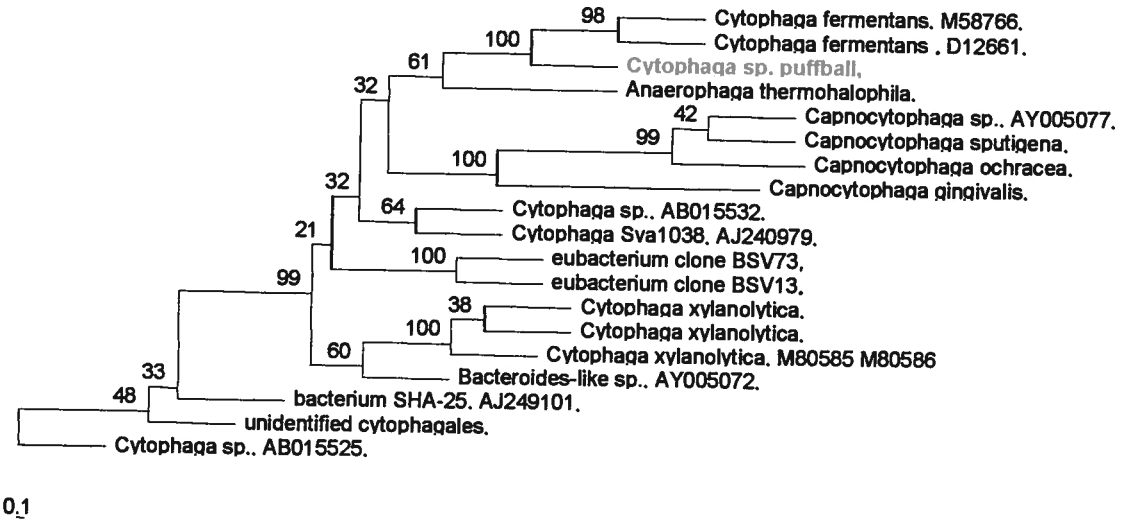


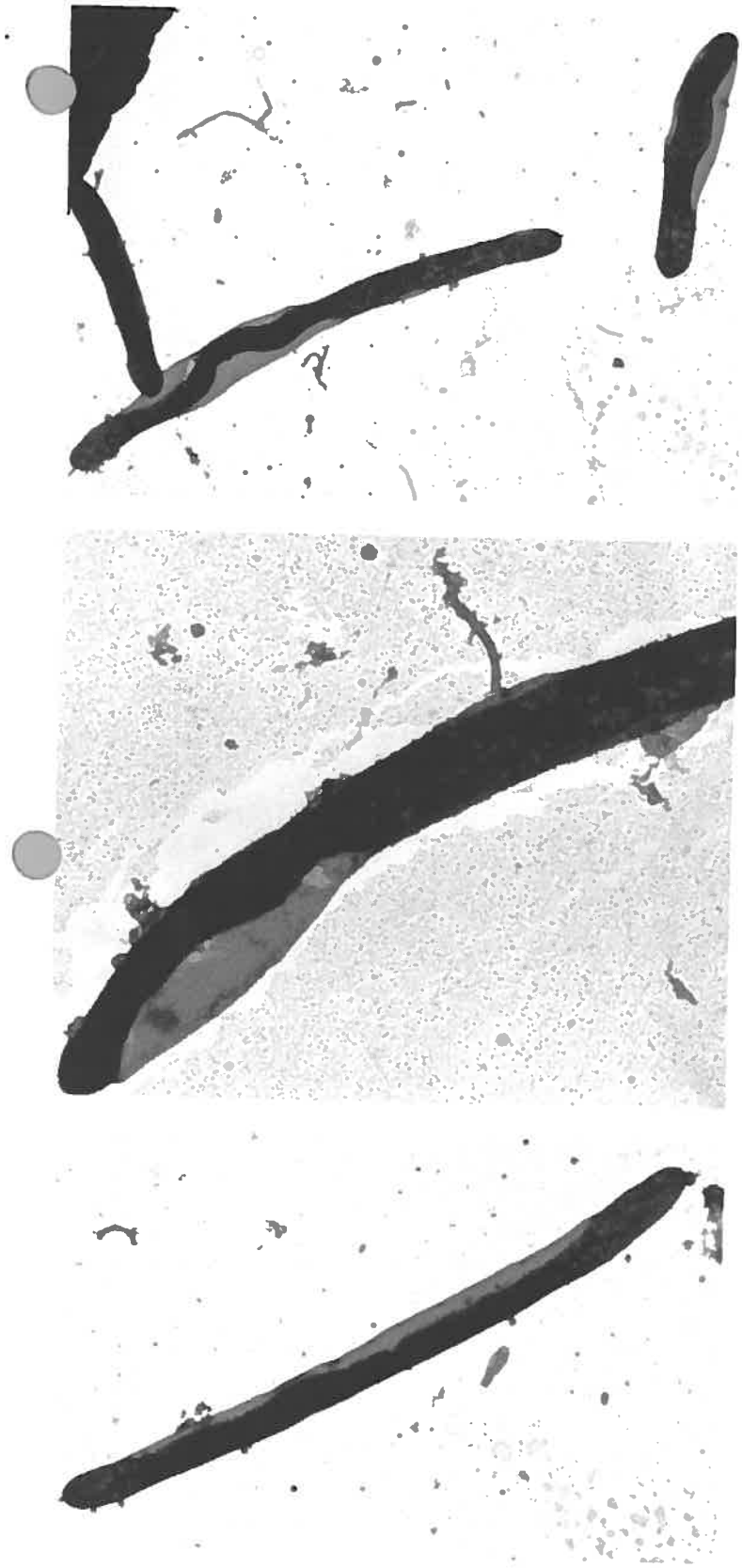
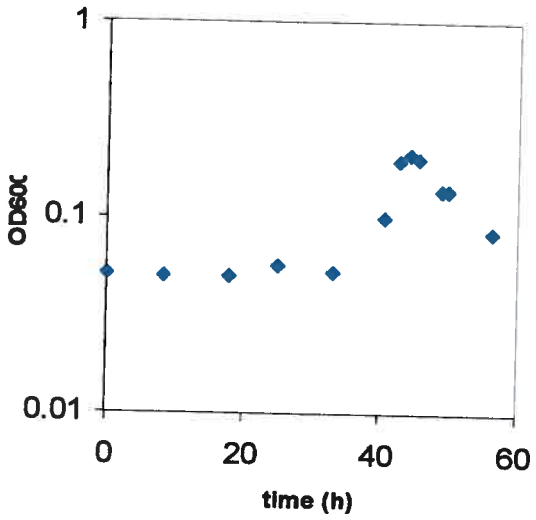


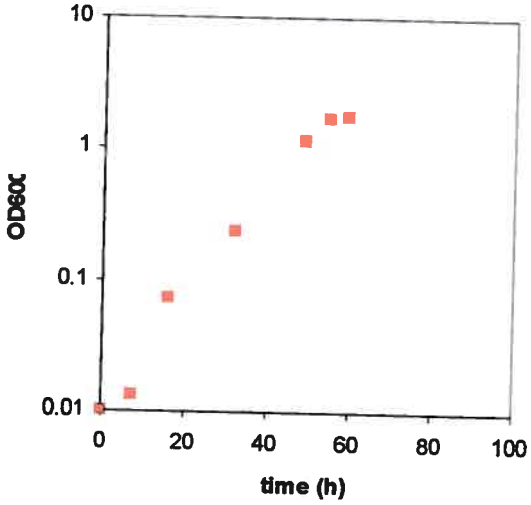
Figure 3. Transmission electron micrographs of *Cytophaga sp. puffball.* Cells appear to have exopolysaccharide on the outside of the cells.

Figure 4. Anaerobic and aerobic growth of *C. puffball* on 20mM (non limiting) glucose. A. Anaerobic growth in anerobic HS medium at 30°C. First transfer of cells usually had ~2 days lag. B. Aerobic growth on HS aerobic medium shaking at 120rpm at 30 degrees, second transfer from cells growing in glucose aerobically. C. Yields and doubling times from several aerobic and anaerobic cultures.

A. Anaerobic growth



B. Aerobic growth



C.

Anaerobic growth
Td: 5.69, 6.55
Yields (OD600):1.59, .092

Aerobic growth
Td (h): 6.8, 4.2
Yields: 1.77, .557

products from growth on glucose (mM)

products	anaerobic	anaerobic products normalized to yield	aerobic
propionate	1.601	5.609	0.077
succinate	0.559	1.958	0.020
formate	0.476	1.667	0.010
acetate	22.700	79.521	0.103

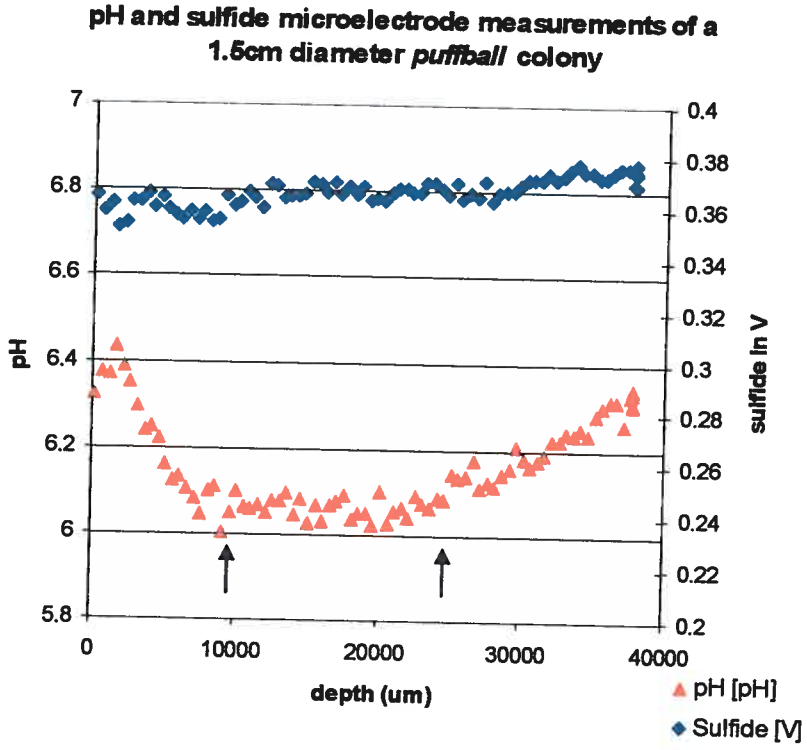


Figure 5. pH and sulfide microelectrode study of a single *C. puffball* colony 1.5cm in diameter. Arrows indicate when the electrodes entered and exited the colony.

Table 1. Ability of *Cytophaga sp. puffball* to utilize different compounds as sole sources of carbon and energy. Compounds were supplied at either 10mM or .2-1% concentrations and cultures were incubated under semi-aerobic conditions (aerobic HS medium, not shaking at 30°C). Growth was confirmed by increase in OD600 or microscopic examination of insoluble particles.

	C. puffball (marine isolate)	*C.fermentans* (marine isolate) Bachmann, 1955	*C. xylanolytica* (freshwater isolate) Haack and Breznak, 1993
Hexoses			
Glucose	+	+	+
galactose	+	-	+
mannose	+	+	+
pentoses			
ribose	-	-	-
rhamnose	+ wall growth	-	-
arabinose	+	+	+
xylose	+	+	+
Dissacharides			
cellobiose	+	+	+
maltose	+	+	+
melibiose	-		
sucrose	+	+	+
Trisaccharides			
raffinose	+	+	
alcohols			
glycerol	-	-	-
mannitol	+		
Polysaccharides			
agar	+	-	
agarose	+	-	
Gelrite	+		
pectin	+		
starch	-	+	
chitin	-	-	
xylan	+		+
cellulose	~ increase in OD, but no visible degradation of filter paper	-	-
alginic acid	+	-	
methyl cellulose	+		
alginic acid	-	-	
organic acids			
malate	-		
succinate	-	-	
amino acids			
asparagine	-		
alanine	-	-	
glutamine	-	-	

other carbon sources			
glucuronic acid	+ wall growth		+
LB	-		
.05% YE Tryp	+ biofilm formation in standing tubes		
DNA	-		
gelatin	-		
peptone	+		
Fermentation products	acetate propionate succinate formate	acetate propionate succinate	acetate propionate Succinate CO2 H2
oxidative phosphorylation	+	+	aeroduric

Appendix

Media recipes:

Marine anaerobic HS medium (per liter)

Base medium:
Mix A:

NaCl	23.4g	400mM
NaHCO3	3.8g	45mM
KCl	1.0g	13mM
.1% resazurin	1.0ml	4uM

Dissolved in ~700ml ddH2O and boiled for 10 minutes under N2/CO2 gas. When cooled, medium was brought into the anaerobic chamber.

Mix B:

MgCl2*6H2O	11.0g	54mM
CaCl2*2H20	.3g	2mM

Dissolved in ~350ml ddH2O and boiled for 10 minutes under N2/CO2. When cooled, medium was brought into the anaerobic chamber.

In the anaerobic chamber, mix A and B were combined and the following components were added:

Trace elements	10ml	
Vitamins	10ml	
5.0ml of 1.0 M KH2PO4 (anaerobic, pH=6.8)		5mM
NH4Cl	1.0g	19mM
Cysteine*HCl	.5g	2.8mM
.2M Na2S*9H2O	2.0ml	.4mM

The medium was brought to 1L and autoclaved for 25minutes.

Amendments:
SW#6 medium consisted of HS Anaerobic medium with 20mM TMA and a headspace containing N2/CO2 (80%/20%). Puffball was originally enriched in 1% agar SW#6 medium shake tubes. Anaerobic HS medium without either TMA or cysteine, or both was also made to test the bacterium's dependence on these compounds.

1XSW medium – similar medium to anaerobic HS, but with reduce amts of several components.

per liter:

NaCl	20.g
MgCl2*2H2O	3.0g
CaCl2*2H2O	.15g
Na2SO4	2.84g
NH4Cl	.25g
KH2PO4	.2g
KCl	.5g

Medium was heated and brought into the anaerobic hood when cooled. 1ml trace elements, 60ml of 1M NaHCO3 and 1ml of vitamins 2ml of .5M Na2S solution was added after the medium had been autoclaved, cooled and pH adjusted to pH 7.3.

Aerobic media:

Aerobic HS: same medium as anaerobic HS medium leaving out Na2S*9H2O
Aerobic 1XSW: same medium as anaerobic 1XSW medium leaving out Na2S*9H2O

NaHCO3 buffer was substituted with 10mM Mops buffer, pH 7 for experiments to test absence of CO2 on growth.

Modified Trace Elements Solution:

Compound	**Amt./Liter**	**MW**	**Conc.**	**Media Conc.**
Nitrilotriacetic acid (Trisodium salt)	1.5 g	257.1	5.8 mM	58 uM
$Fe(NH_4)_2(SO_4)_2$	0.8 g	392.1	2 mM	20 uM
Na_2SeO_3	0.2 g	172.9	1.1 mM	11 uM
$CoCl_2 \cdot 6H_2O$	0.1 g	237.9	0.4 mM	4 uM
$MnSO_4 \cdot H_2O$	0.1 g	169	0.6 mM	6 uM
$Na_2MoO_4 \cdot 2H_2O$	0.1 g	241.9	0.4 mM	4 uM
$Na_2WO_4 \cdot 2H_2O$	0.1 g	329.9	0.3 mM	3 uM
$ZnSO_4 \cdot 7H_2O$	0.1 g	287.5	0.3 mM	3 uM
$NiCl_2 \cdot 6H_2O$	0.1 g	237.7	0.4 mM	4 uM
H_3BO_3	0.01 g	61.83	0.16 mM	1.6 uM
$CuSO_4 \cdot 5H_2O$	0.01 g	249.7	40 uM	0.4 uM

Vitamin Solution:

Compound	Amt./Liter	MW	Conc.	Media Conc.
p-Aminobenzoic acid	10 mg	137.1	73 uM	729 nM
Nicotinic acid	10 mg	123.1	81 uM	812 nM
Ca pantothenate	10 mg	238.3	42 uM	419 nM
Pyridoxine HCl	10 mg	205.6	49 uM	486 nM
Ribflavin	10 mg	376.4	27 uM	266 nM
Thiamine HCl	10 mg	337.3	30 uM	296 nM
Biotin	5 mg	244.3	20 uM	204 nM
Folic acid	5 mg	441.4	11 uM	113 nM
α-Lipoic acid	5 mg	206.3	24 uM	242 nM
Vitamin B_{12}	5 mg	1355.4	3.7 uM	37 nM

Filter sterilize using a 0.22 micron filter
Store in the dark at 4° C.