IDENTIFICATION OF AN AEROMONAD AND A HOMOACETOGEN FROM THE INTESTINE OF A GRASS CARP (*CTENOPHARYNGODON IDELLA*)

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ABSTRACT

Herbivorous fish, such as grass carp (*Ctenopharyngodon idella*), may contain anaerobic microbial communities based on cellulose catabolism. These communities are expected to contain terminal H_2-consumers such as methanogens and/or homoacetogens. In this study to determine whether homoacetogens could be isolated from the intestinal tract of *C. idella*, we added the contents of a *C. idella* intestine to a minimal medium broth with an 80% H_2/20% CO_2 headspace (20 psi total pressure). Sodium 2-bromoethanesulfonate (BES) was added to inhibit methanogenesis at a concentration of 5 mM. Four subsequent isolation attempts were done using the anaerobic roll tube technique with minimal agar medium containing 10 mM fructose (another substrate homoacetogens are known to grow on) with an 80% N_2/20% CO_2 headspace (20 psi). After isolation of colonies, DNA was extracted, sequenced and the initial isolate was identified as an aeromonad. Subsequent ten-fold, serial dilutions of the original enrichment in minimal medium with a H_2/CO_2 headspace to provide the carbon and energy sources resulted in growth thought to be a pure culture of homoacetogens. To determine what organisms were present in this culture, we extracted DNA from the 10^{-1} and 10^{-5} dilutions of the presumed homoacetogen culture, amplified using primers for the 16S rRNA gene and cloned the amplicons in the pGEM®-T Easy Vector. Inserts from random clones were sequenced and analysis indicated that the organisms were *Acetobacterium* sp. The growth of aeromonads on fructose is not uncommon; however, its presence in an environment with only H_2/CO_2 was unexpected. While the presence of the homoacetogen was shown, it was obtained late in the project and consequently has not been characterized. The relationship between the homoacetogen and the aeromonad, if any, is also undetermined.

Keywords

Homoacetogen, aeromonad, microbial cellulose degradation

INTRODUCTION

Anaerobic, cellulosic environments can occur in a variety of locations: landfills, soils, aquatic environments and in the rumen or intestinal tract of various herbivorous animals, potentially including the intestinal tract of herbivorous fish such as grass carp (*Ctenopharyngodon idella*) (Leschine 1995). In these habitats, cellulose degradation is facilitated by a variety of microorganisms, where diverse groups are responsible for different steps of the degradation process (Saha et al. 2006; Leschine 1995). This diversity is necessary due to the varied substrates and electron acceptors available in the anoxic environment as well as the limited metabolic repertoire available to each trophic group. This complex relationship between available substrates, electron acceptors and microorganisms forms a microbial food web (Leschine 1995). Cellulolytic microorganisms hydrolyze cellulose and the resulting sugars are fermented. Fermentations produce alcohols, organic acids (such as acetate, propionate, butyrate), CO_2, and H_2 (Leschine 1995). The resulting H_2 is consumed by terminal electron accepting organisms such as methanogens and homoacetogens, which reduce the CO_2 using H_2 to form methane and acetate respectively (Diekert and Wohlfarth 1994; Thauer et al. 1977; Fenchel and Finlay 1995). Syntrophic bacteria may also be present and grow in a relationship with the H_2 consumers to ferment fatty acids or alcohols (Leschine 1995).

Homoacetogens are strict anaerobes that can produce acetate by catalyzing single carbon units (Diekert and Wohlfarth 1994). The Wood-Ljungdahl pathway demonstrates how homoacetogens convert CO_2 to acetate (Müller 2003). Methyl compounds (methanol, methoxylated aromatic compounds), two carbon compounds and sugars can also be used as an energy source for homoacetogens (Diekert and Wohlfarth 1994). *Acetobacterium hydrophila* has been shown to metabolize both fructose and mannose sugars using phosphotransferase systems (Binet et al. 1998). Anaerobically, *A. hydrophila* has also been shown to utilize nitrate, fumarate, Fe(III), Co(III), and Se(VI) as terminal electron acceptors (Knight and Blakemore 1998).

Homoacetogens and methanogens, because they both use H_2, are competitors in these communities. However, the process of methanogenesis has a more negative ΔG than the process of homoacetogenesis (Thauer et al. 1977). The reaction to produce methane has a $\Delta G^{\circ\prime}$ = -131 kJ/mole reaction and for homoacetogenesis the $\Delta G^{\circ\prime}$ = -95 kJ/mole (Fenchel and Finlay 1995). If both groups are present, with H_2 as the sole energy source and CO_2 as the electron acceptor, methanogens will often out-compete the homoacetogens. In some circumstances, homoacetogens have the upper hand because they are mixotrophs and can grow both autotrophically and heterotrophically by either switching between the two, or potentially utilizing both at the same time (Fenchel and Finlay 1995). This could enable the homoacetogens to dominate an environment over a methanogen. A known environment where homoacetogenesis is prominent is the hindgut of a termite (Warnecke et al. 2007). For the isolation of homoacetogens from a community with both types potentially present, sodium 2-bromoethanesulfonate (BES) can be added to inhibit methanogenesis (Zinder et al.

1984; Kamlage et al. 1997). This was employed for this project since the goal was to isolate a homoacetogen from the intestinal contents of a *C. idella*.

METHODS

Sampling – An intestine from a grass carp was obtained and divided into five sections: the stomach, an anterior section, two mid-gut sections and a posterior section, and clips were placed on the ends to reduce oxygen exposure. The intestinal segments were placed into a rectangular AnaeroPack® container with an anaerobic gas generating sachet (Mitsubishi Gas Chemical America) for transport to an anaerobic chamber (Coy Laboratory Products, Inc.). The contents of the intestine were anaerobically transferred into 29 mL of sterile, anaerobic buffer containing 10 mM phosphate buffer with 0.4 mM sodium sulfide and 0.0001% resazurin with a pH of 7.

Basal Media – Addition of the anaerobic medium was done in the anaerobic chamber (Coy Laboratories) with 10 mL added to each 20 mL headspace vial and 30 mL to each 125 mL serum bottle. The anaerobic basal medium consisted of the following: 20 mL mineral solution (per liter: NaCl – 40 g; NH_4Cl – 50 g; KCl – 5 g; KH_2PO_4 – 5 g; $MgSO_4 \cdot 7\ H_2O$ – 10 g; $CaCl_2 \cdot 2\ H_2O$ – 2 g, 5 mL trace metal solution (per liter: nitrilotriacetic acid – 2.0 g, adjust pH to 6 with KOH): $MnSO_4$ – 1.0 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$ – 0.8 g; $CoCl_2 \cdot 6\ H_2O$ – 0.2 g; $ZnSO_4 \cdot 7\ H_2O$ – 0.2 g; $CuCl_2 \cdot 2\ H_2O$ – 0.02 g; $NiCl_2.6\ H_2O$ – 0.02 g; $Na_2MoO_4 \cdot 2\ H_2O$ – 0.02 g; Na_2SeO_4 – 0.02 g; Na_2WO_4 – 0.02 g), 10 mL vitamin solution (per liter: pyridoxine·HCl – 10 mg; thiamine·HCl – 5 mg; riboflavin – 5 mg; calcium pantothenate – 5 mg; thioctic acid – 5 mg; p-aminobenzoic acid – 5 mg; nicotinic acid – 5 mg; vitamin B_{12} – 5 mg; biotin – 2 mg; folic acid – 2 mg; mercaptoethanesulfonic acid – 10 mg), 0.34 g KH_2PO_4, 1.31 g K_2HPO_4, 0.001 g resazurin, 3 g $NaHCO_3$, 1 mM $Na_2S \cdot 9\ H_2O$ and sufficient reversed-osmosis water to bring the solution to a volume of 1000 mL. Bottles were capped inside the chamber with 1 cm thick aluminum seal stoppers (Bellco Biotechnology, Inc.), secured with aluminum crimp seals and degassed by alternately pressurizing with either 80% N_2/20% CO_2 or 80% H_2/20% CO_2 (20 psi total pressure) and then evacuating three times and leaving under pressure. Media were sterilized at 121°C for 30 minutes. Stock solutions of fructose, BES, and the various electron acceptors were sterilized, separated and added aseptically after the bottles were autoclaved. All cultures were incubated statically in the dark and at room temperature.

Isolations of DNA from Samples – Contents from the fish intestine were initially added to the anaerobic basal media amended with 5 mM BES and an 80% H_2/20% CO_2 headspace (20 psi) in a 25 mL test tube. After growth was observed in this sample, 0.1 mL was transferred to 10 mL of the basal media amended with 10 mM fructose and an 80% N_2/20% CO_2 headspace (20 psi) in a 25 mL test tube. Isolations were carried out using the anaerobic roll tube technique within a 25 mL test tube in basal media amended with 10 mM fructose and an 80% N_2/20% CO_2 headspace (20 psi). DNA extraction, purification,

PCR (using forward primer 27f (5- AGA GTT TGA TCCTGG CTC AG -3) and reverse primer 1492r (5- TAC CTT GTT ACG ACT T -3) and sequencing was done by the Nevada Genomics Center, and the National Center for Biotechnology Information's (NCBI) BLASTn program was used to determine identity of the isolate. After the isolate was determined to be an *Aeromonas*, the isolate was streaked onto Lysogeny Broth - Miller (LB) agar plates and incubated aerobically.

In a second attempt to identify a homoacetogen, the contents from the fish intestine were added to 30 mL basal media amended with 5 mM BES and an 80% H_2/20% CO_2 headspace (20 psi) in 125 mL bottles serum bottles. A ten-fold, serial dilution was done and samples from the 10^{-1} dilution and 10^{-5} dilution (the highest dilution with visible growth) were used for DNA extraction. DNA was extracted from the 10^{-1} and 10^{-5} dilution as well as the *Aeromonas* sample from the LB agar using the UltraClean® Microbial DNA Isolation Kit (MoBio).

PCR and Cloning of Samples – PCR was done with the DNA from the 10^{-1} and 10^{-5} dilutions of the homoacetogen culture as well as the *Aeromonas* sample using the forward primers: 27f1 (5′-AGA GTT TGA TCC TGG CTC AG-3′), 27f2 (5′-AGA GTT TGA TCA TGG CTC AG-3′), 27f3 (5′-AGA GTT TGA TTC TGG CTC AG-3′), 27f4 (5′-AGA GTT TGA TCC TGG CTT AG-3′), 27f5 (5′-AGA GTT TGA TTA TGG CTC AG-3′), and 27f6 (5′-AGG GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492r (5′-TAC CTT GTT ACG ACT T-3′) (obtained from Integrated DNA Technologies). Two master mixes were used and each contained: PCR buffer (1X), $MgCl_2$ (1.5 mM), dNTPs (200 nM), three forward primers (100 mM of each primer; 27f1, 27f2 and 27f3 in one and 27f4, 27f5, and 27f6 in the other), reverse primer (300 nm; 1492r) (Frank et al. 2008) and Platinum® Taq DNA Polymerase (1 unit) (Invitrogen). Reactions were 50 µL, containing 49 µL of the master mix and 1 µL of DNA template. Amplification was done for 35 cycles at 94 °C, 1 minute; 48 °C, 0.5 minute; and 72 °C for 2 minutes in a thermocycler. PCR products were cleaned up using Wizard® SV Gel and PCR Cleanup System (Promega). Ligation of the products was done using the Promega pGEM®-T Easy Vector System. JM109 high efficiency competent cells were used for transformation. LB agar plates with 100 µg/mL ampicillin, 2 µM IPTG and 80 µg/mL X-Gal were inoculated with the resulting transformed cells. Twelve white colonies from each of the 10^{-1} dilution, the 10^{-5} dilution and the *Aeromonas* samples were re-streaked and isolated colonies were grown in LB broth with 100 µg/mL ampicillin. The plasmids from each clone were purified using the QIAprep® Miniprep (Qiagen). These plasmids were then sent to the Nevada Genomics Center for sequencing. Again, NCBI's BLAST program (http://www.ncbi.nlm.nih.gov/) was used to determine identity of the isolates. Phylogenetic trees were constructed to determine the relationship between the clones from each of the samples using the San Diego Supercomputer Center (SDSC) Biology Workbench (http://workbench.sdsc.edu/).

Testing Electron Donors and Electron Acceptors – The *Aeromonas* isolate was tested with various electron donors to determine its ability to utilize these compounds. *Aeromonas* was inoculated into 30 mL basal medium in 125 mL bottles supplemented with Na-acetate (30 mM) and an 80% N_2/20% CO_2 headspace

(20 psi), Na-acetate (30mM) with an 80% H_2/20% CO_2 headspace (20 psi) and an 80% H_2/20% CO_2 headspace (20 psi) without any organic molecules added. Growth with each of these potential electron donors was evaluated using Na-Nitrate at 20 mM or 2 mM, Na-Nitrite at 2 mM or Na-thiosulfate at 20 mM as potential electron acceptors. Samples were observed for growth after five days and the turbidity was measured using a spectrometer at 600 nm.

Growth of the *Aeromonas* isolate using fructose (10 mM) as an organic carbon and energy source with the electron acceptors describe above was evaluated in an 80% N_2/20% CO_2 headspace (20 psi). These samples were done in triplicate with 30 mL of media in 125 mL serum bottles. The turbidity was measured at 600 nm using a spectrometer after five days incubation at room temperature. Absorbances were averaged and error bars were calculated using standard deviation.

Aeromonas Growth Aerobically and Anaerobically – Comparison of *Aeromonas* growth both aerobically and anaerobically was also measured. *Aeromonas* was added to 10 mL of basal medium amended with 2.5 mM fructose in 30 mL serum bottles either sealed with aluminum seal stoppers for anaerobic growth or with foam stoppers for aerobic growth. Turbidity was measured using a spectrometer at 600 nm after five days incubation at room temperature. Absorbances were averaged and error bars were calculated using standard deviation.

Mimicking Conditions with a Homoacetogen – Environmental conditions that might occur when a homoacetogen is present were created by adding acetate (20 mM) to 30 mL basal media in 125 mL serum bottles, and to trials that also contained BES (5 mM) to copy the original conditions, and an 80% H_2/20% CO_2 headspace (20 psi). Only the *Aeromonas* was added to see if it was able to grow in these environments without the homoacetogen. As a control the *Aeromonas* was inoculated alone into the basal media and an 80% H_2/20% CO_2 headspace (20 psi).

RESULTS

Results from DNA Isolation – The first isolation showed high similarity to *Aeromonas veronii*. Trimmed sequences ranged from 9 bp to 60 bp. Because of the poor quality of the sequence, these results could not be considered conclusive.

Results from PCR and Cloning – Twelve clones from each sample, the 10^{-1} and 10^{-5} dilutions of presumed homoacetogens and the pure culture of *Aeromonas*, were compared. All 24 samples from the homoacetogen dilutions grouped together in the phylogenetic trees (data not shown). Results from NCBI for these dilutions showed a high degree of similarity to *Acetobacterium* sp., which are usually homoacetogenic. Clones from the *Aeromonas* sample also grouped together in the phylogenetic trees (data not shown). NCBI results showed all the clones to be *Aeromonas* sp. with a high degree of similarity. Trimmed sequences for all these samples ranged from 700 bp to 850 bp.

Results from Electron Donors and Acceptors – There was no visible growth observed in the tests done with the various electron acceptors and acetate and 80%

N_2/20% CO_2 headspace (20 psi), acetate and the 80% H_2/20% CO_2 headspace (20 psi) or with the 80% H_2/20% CO_2 -headspace (20 psi) alone.

The turbidity results from the tests done with the potential anaerobic electron acceptors and fructose are shown in Figure 1. From the absorbances, there was much lower turbidity with nitrite while other electron acceptors and the control were comparable.

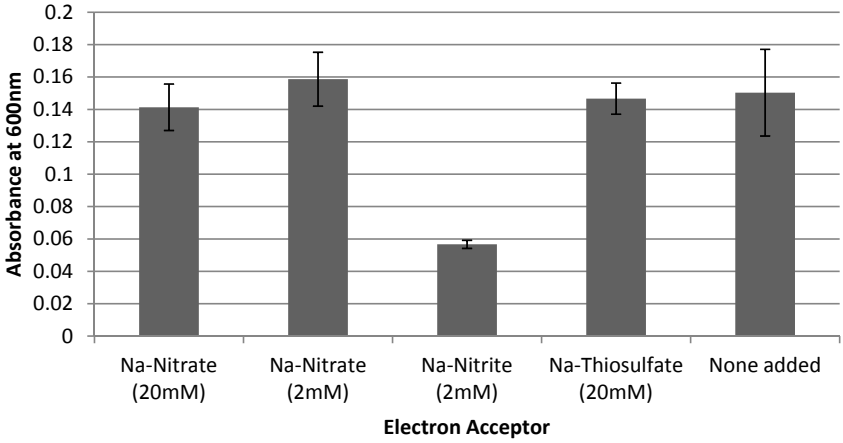

Figure 1. Absorbance at 600 nm measured after a five day incubation of Aeromonas at room temperature in minimal medium supplemented with 2.5 mM fructose, the listed electron acceptor and an 80% N_2/20% CO_2 headspace (20 psi). Averages were determined from triplicate samples, and error bars represent standard deviation.

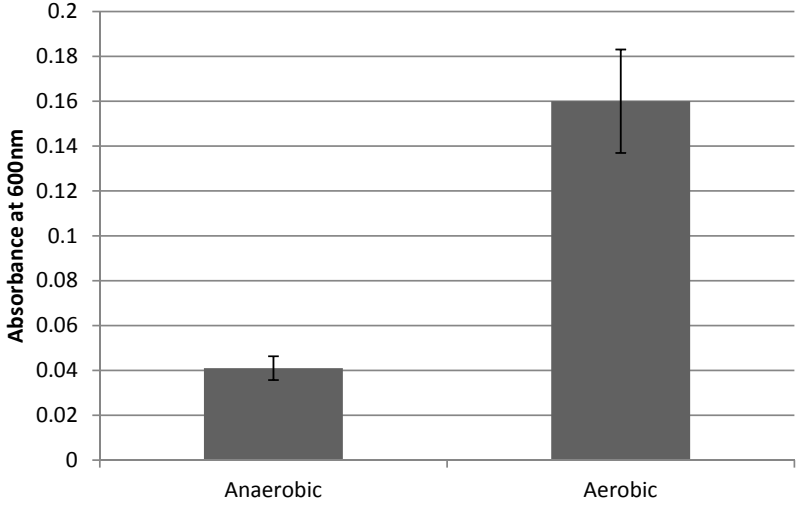

Figure 2. Absorbances at 600 nm measured after a five day incubation of Aeromonas at room temperature in minimal medium supplemented with 2.5 mM fructose. Anaerobic incubations were done using an 80% N_2/20% CO_2 headspace (20 psi) in a 30 mL serum bottle while aerobic incubations were done in similar sized serum bottles with foam stoppers. Averages were determined from triplicate samples, and error bars represent standard deviation.

Aeromonas Growth Aerobically and Anaerobically – Absorbances from *Aeromonas* growing aerobically and anaerobically are shown in Figure 2. Aerobic growth on fructose yielded a biomass approximately four times greater than anaerobic growth.

DISCUSSION

The presence of 16S rRNA gene sequences closely related to *Acetobacterium* sp. is evidence that there is a homoacetogen inside the *C. idella* intestine, but an isolated colony was not obtained. The initial isolation of the *Aeromonas* was unexpected and preliminary work focused on trying to determine whether this organism was simply maintaining itself in the initial enrichment or was actually growing syntrophically with the homoacetogen.

Aeromonas species are known to be associated with both marine and fresh water as well as aquatic animals, including fish, therefore, its presence in our sample is not altogether surprising (Dügenci and Candan 2003; Trust 1975). However, while *Aeromonas* is known to grow on hexose sugars, such as mannose and fructose, what it was metabolizing in the original enrichment, which only contained H_2, CO_2 and BES, is not apparent (Binet et al. 1998). As of now, it is unclear if or how the *Aeromonas* was growing in a co-culture with the homoacetogen. It may have been dormant while in the minimal media with an 80% H_2/20% CO_2 headspace and started growing again when transferred into the fructose medium. Alternatively, *Aeromonas* might have been growing syntrophically with the homoacetogen. It may be that the *Aeromonas* is growing at the end of the homoacetogen cycle. As the homoacetogen grows, the amount of hydrogen present will decrease and the amount of acetate and other potential products of homoacetogen metabolism (butyrate, fatty acids) will increase (Leschine 1995). It is possible that under these conditions, the *Aeromonas* is able to grow. Also, dehalogenating behavior has been observed in *Acetobacterium* sp. so another possibility is that the homoacetogen is able to debrominate the BES, making it a usable substrate for the aeromonad (Damborsky 1999).

The increased biomass of the *Aeromonas* in the aerobic cultures as compared to the anaerobic cultures resulted from the greater energy yield provided by substrate oxidation in aerobic respiration rather than from fermentation or anaerobic respiration. Because *A. hydrophila* has been shown to utilize nitrate as an electron acceptor, an increase in biomass was expected, but not observed in this experiment (Knight and Blakemore 1998). This may be a result of small variations in the amount of fructose added to the samples, while it was added to individual bottles instead of to one batch of media. Inhibition of *Aeromonas* growth with the nitrite was expected since nitrite inhibition of *A. hydrophila* growth has been previously reported (Lambert and Bidlas 2007).

When conditions that occur during growth of the homoacetogen were created and inoculated with only the aeromonad, no growth was observed, suggesting that the homoacetogen is needed for the aeromonad to grow in the minimal conditions originally provided. Further tests will need to be done to determine the exact relationship between the two organisms.

Identification of other organisms present inside the intestine will provide a more complete picture of the anaerobic, cellulosic degrading microbial food web contained inside *C. idella*. Characterizing the relationship between two known contributors, homoacetogens and aeromonads, will also advance the knowledge of this overall, complex relationship. Determining the relative numbers of these organisms, as well as other players, would also help to describe this environment.

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