COMPARING THE GENOME EXPRESSION PROFILES OF *VERRUCOMICROBIUM* SP. STRAIN TAV2 CELLS GROWN UNDER TWO DIFFERENT OXYGEN CONCENTRATIONS

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ABSTRACT

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Termites are significantly different in their lifestyles and organization but most are linked by the common trait in degrading plant material, whether it is leaf litter, wood, soil or animal waste. They consume the cellulose, hemicelluloses and lignin that make up the plant material but rely on a food web consisting of about 270 different phylotypes of microorganisms to break down the biomass into usable nutrients. Isolated from the hindgut of the termite *Reticulitermis flavipes*, one such organism is *Verrucomicrobium* sp. strain TAV2. Members of this relatively new bacterium phylum have rigid peptidoglycan with some possessing prosthecae. They have been detected in soils, aquatic habitats, as well as symbionts in termites. Within the interior of the termite's hindgut, oxygen concentrations range from 2% oxygen near the interior lumen, to an anoxic environment at the core of the hindgut. TAV2 is thought to be found in the 2% oxygen range although little else is known about the organism. Determining the bacteria's genomic expression profile when grown under two different oxygen concentrations provides information into the organisms ability to survive and thrive within the termite as well as elicit information

concerning TAV2's activated genes when in differing oxygen concentrations. TAV2 was grown under 2% oxygen, its presumed natural environment, and 20% oxygen concentration, near atmospheric oxygen concentration, and had its RNA extracted while growing at exponential growth to use for microarray analysis. Growth curves provide evidence that genomic regulation is affected by the oxygen concentrations in that TAV2 has a doubling time approximately eight hours faster when grown under 2% oxygen verses 20% oxygen. Microarray results convey that 75 genes are up and down regulated when comparing the two oxygen conditions. Examples of affected genes include malate/L-lactate dehydrogenase genes as well as hypothetical genes within the genome. Using TAV2 as a model organism, information gathered can be applied to the other TAV strains within the hindgut illuminating how oxygen concentrations affect the ability to breakdown plant material effectively.

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INTRODUCTION

LITERATURE REVIEW

As the world's energy consumption continues to increase and the oil reservoirs diminish, societies are looking to new methods from which they can generate renewable fuel to supply energy needs. Bio-fuels are a possibility whose many facets are being researched daily. From these possibilities, plant biomass conversion to ethanol is one of the largest resources not being utilized to its fullest potential. Ethanol from corn grain distillation accounted for only 2% of total gasoline and diesel consumption in America in 2004 [1]. With the sustainability of fossil fuels in question, other forms of consumable energy must take the forefront.

Converting plant material into ethanol, in simple terms, can be divided into two major steps. The first step consists of the physical breaking up the plant material into glucose subunits and then the second step, fermenting the glucose into ethanol. Plant biomass comes from various sources as fibrous, woody, and generally inedible portions of plant matter [1]. Although humans utilize many various parts produced from plants such as fruits, nuts, and even the wood for structural components, a large percentage of the plant material is not manipulated for potential use. A problem that prevents the use of plant biomass for energy needs is deconstructing the lignocellulosic material. In the year 2004, ethanol production from plants was not only cost ineffective but also unable to provide enough resources per unit weight [1]. Enzymes, such as cellulases, have been isolated from microorganisms and applied to processes which degrade plant material, but it has been found that when the bacteria themselves are taken out of the equation, the degradation process can take twice as long [2]. Therefore a shift of focus to microbial patterns in nature could aid with the extraction of usable energy from plant material.

Information gathered when microorganisms are cultivated under differing environments provides insight into their genetic control. One factor that can be taken into effect is the oxygen gradient. Because oxygen is an oxidizing reagent and is present in the atmosphere of everyday life for humans, it has a profound impact on the microbes which we wish to manipulate for societies benefit. To understand how this affects the microbial processes, transcriptional variation analysis was completed on the bacteria *Termite Associated Verrucomicrobium* strain (TAV2).

1.1 Plant Biomass as an Energy Source

Plant biomass, termed lignocellulose, mainly consist of three subunits, cellulose (28-50%), hemicelluloses(20-30%), and lignin(18-30%) [3]. Currently these molecules are extremely difficult to degrade in a cost effective manner for the extraction of useable energy. Cellulose is comprised of beta (1,4) linked glucose molecules existing in a highly ordered crystalline state with a tensile strength comparable to steel [3].

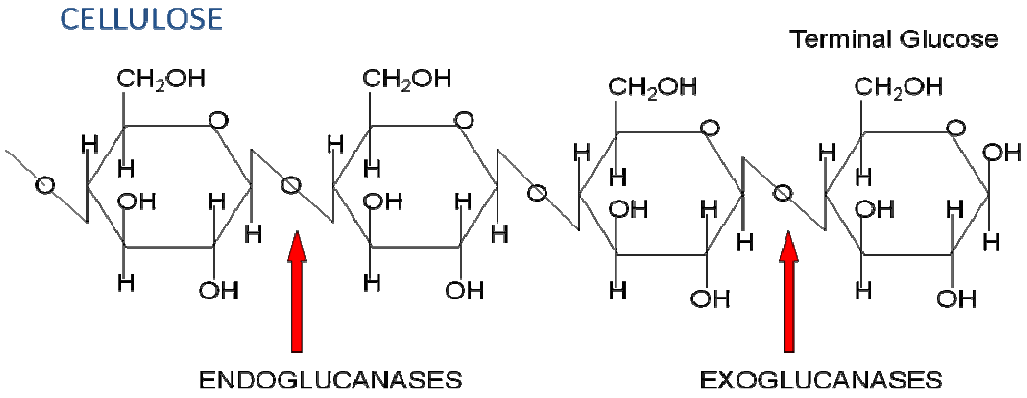

Figure 1.1 Chemical structure of glucose subunits in beta (1,4) linkage. Endoglucanases attack interior glucosidic bonds and exoglucanases attack the terminal bond in a glucose chain.

Enzymes that hydrolyze the cellulose material possess the generic name of cellulases. These enzymes include endoglucanases which cleave internal glucosidic bonds, exoglucanases which preferentially cleave the terminal end of a polyglucan chain, and beta-1,4-glucosidases which cleave cellulobiose and water soluble cellodextrins to glucose [3].

Hemicellulose is composed of heterogeneous branched polysaccharides made of D-xylose, D-mannose, L-arabinose, and D-galactose and any combination thereof [3].

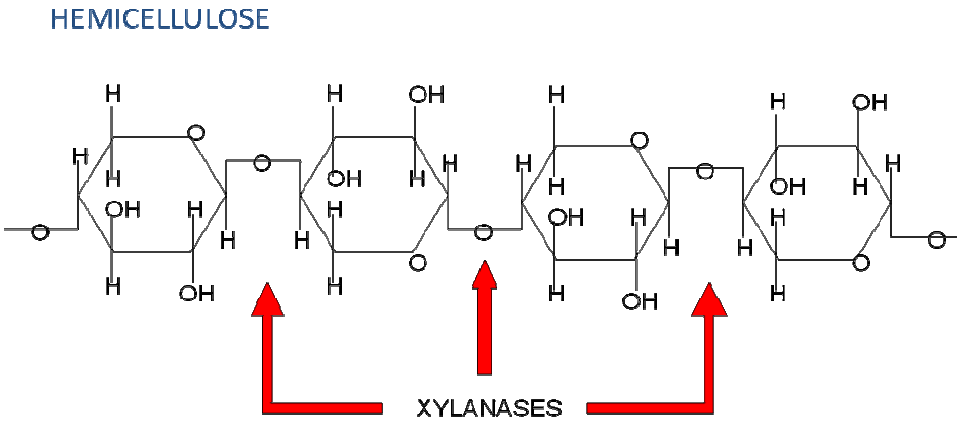

Figure 1.2 Chemical structure of xylan composed of hexose sugar residues linked by beta (1,4) linkages. Xylanases are the enzymes that hydrolyze the interior xylan bonds.

Because the hemicelluloses' are mainly composed of a beta 1,4 xylose backbone, enzymatic hydrolysis of these molecules are accomplished by endo – beta-1,4 xylanases. The other branches made of other sugar residues are broken down by various corresponding hydrolytic enzymes.

Lignin is the third component that makes up the majority of plant biomass. Although cellulose is the most common component, lignin follows behind as the second most common component in plant biomass material and is one of the most important constituents of woody material [4]. This molecule is extremely hard to cleave by hydrolysis because of its chemical makeup as well as a three dimensional, non-repeated subunit structure. Chemically it comprises of phenylpropane residues linked by carbon-carbon and ether bonds that are chemically cross-linked to the other components of the plant cell wall [4, 5].

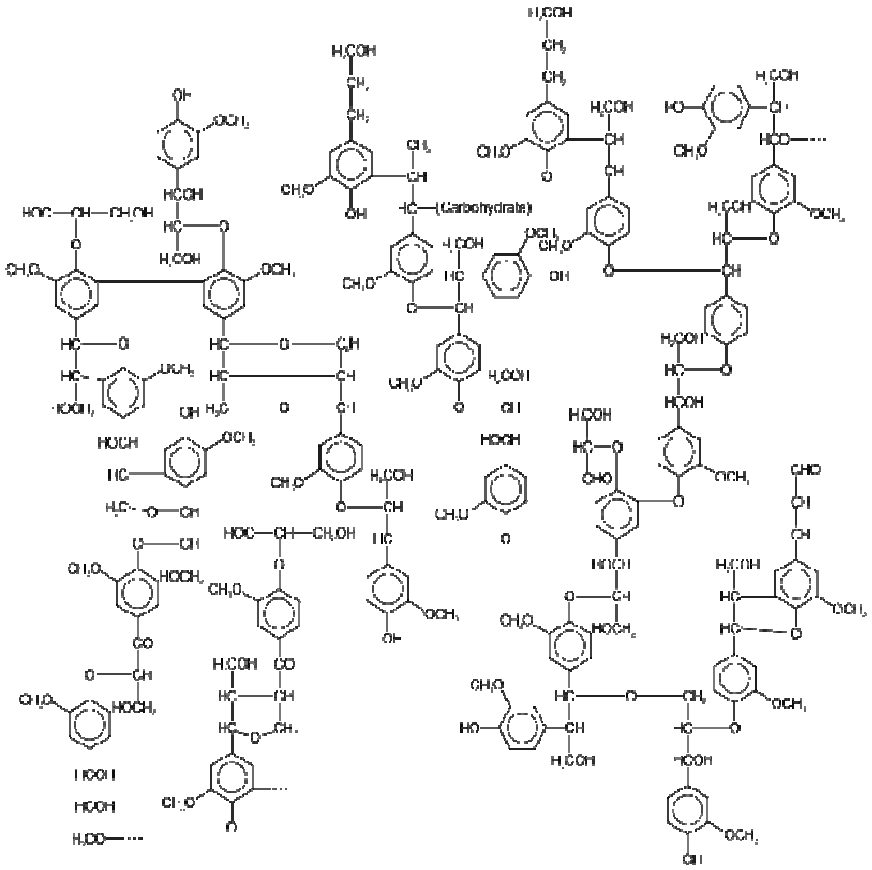

Figure 1.3 Proposed chemical structure of a fragment of softwood lignin composed of phenylpropane residues randomly linked carbon-carbon and ether bonds. (Adapted from [6])

In addition, to degrade lignin, organisms must have access to oxygen as a prerequisite for lignin peroxidases as there are no known mechanisms to degrade lignin anaerobically [4, 3, 5]. Lignin makes up a greater and greater percentage of the plant material as one observes woodier biomass [3]. Plant material with higher lignin composition therefore is more resistant to decomposition.

1.2 Biological Systems for Lignocelluloses Degradation: The Termite

To provide insight into ways societies may convert plant biomass into ethanol, we can look to biological systems that already accomplish the task. Termites are a biological system which can break down plant material into usable energy. Their ability to efficiently digest plant

biomass with the aid of microorganisms has gained recognition as a model bioreactor [7, 8, 3]. Lignocellulose material is broken down into useable nutrients such as acetate and lactate to provide energy for the microorganisms as well as the termites they inhabit. The termite gut provides a self contained bioreactor where these enzymatic actions can be studied and applied to new methods for energy enrichment from microbial processes.

More than 2,000 species of termites have been described. They inhabit about two-thirds of the worlds land surface between the latitudes 48°N and 45°S [9]. Although significantly different in their lifestyles and organization, most termite species are linked by the common trait of degrading lignocellulosic plant material, whether it is leaf litter, wood, soil or animal waste [10, 11]. Termites ingest about 3-7 x 10^{15}g of the 136 x 10^{15}g of the dry plant material (~2-5% annually) [3].

Termites are divided into two orders, higher and lower termites. Lower termite families (*Mastotermitidae*, *Kalotermitidae*, *Hodotermitidae*, *Rhinotermitidae*, *Thermopsidae*, and *Serritermitidae*) are distinct because along with the bacteria they possess in their gut they also harbor plant biomass degrading protists in their midgut. The symbiosis that occurs between termites and the microorganisms inhabiting their digestive tract allow enormous amounts of plant material to be digested into useable nutrients.

Higher termites are classified differently than lower termites on the basis of gut configuration and gut microbial diversity. Higher termites are classified into one single family, *Termitidae.* This group contains about 85% of all termite species [12]**.** Higher termites do not contain hindgut flagellate protozoa, but instead contain fungi and prokaryotes [3, 7, 11]. This work will focus on the lower termite and their microbial inhabitants, specifically on the wood-feeding *Reticulitermis flavipes* in the *Rhinotermitidae* family.

The *Reticulitermes* genus has species found in every state in America except for Alaska. Of these, *Reticulitermes flavipes* is the most widespread and is found from Ontario, Canada to Key Largo, Florida. In practical economic terms, $2.2 billion is spent yearly on

termite control of which the majority is thought to be due to *R. flavipes* and its relative *R. virginicus* [13]. *R. flavipes* colonies can have 100,000 to 1,000,000 individuals made up of three primary types of castes: soldiers, workers, and the reproductives (these contain the king, queen, and supplementary reproductives) [13, 14]. It can take five to ten years in order for a mature colony to form from a new mating pair, of which the workers can live up to five years [13].

Studies have revealed an abundance of microorganisms within the digestive system of the termite *Reticulitermis flavipes*. It is estimated that approximately $5.0x10^8$ cells are present per mL of hindgut fluid [15]. A study carried out by Hongoh and coworkers focused on the bacterial community diversity in the termite *Reticulitermes speratus*, a relative of *R. flavipes*. Results indicated that 268 phylotypes were present through 16S rRNA clone libraries and represented 11 bacterial divisions [16]. They estimated approximately 2.7×10^6 bacteria, of the divided 270 phylotypes, inhabit the hindgut of these termites and only about 10% of the cells observed by microbial counts have been cultured [15, 16, 10, 7]. Among these, *Spirochaeta* bacteria make up as much as half of the sequences [3]; [17]; [16].

The breakdown of lignocellulostic material can be an intricate process involving many organisms. Therefore a prolonged digestion time of up to 26 hours has been observed in *R. flavipes.* This duration supplements the microorganisms ability to breakdown the tough plant material [3]. The lower termite digestive system can be divided up into individual parts, each with its own function in the degradation of lignocelluloses material. Beginning with the physical breakdown of material in the mouth of the termite, the food then enters the foregut where most of the oxygen is removed by the termite [18]. From there, the material proceeds to the midgut where it is acted upon by protists and the termites own cellulases [8, 19]. The termite can secrete a small fraction of its own cellulases to aid in the enzymatic breakdown of the plant material [19]. However it has been found that these secreted cellulase enzymes are not enough for the termite to survive on solely [19].

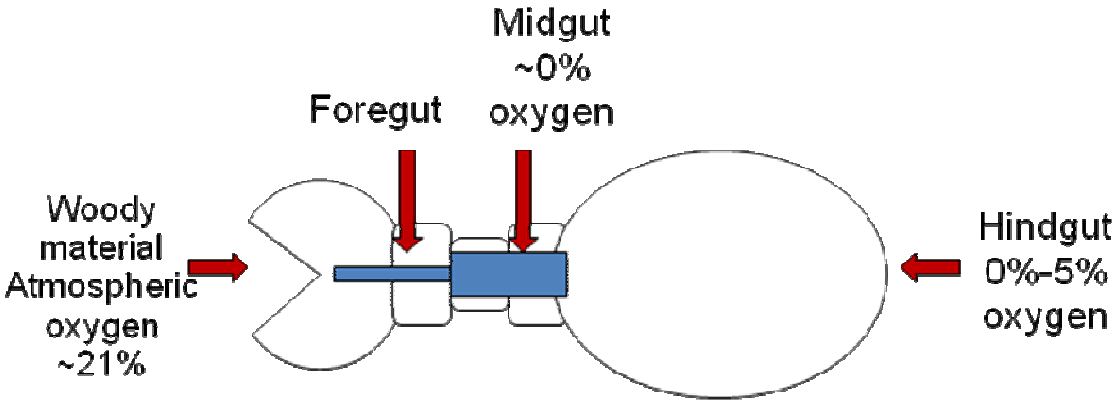

Figure 1.4 Diagram of termite digestive tract and oxygen content in each respective area. Lignocellulosic material enters the mouth and is physically broken down by termite mastication. From here the material enters the foregut where most of the oxygen is removed. Next the bolus enters the midgut where protists act on the molecules in a near anoxic environment (~0%). Lastly the subunits of lignocellulose decomposition enter the hindgut and are acted on by organisms in anoxic (0% oxygen) to microoxic (2-5%) conditions.

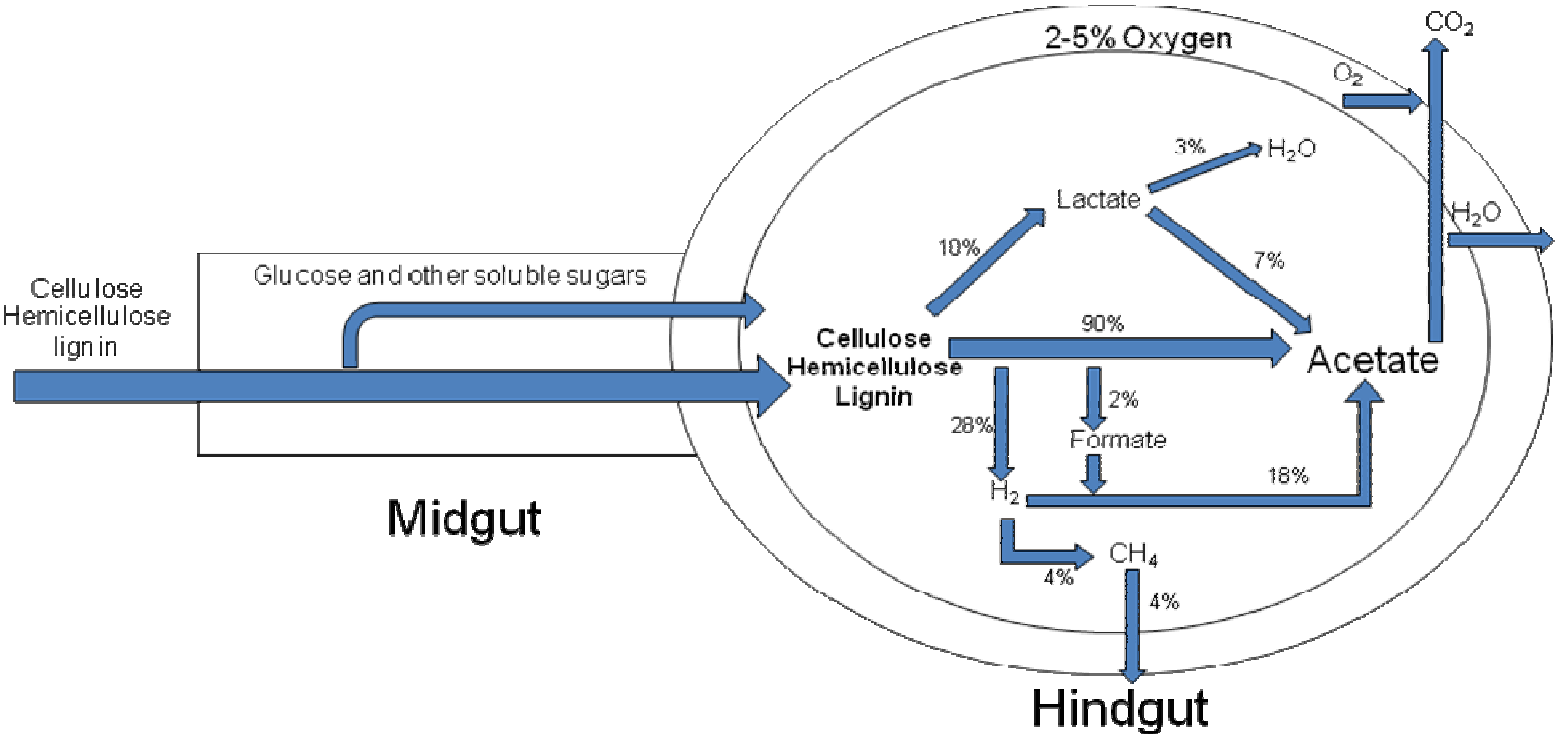

Figure 1.5 Depiction of chemical breakdown of lignocellulose material within the digestive system of the termite. Percentage values represent proposed hydrogen metabolism. Plant biomass enters the midgut and begins to be broken down into individual glucose molecules, lactate, formate, acetate, CO_2, and H_2. CO_2 and H_2 are then combined by methanogens into methane whereas acetogens combine CO_2 and H_2 to form acetate. The acetate is oxidized by the termite for its energy needs. The hindgut has an oxygen gradient that is microoxic near the outer edges of the paunch and decreases to an anoxic environment at the interior. Hydrogen gas concentrations are the highest near the core and decrease as one advances to the exterior of the hindgut. (Adapted from [20]

The symbiotic relationship between the termite and protists is vital to the degradation of lignocellulose as well as to the termite's survival. It has been shown that cellulose degrading bacteria are not present in significant numbers within the termite midgut that contain protists. Most of the cellulose material breakdown is done by protists [21, 8]. The termites provide a desirable habitat for protists to thrive because of their own consumption of oxygen for metabolic purposes as well as spiracle-control strategies that limit the amount of internal oxygen [18]. This atmosphere thereby provides an anaerobic environment and consistently supplies small fragments of cellulose to protists. Protists in turn will degrade the cellulose into useable substrates which the termite can then convert into energy. Termites that have had protists removed perish from starvation when fed a diet of wood. Protists degrade the cellulose to acetic acid, hydrogen, and carbon dioxide [22, 8, 21]. One way to imagine the degradation of cellulose is to look at each glucose subunit. Glucose is not absorbed directly by the termite. The glucose molecule, when catabolized, can theoretically produce three molecules of acetate. Protists degrade the glucose to two units of acetate to be used by the termite as well as hydrogen and carbon dioxide to be later handled by the hindgut microorganisms [3]. The termites absorb the large amount of acetic acid generated to oxidize for energy [8, 22]. In addition, the mutualism is furthered in that if the end products of cellulose degradation are allowed to accumulate around the protist they can reach high concentrations and kill the protist. Equation 1 demonstrates how glucose compounds are turned into acetate for the termite.

Equation 1: $C_6H_{12}O_6 + H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$

From the midgut, the end products of cellulose breakdown and additional lignocellulosic material enter the hindgut region of the digestive track. As in the midgut, protists inhabit the anoxic inner core of the hindgut [23]. The hindgut can be thought of as a large pouch, similar to a stomach in appearance and is the widest point on the termite worker body, approximately 0.5 to 1 mm thick in *Reticulitermis flavipes* [17]. Along the inner membrane of the termite hindgut there also are cup shaped depressions, phoresis, (2-4 µm in width and 1-2 µm in depth) which

provide sites for attachment for bacteria [17]. These phoresis could take on added benefits in survivability and protection for slow growing bacteria that reside on the outer regions of the paunch [17]. Given that the hindgut of a termite is spatially structured, it is expected that different microorganisms will position themselves accordingly [24].

Importantly the paunch also has an oxygen gradient whose outer edge has an oxygen concentration around 2-5 % and an inner region that is anoxic (Figure 1.6) [25, 10]. The outer microoxic ring usually is 50-200µm thick and exists as a result of simple diffusion of atmospheric oxygen through the thin outer membranes of the termite [25, 10]. Thus, in proportion to the total volume of the hindgut, only 25-40% of the paunch is completely anoxic [25]. This oxygen gradient has a significant impact on the carbon and electron flow within the hindgut microbial community [26]. The oxygen that diffuses through the thin outer layers of the termites hindgut is used as a final electron acceptor for metabolic reactions and also plays a key role in degradation of lignin [10]. Although lignin degradation rates are negligible under anaerobic conditions, aromatic rings, like those found in the components of lignin, can be degraded aerobically by bacteria isolated from the gut [5]. This process specifically occurs within the *Reticulitermes flavipes* and studies have shown that only five percent of the material was being excreted in the feces [4]. In addition, degradation rates increased when lignin molecules are catabolized with a cosubstrate such as the components of cellulose, hemicelluloses, glucose, or xylan [5]. This is not surprising considering that these components are found together alongside lignin within the plant cell wall material.

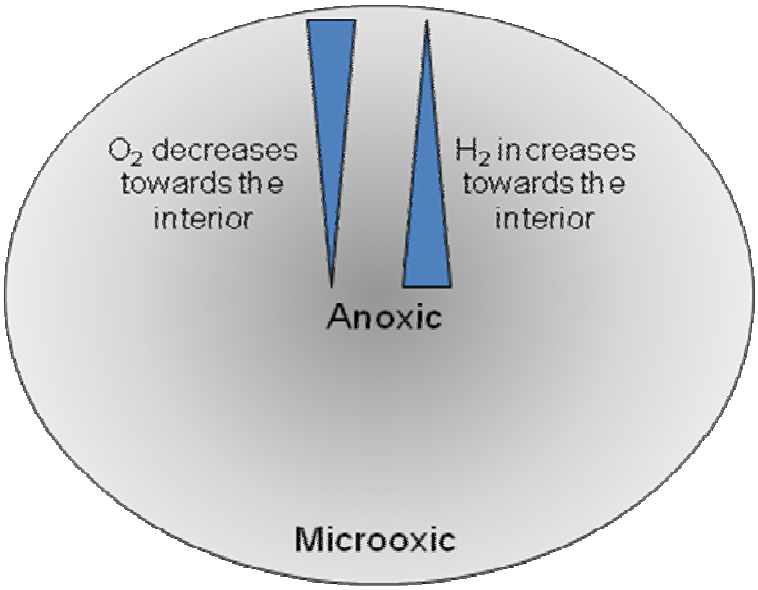

Figure 1.6 Radial view of hindgut of *R. flavipes*. Oxygen profile in microoxic ring is 2-5% oxygen and decreases to an anoxic environment within progressing 50-200µm inwards. Conversely, the hydrogen concentration is 28% in the anoxic core and gradually decreases towards the outer periphery.

The cellulose material is broken down into H_2, CO_2, and acetate in the midgut as well as within the anoxic region of the hindgut (Figure 1.5) [27, 3, 10]**.** As stated, protists are found in the inner anoxic core and continue to produce hydrogen gas and carbon dioxide from the breakdown of lignocellulose in the inner core of the paunch [23]**.** Importantly the hydrogen concentration has the highest concentration at the anoxic center and decreases in concentration when proceeding to the exterior (Figure 1.6) [23]. Hydrogen concentrations in *R. santonensis*, a relative of *R. flavipes*, have been detected at 28% in the core. Because the oxygen is actively consumed in the outer edges to provide an anoxic core, methanogens and acetogens thrive to combine hydrogen and carbon dioxide to generate acetate and methane [3]. Consequently, acetogens combine a majority of the hydrogen with carbon dioxide to produce additional acetate molecules which can provide the termite with up to one third of its respiratory needs [23, 20, 3]. This can be regarded as the third part of the glucose molecule that is metabolically altered into acetate. Without the anoxic environment acetogenesis would not be able to proceed because it is a strictly anaerobic activity, thereby decreasing the useable

amount of energy that can be extracted from the plant material for the termite and its microbial community [10]. It has been found that the spirochete bacteria make a substantial contribution to the termite nutrition in this manner [27]. These bacteria are thought to reside in and/or near the protists in the anoxic core of the midgut and form a mutualistic relationship within the food web of the hindgut [23, 27]**.** Equation 2 shows the conversion of hydrogen and carbon dioxide to acetate and water.

Equation 2: $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$

Additionally methanogenic bacteria use the escaping hydrogen gas and carbon dioxide to form methane and water [3]. Traditionally it is thought that methanogenesis occurs in anoxic low sulfur environments, yet in the study by Leadbetter and Breznak bacteria that perform this function were found in the microoxic periphery of the hindgut. Isolates of these bacteria have been found on the outer periphery of the lumen in the hindgut both attached directly to the hindgut epithelial surface, in phoresis, and within clumps of other prokaryotes that are attached to it [28]. Although it is currently unknown how these isolates are able to produce methane in such conditions, their catalase activity may provide information to future metabolic pathways within the termite hindgut. The methane that is produce by methanogenic bacteria is not utilized by the termite. This means that all the methane produced in the hindgut is emitted by the termite [20]. Equation 3 demonstrates how methanogenesis occurs with hydrogen and carbon dioxide being transformed into methane and water.

Equation 3: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

It is thought that the acetogenic and methanogenic bacteria completely use the hydrogen that is produced because no hydrogen is detected near the rectum or emitted to the atmosphere [10, 20]. The acetogens have been shown to out-compete the methanogens for the hydrogen gas within the *R. flavipes* termite hindgut [29]. This is contradictory to other environments that are anoxic and low in sulfate and nitrate. These environments generally have carbon dioxide and hydrogen gas converted to methane rather than acetate [30, 3]. This

is because, energetically, methanogenesis is more favorable than acetogenesis [30]. It has been suggested that mixotrophic use of organic and inorganic substrates may allow the acetogens to outcompete the methanogens in the hindguts of wood-feeding termites [29]. Another theory considers that the acetogens are positioned around the anoxic core near or attached to the protists while the methanogens use the escaping hydrogen gas at the inner lumen periphery of the termite [23]. This idea would have the acetogens and methanogens not in competition, but affected by spatial distribution for resource usage [23, 28, 27]. Since up to one third of the acetate that is used by the termite for metabolic processes is supplied in this manner, acetogenic activity is advantageous for the termite.

In addition to the cellulose, hemicellulose, and lignin degradation into acetate, hydrogen and carbon dioxide, other substrates are also produced. These include lactate and formate (Figure 1.5) [15]. Equation 4 shows the conversion of the lactate to acetate.

Equation 4: $2C_3H_6O_3 \rightarrow 3CH_3COOH$

Interestingly, when glucose from cellulose breakdown is consumed directly in an anoxic environment formation of lactate is favored over acetate. Yet in the presence of a microoxic environment, acetate production shifts to favor acetate over lactate [31]. The microoxic oxygen ring that forms on the inner lumen of the hindgut provides an ideal habitat where this may take place. In addition, lactate consumed directly by the termite or produced as a by-product of lignocellulose degradation is broken down into acetate and carbon dioxide in equimolar ratios, of which the acetate is used by *R. flavipes* [26, 20]. Once again demonstrating how important acetogenesis is to the survival of the termite. Formate, although present in smaller concentration, also metabolizes down into acetate and CO_2 [26, 32]. It has been shown that when hindguts were injected with labeled formate it was oxidized to CO_2 but not as well as lactate or acetate [26]. Acetate, lactate, hydrogen and other metabolites are formed within the hindgut of the *R. flavipes* and create an integrated food web between the bacteria that reside there.

1.3 The Phylum *Verrucomicrobium*

It is in the outer region of 2-5% oxygen concentration that *Verrucomicrobium* sp. strain TAV2 is believed to reside. Originally the contents of a *Reticulitermes flavipes* termite worker were removed and its contents plated to isolate microorganisms. Five strains of the phylum *Verrucomicrobium* sp. were isolated from plates incubated under 2% oxygen in a microoxic chamber [32].

Since being designated a phyla in 1987, new members of *Verrucomicrobia* have been discovered in rice paddies, soils, aquatic habitats as well as symbionts with multiple species of animals including humans [33, 34, 35]. Since then, the phylum has been divided into four genera and one proposed genera. The genus *Verrucomicrobium* contains only one known species, *Verrucomicrobium spinosum,* and is described as a heterotrophic bacterium that possess bundles of fimbriae which extend from the tips of the prosthecae [35]. The *Verrucomicrobium* phylum derives its name from this species. Genus *Prosthecobacter* contain nonmotile fusiform-shaped bacteria and have only a single polar prostheca [35]. Species in this genera are the only known bacteria so far to contain the genes *btuba* and *btubb*, which are homologs for α- and β- tubulin genes [36]. These genera, the *Verrucomicrobiae* and *Prosthecobacter,* have appendages that extrude from their bodies which are thought to increase the surface to volume ratio of the cell thus improving the ability to uptake nutrients [36, 35]. Additionally, these bacteria replicate either by binary fission or by unequal cell division [35]. The genus *Opitutus* has been isolated from rice paddy soil and unlike the *Verrucomicrobium* and *Prosthecobacter* genera because they do not possess prosthecae [37, 38]. They appear spherical in shape and are most commonly found as diplococci. The last characterized genus of the phylum *Verrucomicrobium* is the genus *Victivallis*. This genus contains one species *V. vadensis* that was isolated from human feces [39]. Additionally, there is a candidate genus *Candidatus xiphinematobacter* which is proposed to include three species that were discovered to reside in the ovarial epithelial cells of the nematode, genus *Xiphinema* [40]. These cells are

rod-shaped, non-motile, and are non-prosthecate [40]. The bacteria present in the phylum *Verrucomicrobium* are diverse and possess many interesting characteristics that vary between genus groups.

The bacteria present in the *Verrucomicrobium* phylum are diverse and distinctive, but interestingly, when compared to other bacteria phyla, some posses similar characteristics such as cell wall makeup, DNA similarities, and unique cell compartments. In recent years there has been debate whether *Verrucomicrobia* should be grouped into a superphyla containing *Planctomycetes, Chlamydiae*, *Poribacteria, Lentispaerae,* and the OP3 candidate phylum [9].

Although bootstrap values have been found to be poor among the groups, biological processes and structural similarities aid in linking these bacteria together [9]. Similarities include a lack of the bacterial homologue FtsZ in members of *Chlamydiae*, *Planctomycetes* and *Verrucomicrobia* [41]. Instead it appears that these bacteria have a homologue similar to the eukaryotic protein tubulin [41]. Comparisons between members of *Verrucomicrobia* and *Chlamydiae* also reveal that there are shared rare genomic changes such as conserved inserts or deletions that are exclusive from all other bacteria [42]. This information therefore suggests that *Verrucomicrobia* is the closest known relative to *Chlamydiae* [42].

Cell compartmentalization links *Verrucomicrobia* to the superphlya in another unique manner. Members of *Verrucomicrobia* and *Planctomycetes* were found to possess a condensed nucleoid and ribosome like particles encased by an intracytoplasmic membrane [43]. This intracytoplasmic membrane, termed a “pirellulosome” in *Planctomycetes*, has now only been discovered in these two phyla’ and is characterized by compartmentalization of the cytoplasm by a major cell organelle bound by a single membrane which contains the DNA as a fibrillar condensed nucleoid, in addition to ribosome-like particles [43]. Interestingly, *Planctomycetes* and *Verrucomicrobium* also have been detected in similar environments by RFLP (restriction fragment length polymorphisms) within the termite gut of *Coptotermes formosanus* [34].

Bacteria in the phylum *Verrucomicrobium* are described as Gram-negative and can occur as either coccoid or rod-shaped, contain diaminopimelic acid and are sensitive to ampicillin. They replicate by binary fission or unequal cell division [35, 36]. While found in numerous natural soil and aquatic habitats, they also are associated with symbiosis within lower orders of termite hindguts.

The Termite Associated *Verrucomicrobia* (TAV) strains were isolated from the hindgut of *Reticulitermis flavipes* termite workers collected near Dansville, Michigan. These strains all belong to subdivision 4, *Opitutus.* Their nearest cultivated relative is the species *Opitutus terrae* strain PB90-1 which was isolated from anoxic rice paddy soil and has the ability to hydrolyze cellobiose [44, 37]. Also belonging to the *Opitutus* subdivision, "ultramicrobacteria" strains VeCb1, VeGlc2, and VeSm13 were isolated from anoxic rice paddy soil. These strains have the ability to utilize some sugar polymers released during the degradation of plant polymers such as xylan, starch, and pectin [38, 44, 37]. The above strains are known for their oxygen tolerance. This could be an opportunistic advantage as rice paddies are flooded and anoxic during the growing season, but once drained they can become aerobic after the rice is harvested [38]. In light that members of the same order degrade cellulose and/or its catabolized by-products in changing aerobic conditions, further studies should be performed to verify whether microorganisms belonging to this subdivision can degrade plant material under varying oxic conditions.

Five separate strains of Termite Associated *Verrucomicrobium* (TAV 1-5) were Isolated from hindgut of the wood-consuming termite *Reticulitermis flavipes*. The termite guts from *Reticulitermis flavipes* larvae workers were extracted under 2% hypoxic conditions and plated. As stated, these strains have been classified into the *Opitutus* subdivision within the phylum *Verrucomicrobium*. Based on the 16S rRNA gene, it has been found that strains TAV-3 and TAV-4 are the closest related strains with 99.9% identity. Next, TAV-2 shares 99.6% identity relatedness to these two strains. The strain TAV-5 has 97.5% shared identity. Lastly, TAV-1

shares 95.3-95.8% similarity to the above strains. Among these five strains, TAV-2 was chosen to be studied the most thoroughly. TAV-2 form small (<0.5 mm diameter), white, round colonies with cells themselves of 0.25-0.5 µm in diameter and found mostly in pairs [32]. TAV-2 has its genetic sequence established and thus far has been found to have 5.2 Mb nucleotides with 60.7% GC content. This sequence revealed approximately 4,827 coding gene sequences and of these 4,036 protein coding genes, yet a mystery remains as to which genes are activated under varying environmental conditions and how strongly they are activated in comparison to other genes (http://genome.ornl.gov/microbial/verr/). It has been shown that *Verrucomicrobium* species have been found in environments important in plant material degradation. Members of their genus have also been found to degrade a variety of polysaccharides. Since TAV-2 is thought to reside in a termite's hindgut at microaerophilic conditions (2% O_2), we can compare and contrast the gene expression profile under controlled oxygenic conditions. A profile of their metabolism and oxygen degradation characteristics can be observed through gene profiling with microarray data.

CHAPTER 2

COMPARING THE WHOLE CELL TRANSCRIPTIONAL PROFILES OF THE *VERRUCOMICROBIUM* SP. STRAIN TAV2 GROWN UNDER TWO DIFFERENT OXYGEN CONCENTRATIONS

2.1 Introduction

As the search for renewable energy continues, scientists are looking to new areas for ideas. One such possibility is the use of microorganisms to convert plant biomass into useable energy such as ethanol. Termites contain microbial symbionts which aid them in extracting usable nutrients from plant biomass [8]. Hindguts of termites are thought to carry over 270 different phylotypes of microorganisms [7]. A bacterium of interest isolated from inside the termite hindgut is the Termite Associated *Verrucomicrobium* (TAV). Although the genome has been sequenced, the genes that control the microorganism's ability to turn plant material into useable energy are open to regulation depending on the environmental conditions. Termite hindguts range from anoxic conditions to low oxygen concentrations of 2% [25]. By comparing the expressed transcriptional profile of strain TAV-2 under two specific oxygenic conditions, we observed genes which are up and down regulated. After isolating total RNA from the strain TAV-2, enriching the mRNA, making a cDNA library, and hybridizing it to a microarray chip, TAV-2 cells grown under 2% oxygen concentrations displayed a different transcriptional profile than cells grown under 20% oxygen.

Microarrays have been used for years to assess transcriptional variation under different circumstances both in eukaryotic and prokaryotic organisms. Specific experiments involving microarrays have been used to study whole genome profiling of bacteria when grown autotrophically vs. heterotrophically as well as specific gene regulation dealing with pathogenicity when oxygen concentrations have been varied [45, 46]. Eukaryotic examples

include studies on changes in ovarian cancer cells with a microarray platform [47]. When applied, microarrays can distinguish genetic expression variability between species as well as within a particular organism exposed to varying conditions. Studies have shown that microarray data can differentiate genes that are being expressed under different conditions effectively and provide sound results when appropriate statistical analysis and normalization is applied.

2.2 Materials and Methods

2.2.1. MYcroarray slide format

To compare the transcriptional profile of *Verrucomicrobium* TAV2 grown under two different oxygen concentrations a microarray platform was used. Custom glass slides spotted with 40,000 45-62mer oligonucleotide primers were used from MYcroarray (Michigan, USA). The synthesis of the slides consist of MYcroarray using phosphoramidite chemistry assembling one nucleotide at a time directly onto the slide with the terminal end of the growing strand treated with a photo-generated acid. 11,552 probes were triplicated on the slide which corresponded to 3,775 genes, or about 92% of the genome.

2.2.2. RNA Extraction and Isolation

TAV-2 cells were grown in 250mL flask containing 100mL of R2B liquid medium to an OD_{600} between 0.298 and 0.317 for five days at a temperature of 25℃. Cells were grown under 2% and 20% controlled O_2 conditions in a hypoxic chamber. Total RNA was isolated using recommendations provided with the Ribo-Pure Bacteria kit (Ambion Inc. Austin, TX, Cat #AM1925). mRNA samples were purified using the protocol set forth by MICROBExpress kit (Ambion Inc., Austin, TX, Cat #AM1905). Samples were stored at -80℃ until use.

2.2.3. cDNA synthesis and aRNA labeling

mRNA was synthesized into cDNA, and then converted into aRNA using the MessageAmp II-Bacteria Prokaryotic RNA Kit (Ambion Inc. Austin, TX, Cat #AM1790). The nucleotides added for assembly of the aRNA contain 50% unmodified UTP's and 50% modified 5-(3-aminoallyl)-UTP's (Ambion Inc. Austin, TX, Cat #AM8437). Steps in amplification follow

protocol of the Ambion amino-allyl master kit calculator (http://probes.invitrogen.com/resources/calc/basedyeratio.html). aRNA samples for TAV-2 cells, grown under 2% and 20% O_2 conditions, were labeled with different color fluorescent tags, Alexafluor 555 and Alexafluor 647, respectively. Labeling of the aRNA followed protocol according to Alexa Fluor Reactive Dye Decapacks for Microarray Applications (Molecular Probes, Inc. Eugene, OR, Cat #A32755). Excess dye was removed using the RNeasy Mini Kit (Qiagen, Valencia, CA, Cat #74104). After removing the excess dye, the aRNA was vacuum centrifuged and stored at -20℃ until use.

2.2.4. Hybridization

Microarray slides were incubated and prepared in prehybridization solution and washed with nuclease free water. The aRNA were resuspended with 1X hybridization solution. All pre-hybridization, hybridization, and post hybridization solutions were assembled according to protocol provided by Dr. Woo-Suk Chang, UTA (personal communication) with some changes made in the hybridization protocol (see supplemental material). The aRNA was incubated for 3 min at 95℃ and immediately placed on ice. Samples containing equal concentrations of labeled aRNA from TAV-2 grown under 2% and 20% O_2 were added to the microarray slides and then the slides were placed in a hybridization chamber. The chamber was wrapped in aluminum foil and placed in a 50℃ water bath and incubated for 17 h. After this time, the slides are removed and washed with post hybridization buffer to remove any non-specific binding that could have occurred.

2.2.5. Data Analysis

Three biological replicates were used for each condition. In addition, three technical replicates were included to verify consistent readings [45]. Concurrently a dye swap was preformed to ensure that one dye does not preferentially bind to the aRNA stronger than another dye. Controls involving a non-coding nonsense positive strand as well as a negative nonsense strand were also applied to verify that stringent hybridization techniques were

followed. Hybridized slides were scanned using a 4200a GenePix laser scanning for Alexafluor 555 and Alexafluor 647. From the GenePix scanner, slide data was input into the GeneSpring 11 microarray analyzing software. The data was normalized using Lowess intensity-dependent normalization both between chips and between genes. From here normalized data was filtered on a 20-100% expression ratio, making sure only spots showing over 20% intensity above the background noise were included. Data was filtered on error minus the coefficient of variation >= 50.0%. Since there are multiple spots on each array for a single gene, the replicate spots are averaged across the slide. This correction excludes genes that were considered up or down regulated but their spots across the slide varied more than 50%. Lastly, statistical analysis was performed using the Benjamini-Hochberg method for multiple testing across the slides. This involved a t-test against zero with a corrected p-value <= 0.05. Each slide was given the same weight in the experiment because all slides are replicates of a given comparison of conditions, whether biological or technical.

2.2.6. Real Time PCR

To confirm microarray data, real time-PCR was carried out for a selected group of genes. Primers were designed for five genes that are consistently expressed. RT-PCR was performed using the 7300 RT-PCR System (Applied Biosystems Foster City, CA). The RT-PCR was done on previously isolated aRNA samples from cells grown under 2% and 20% O_2 following Ambion's tech notes 14(1) (http://www.ambion.com/techlib/tn/141/10.html). Forward and reverse primers were constructed using the Integrated DNA Technologies website (http://www.idtdna.com/Scitools/Applications/RealTimePCR/). Genes selected, gene function, primer sequence, annealing temperature, and oxygen concentration under which each gene is up-regulated is shown in Table 2.1. See Appendix B for specific concentrations of RT-PCR mixture and cycle information. Triplicate reactions were performed on each gene under a three dilution configuration. Each replicate had three concentrations of aRNA, 2 µL, 1 µL, and 0.5 µL, analyzed for cycle threshold (Ct) values. Values measured were calculated at an aRNA

concentration of 1µL for all genes. After applying an unpaired t-test to triplicate samples, microarray results were verified (Figure 2.6). When observing the housekeeping gene expression, median values of three biological replicates were taken and analyzed using an unpaired student t-test.

Table 2.1 Genes chosen to perform RT-PCR to verify microarray results.

Gene ID	Gene Function	Accession #	Oxygen Concentration Up-Regulated	Primer (forward/reverse)	Annealing Temp
ObacDRAFT_0946	xylose isomerase domain protein TIM barrel	ZP_02013205	20%	ACTTCGGACCATTCTTGCG	58
				GGATCACCTGCAAACCTTG	58
ObacDRAFT_1002	ATP synthase F1, alpha subunit	ZP_02011249	20%	TTCAACCCCATTCCCATCG	58
				ACTTTCTTGATGTCGAGGCC	60
ObacDRAFT_1722	Malate/L-Lactate dehydrogenase	ZP_02013686	2%	ATACATCGCCTACACCAACTG	62
				TGACGATGGGATAACCAATGG	62
ObacDRAFT_2549	DNA Polymerase III Beta Subunit	ZP_02011517	2%	AATGTAGTCCTTTTCCGCCTC	60
				ATCACTTCAGCAACGGACTC	60
ObacDRAFT_1087	DNA Primase	ZP_02011968	housekeeping	GACCATGACGAAGGGCTG	60
				GTTAATTCCACCGAGACCCC	60

2.3 Results

2.3.1. Cell growth and RNA extraction

Growth curves for TAV2 cells grown in R2B medium under different O_2 concentrations demonstrated that the average doubling time of the cells grown under 2% oxygen was 26.3 hours with a growth rate (μ) of 0.026h^{-1} and the average doubling time of cells grown under 20% oxygen was 34.2 hours with a growth rate (μ) of 0.020h^{-1} (Figure 2.1 and 2.2). Cells were grown in R2B medium and harvested at OD_{600} of 0.298-0.317 (average of 0.3). This was determined to be in mid-exponential growth phase.

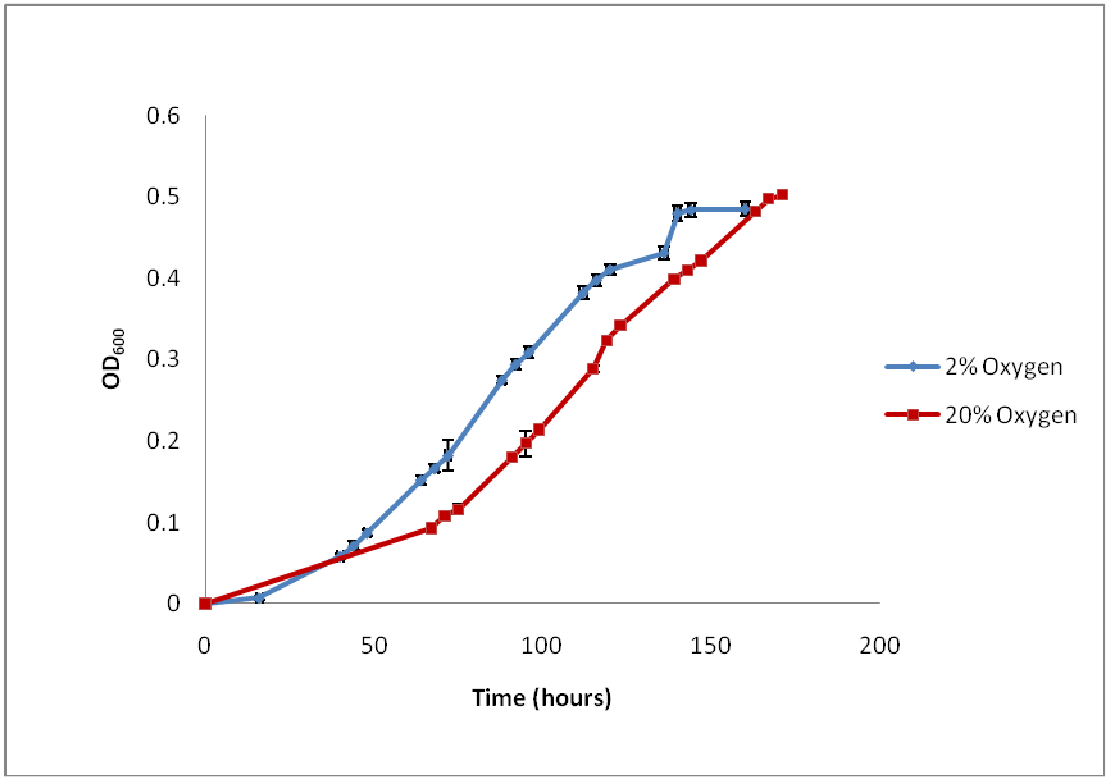

Figure 2.1 Growth curves of the *Verrucomicrobium* sp. strain TAV2 cells grown under two contrasting oxygen concentrations (2% and 20%). Where error bars are not shown, the standard errors from triplicates are smaller than symbols.

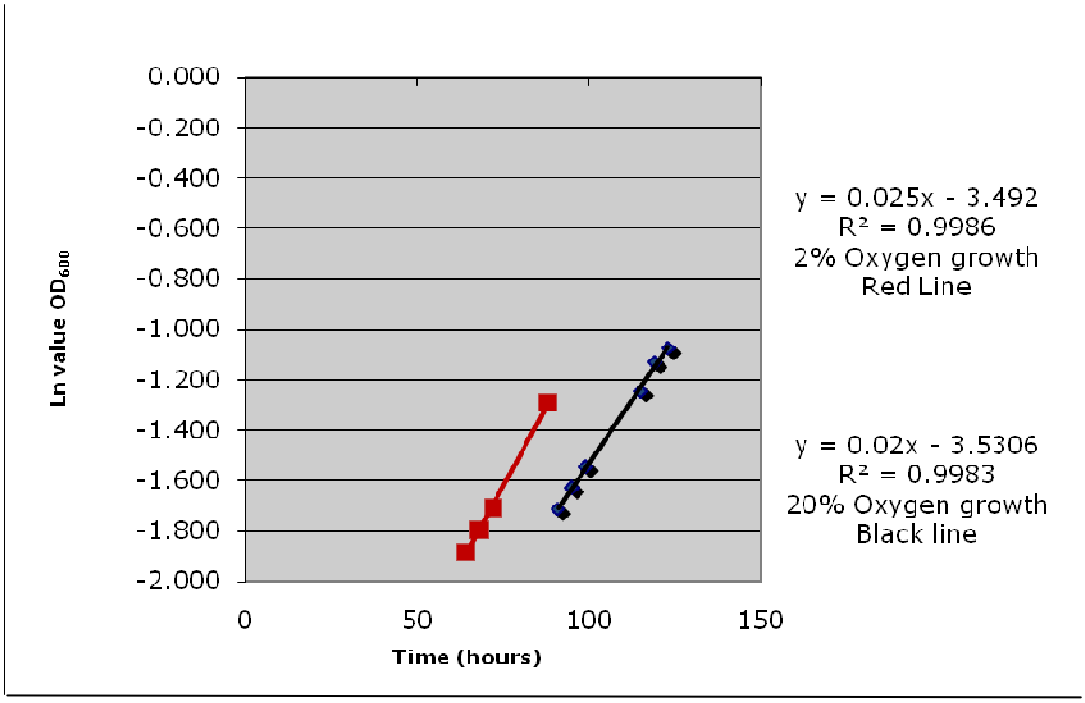

Figure 2.2 Natural log values of exponential growth of the *Verrucomicrobium* sp. strain TAV2 cells grown under two contrasting oxygen concentrations (2% and 20%).

Cells had their total RNA extracted, and mRNA was enriched. After enrichment, approximately 11 to 18% of the total RNA was mRNA that could be used for further manipulation.

2.3.2 Microarray Analysis

75 genes were significantly up-regulated between the TAV2 cells, when TAV2 was grown under 2% and 20% oxygen conditions and a p-value cutoff of 0.05 was applied (Figure 2.3). Of these, 47 genes were up-regulated when cells were grown under 20% oxygen and 26 genes were up-regulated when cells were grown under 2% oxygen condition (Tables 2.2 and 2.3).

Further statistical analysis using a 2.0 fold change cut off value rendered 69 genes as significantly up-regulated between the two growth conditions. Significantly up-regulated in the 20% oxygen concentration were 46 genes, and significantly up-regulated in the 2% oxygen concentration were 23 genes. The 6 genes that were excluded when a fold change of 2.0 was

applied are: ObacDRAFT_0185 and ObacDRAFT_0725 for 2% oxygen concentration and ObacDRAFT_1476, ObacDRAFT_2054, ObacDRAFT_2308 and ObacDRAFT_3094 for 20% oxygen concentration.

Additional investigation was performed using a clustering application on Genespring 11. After averaging the technical replicates together into biological samples from both dye combinations, Hierarchal, Centroid clustering with Euclidian distance analysis was applied. Normalized expression ratios between the two colors were assembled into a tree where similar items are joined by very short branches, and as their similarity decreases the branches elongate (Figure 2.4).

Table 2.2 Genes up-regulated when cells are grown under 20% oxygen and Benjamini-Hochberg analysis using a t-test against zero with a corrected p-value <= 0.05 is applied

Expressed genes when grown under 20% oxygen	
Name	**Gene Product**
ObacDRAFT_0125	Hypothetical Protein
ObacDRAFT_0185	Hypothetical Protein
ObacDRAFT_0260	Hypothetical Protein
ObacDRAFT_0441	Hypothetical Protein
ObacDRAFT_0725	Protein Of Unknown Function DUF323
ObacDRAFT_0741	Hypothetical Protein
ObacDRAFT_0946	Xylose Isomerase Domain Protein TIM Barrel
ObacDRAFT_0997	Putative Methyltransferase
ObacDRAFT_1002	ATP Synthase F1, Alpha Subunit
ObacDRAFT_1211	Hypothetical Protein
ObacDRAFT_1220	Phosphopantetheine-Binding
ObacDRAFT_1312	Hypothetical Protein
ObacDRAFT_1498	Lysyl-tRNA Synthetase
ObacDRAFT_1822	Hypothetical Protein
ObacDRAFT_1841	Hypothetical Protein
ObacDRAFT_1876	Uncharacterized Protein-Like Protein
ObacDRAFT_1900	Alpha-L-Arabinofuranosidase-Like Protein
ObacDRAFT_1903	Regulatory Protein Gntr HTH
ObacDRAFT_2061	Hypothetical Protein
ObacDRAFT_2096	D-Isomer Specific 2-Hydroxyacid Dehydrogenase
ObacDRAFT_2137	Conserved Hypothetical Protein
ObacDRAFT_2168	TPR Repeat-Containing Protein
ObacDRAFT_2172	Na+/Solute Symporter
ObacDRAFT_2179	Putative Secreted Protein, Putative Xanthan
ObacDRAFT_2291	Hypothetical Protein
ObacDRAFT_2305	Putative Issod13, Transposase
ObacDRAFT_2379	Hypothetical Protein
ObacDRAFT_2383	Hypothetical Protein

Table 2.2 - *continued*

ObacDRAFT_2387	Hypothetical Protein
ObacDRAFT_2582	Tyrosyl-rRNA Synthetase
ObacDRAFT_2782	Beta-Lactamase Domain Protein
ObacDRAFT_2915	Type III Secretion Exporter
ObacDRAFT_3119	Hypothetical Protein
ObacDRAFT_3322	Glutamate Dehydrogenase (NADP(+))
ObacDRAFT_3328	Transposase IS4 Family Protein
ObacDRAFT_3534	Protein Of Unknown Function DUF433
ObacDRAFT_3548	Pyridine Nucleotide-Disulphide Oxidoreductase
ObacDRAFT_3553	Conserved Hypothetical Protein
ObacDRAFT_3557	Endoribonuclease L-PSP
ObacDRAFT_3616	Sigma 54 Modulation Protein/Ribosomal Protein
ObacDRAFT_3703	AMP-Dependent Synthetase And Ligase
ObacDRAFT_3854	Alcohol Dehydrogenase Zinc-Binding Domain
ObacDRAFT_3927	Alpha/Beta Hydrolase Fold-3 Domain Protein
ObacDRAFT_3972	Hypothetical Protein
ObacDRAFT_3995	ATP-Dependent DNA Helicase RecQ
ObacDRAFT_4060	Heat Shock Protein Hsp20
ObacDRAFT_4142	Hypothetical Protein
ObacDRAFT_4143	Hypothetical Protein

Table 2.3 Genes up-regulated when cells are grown under 2% oxygen and Benjamini-Hochberg analysis using a t-test against zero with a corrected p-value <= 0.05 is applied.

Expressed genes when grown under 2% oxygen	
ObacDRAFT_0630	CRISPR-Associated Protein Cas1
ObacDRAFT_0784	Exopolysaccharide Synthesis ExoD
ObacDRAFT_0818	Protein Of Unknown Function DUF1458
ObacDRAFT_0880	Na+/Solute Symporter
ObacDRAFT_0988	Helix-Turn-Helix- Domain Containing Protein AraC
ObacDRAFT_1223	Methyltransferase Type 12
ObacDRAFT_1408	DoxX Family Protein
ObacDRAFT_1476	Urease, Beta Subunit
ObacDRAFT_1722	Malate/L-Lactate Dehydrogenase
ObacDRAFT_1803	Hypothetical Protein
ObacDRAFT_2054	Hypothetical Protein
ObacDRAFT_2308	Protein Of Unknown Function DUF34
ObacDRAFT_2443	Regulatory Protein LacI
ObacDRAFT_2549	DNA Polymerase III, Beta Subunit
ObacDRAFT_2810	Efflux Transporter, RND Family, MFP Subunit
ObacDRAFT_2828	Putative Chew Protein
ObacDRAFT_2969	ATPase With Chaperone Activity ATP-Binding
ObacDRAFT_3094	Hypothetical Protein
ObacDRAFT_3116	Hypothetical Protein
ObacDRAFT_3430	Hypothetical Protein
ObacDRAFT_3432	Hypothetical Protein
ObacDRAFT_3526	Dihydropteroate Synthase
ObacDRAFT_3556	6-Phosphogluconate Dehydrogenase NAD-Binding
ObacDRAFT_3861	Radical SAM Domain Protein

Table 2.3 - *continued*

ObacDRAFT_3914	RNA Polymerase, Sigma-24 Subunit, ECF Subfamily
ObacDRAFT_4053	Short-Chain Dehydrogenase/Reductase SDR
ObacDRAFT_4102	Hypothetical Protein

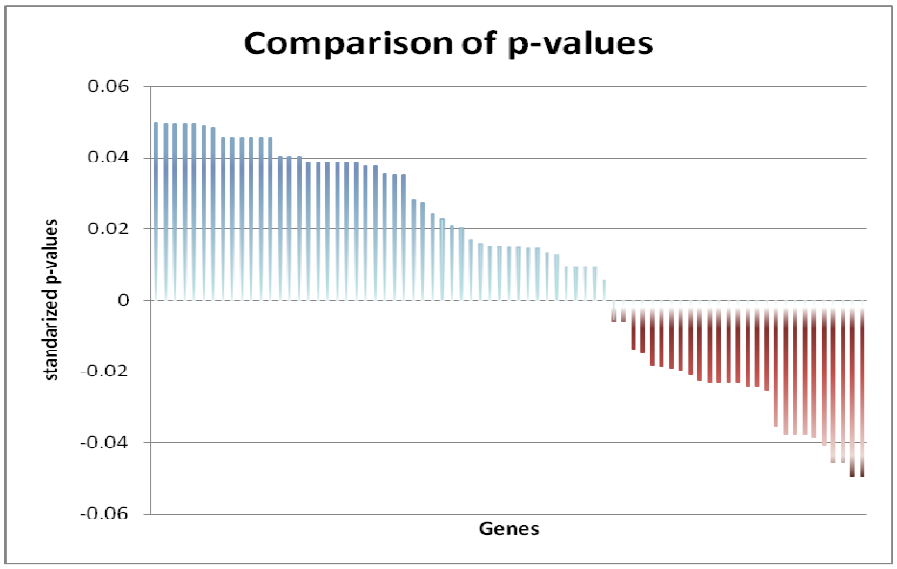

Figure 2.3 Graphical representation of significantly up-regulated p-values with a cut-off of p<=0.05. Blue (positive values) represent genes expressed when grown in 20% oxygen while red (negative values) represent genes expressed when grown in 2% oxygen.

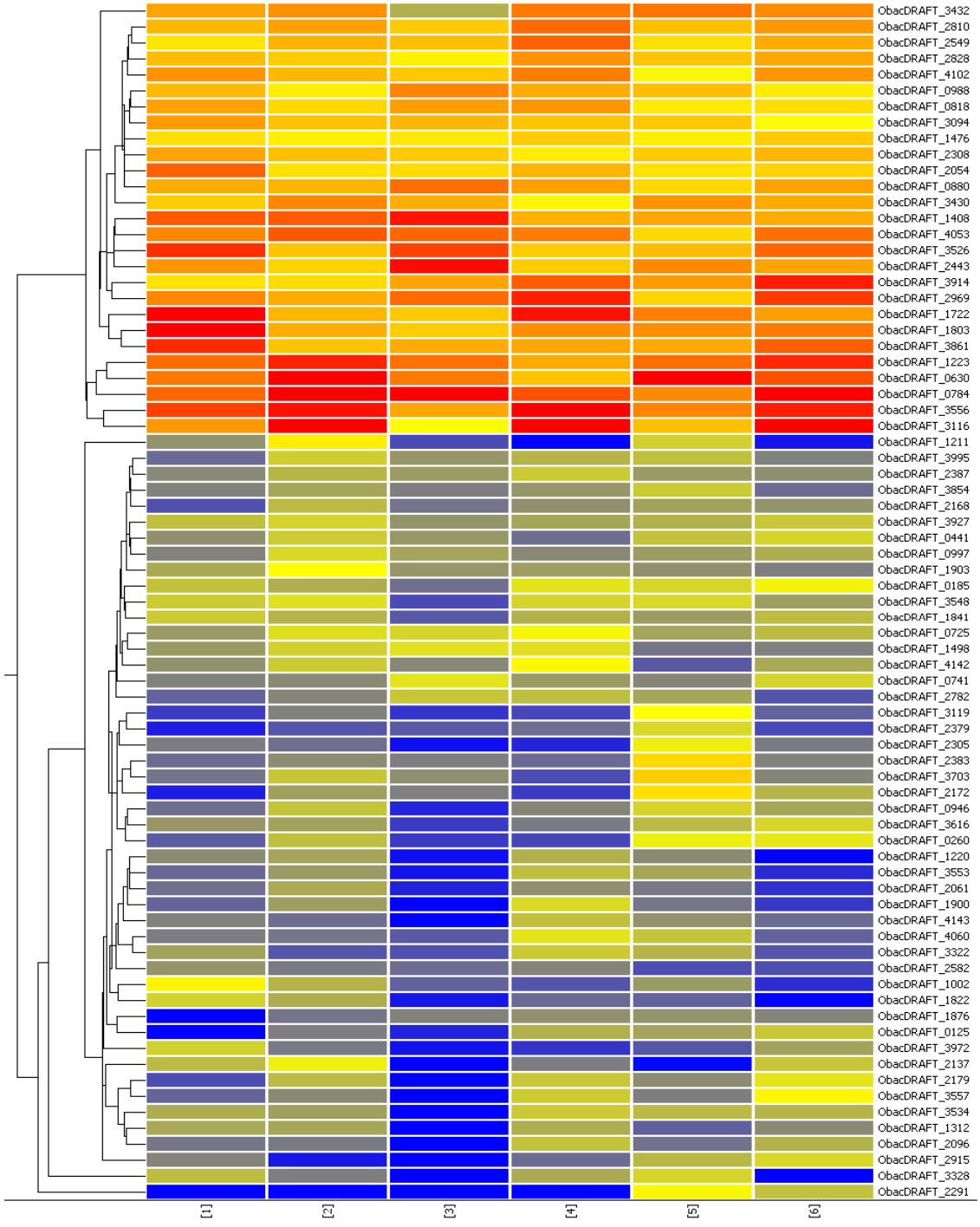

Figure 2.4 Cluster analyses of the 75 up-regulated genes using Hierarchal Centroid clustering method with Euclidian distance. Genes up-regulated in 2% oxygen concentration are red while genes up-regulated in 20% are blue. Equal expression between the two conditions are yellow. Each oxygen condition clustered into two main branches.

Up-regulated genes had different cell functions that ranged from DNA repair to carbohydrate transport and metabolism. Significantly expressed genes were organized according to COG (cluster of orthologous groups) categories to allow for comparisons of cell functions that were affected the greatest when grown under different oxygen concentrations (Figure 2.5). COG groups M and T each had one gene representative and were only up-regulated when grown under 2% oxygen. These systems pertain to the cell envelope biogenesis and outer membrane, and signal transduction mechanisms respectively. COG groups J, N, G, and Q were solely up-regulated under 20% oxygen. These are J - translation, ribosomal structure and biogenesis, N - Cell motility and secretion, G - carbohydrate transport and metabolism, and Q - Secondary metabolites biosynthesis, transport and catabolism. Of these group J had the most genes up-regulated with 4 genes being expressed when cells were grown under 20% oxygen. In addition, certain groups were left unaffected when cells were grown under either condition, such as groups D - cell division and chromosome partitioning, P - inorganic ion transport and metabolism, and F - Nucleotide transport and metabolism. Certain COG groups had an equal number of genes affected by the oxygen concentrations when comparing the number of up-regulated genes. These groups are L - DNA replication, recombination and repair, O - posttranslational modification, protein turnover, chaperones, and H - coenzyme metabolism. The cell system that was most affected under each oxygen condition was group S which contains genes with unknown functions. Cells grown in 20% oxygen had many more hypothetical genes affected when compared to the amount of hypothetical genes up-regulated from cells grown under 2% oxygen, 26 and 10 respectively. Yet group R genes, which contain genes whose general function can only be predicted, had a greater number of genes up-regulated when cells were grown under 2%(4) verses 20%(2).

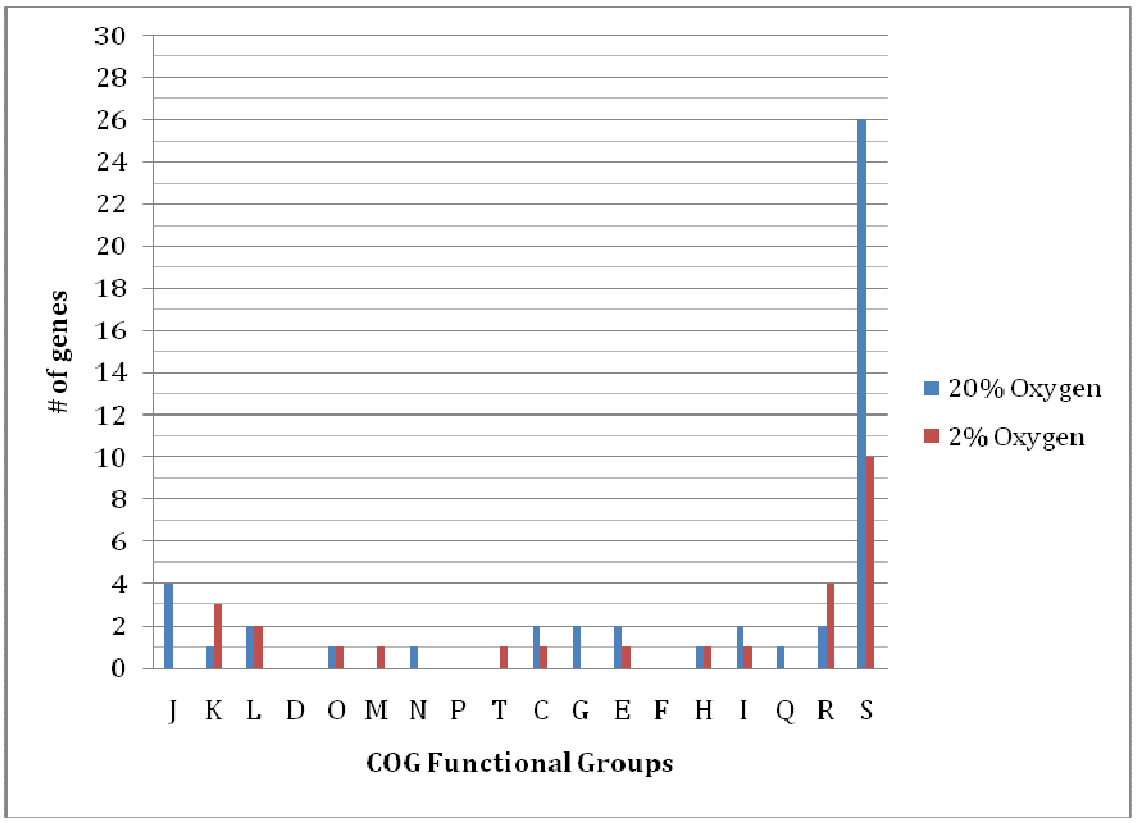

Figure 2.5 Graphical representation of a comparison between the number of up-regulated genes in their respective COG (cluster of orthologous groups of proteins) of cells grown at 2% oxygen and 20% oxygen.

2.3.3 RT-PCR

Of the 75 genes that were up-regulated between the two growth conditions, five genes were selected to verify the microarray data through real-time PCR. Two genes were selected from the group up-regulated in 20% oxygen, two genes were selected from the group up-regulated in 2% oxygen and one gene was selected as a housekeeping gene (Table 2.1). Real-time PCR Ct, cycle threshold, values were collected from the five genes examined. Unfortunately the difference in expression of gene ObacDRAFT1722 with 1 µL of aRNA was not statistically significantly with a Ct value of 24.24 for 2% and a Ct value of 24.33 for 20%. However, averaged Ct values when aRNA concentrations were 0.5µL was 1.11 cycles sooner in cells grown under 2% oxygen, and when aRNA concentrations were 2 µL the Ct value was

1.03 cycles sooner in cells grown under 2% oxygen. It was found that for ObacDRAFT0946 and ObacDRAFT1002 averaged Ct values were lower in cells grown under 20% oxygen conditions. ObacDRAFT0946 had a Ct value of 22.47 when cells were grown under 20% oxygen and a Ct value of 25.50 when grown under 2%. ObacDRAFT1002 had a Ct value of 21.95 when cells were grown under 20% and a Ct value of 24.06 when grown under 2%. ObacDRAFT2549 had a lower Ct value in TAV2 cells grown in 2% oxygen, 16.99, than when cells were grown under 20% oxygen, 18.51. When evaluating the housekeeping gene, aRNA from three biological replicates was analyzed. The housekeeping gene had no statistically significant difference between the level of expression in cells grown in 2% and 20% oxygen concentrations, 23.48 and 23.49 respectively.

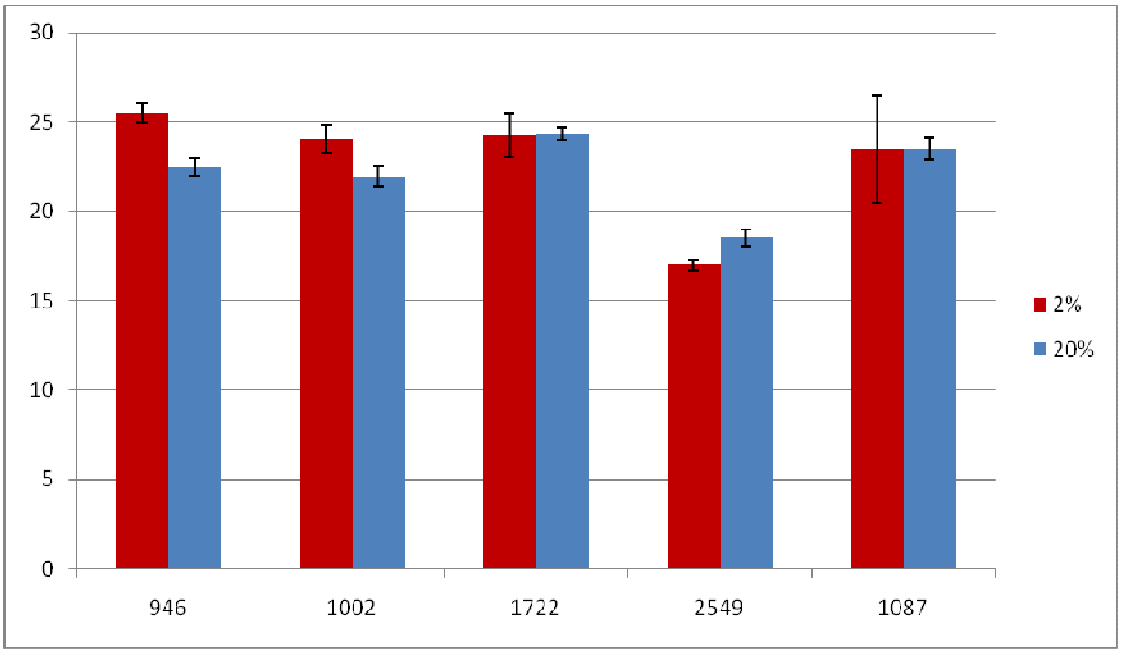

Figure 2.6 Graphical representation of RT-PCR Ct value comparisons between cells grown under 2% and 20% oxygen. X axis values correspond to genes selected from Table 1. Y axis values designate Ct values

Table 2.4 RT-PCR statistical data. Ct values are triplicate replicates in three dilution series

	CT Value	CT Value	CT Value	CT Value	CT Value	CT Value	Statistical work
	2%	2%	2%	20%	20%	20%	two tailed p value of 0.0019
ObacDRAFT0946	2 uL	1uL	0.5uL	2 uL	1uL	0.5uL	very significant
1	23.4	25.16	26.28	21.09	23.09	24.44	t=7.2376
2	24.44	25.28	26.75	20.96	22.19	24.11	df=4
3	23.04	26.06	24.67	21.2	22.14	21.68	sd error of difference 0.418
Avg	23.62667	25.5	25.9	21.08333	22.47333	23.41	
St dev	0.727003	0.488672	1.090825	0.120139	0.534634	1.507282	
ObacDRAFT1002	2 uL	1uL	0.5uL	2 uL	1uL	0.5uL	two tailed p value of 0.0099
1	23.51	24.58	25.25	20.39	21.05	22.8	very significant
2	24.02	24.19	25.07	20.34	22.26	23.11	t=4.6135
3	23.33	23.41	23.89	22.06	22.55	23.09	df=4
Avg	23.62	24.06	24.73667	20.93	21.95333	23	sd error of difference 0.5
St dev	0.357911	0.595735	0.738738	0.978928	0.795634	0.173494	
ObacDRAFT1722	2 uL	1uL	0.5uL	2 uL	1uL	0.5uL	two tailed p value of .2060
1	24.07	24.11	23.88	23.17	23.98	24.23	not statistically significant
2	22.11	24.12	24.57	23.13	24.73	26.66	t=0.3698
3	20.73	24.5	24.26	23.71	24.3	25.15	df=4
Avg	22.30333	24.24333	24.23667	23.33667	24.33667	25.34667	sd error of difference 0.252
St dev	1.678372	0.222336	0.345591	0.323934	0.376342	1.22688	
ObacDRAFT2549	2 uL	1uL	0.5uL	2 uL	1uL	0.5uL	two tailed p value of .0094
1	16.74	16.93	19.17	16.78	18.18	19.82	very significant
2	16.72	16.55	19.23	17.26	18.61	19.42	t=4.6840
3	16.47	17.5	18.85	18.26	18.74	19.64	df=4
Avg	16.64333	16.99333	19.08333	17.43333	18.51	19.62667	sd error of difference 0.324
St dev	0.150444	0.478156	0.204287	0.755072	0.293087	0.200333	
ObacDRAFT1087	2 uL	1uL	0.5uL	2 uL	1uL	0.5uL	two tailed p value of .9972
1	22.65	23.85	24.97	20.94	22.14	22.8	not statistically significant
2	22.71	23.8	24.77	23.62	26.96	26.52	t=0.0037
3	21.5	22.8	23.93	19.18	21.37	23.06	df=4
Avg	22.28667	23.48333	24.55667	21.24667	23.49	24.12667	sd error of difference 1.782
St dev	0.681934	0.592312	0.551845	2.235829	3.02967	2.07676	

2.4 Discussion

The *Verrucomicrobium* sp. strain TAV2 is autochthonous to the *R. flavipes* termite hindgut. Therefore, it is subject to the environmental conditions present inside of the hindgut structure. One such condition is the low partial pressure of oxygen present at the interior surface of the gut wall [25, 10]. My experimental work began by testing the growth response of strain TAV2 when subjected to contrasting oxygen concentrations, 2% and 20%. Results indicated that the cell replication rates varied according to the oxygen concentration (Figure 2.1). When the *Verrucomicrobium* sp. strain TAV2 was grown under 20% oxygen, cells took approximately 91 hours to reach exponential phase. In contrast, when cells were grown under 2% oxygen, they were able to reach exponential phase in approximately 64 hours. These results suggests that the TAV2 isolate is adapted to low oxygen concentrations, strengthening the argument that cells of this microorganism are most likely to reside in the outer edges of the termite *R. flavipes* paunch.

Microaerophilic bacteria provide substantial resource turnover by residing in unique niches at aerobic/anaerobic interfaces. Methanogens found in the hindgut of termites were originally thought to be completely anaerobic bacteria, but studies have shown that the bacteria are thought to reside on the outer microaerophilic regions within the hindgut [28]. Obviously there is still much to be learned about the interactions that occur within the microoxic periphery of the termite hindgut. TAV2 is thought to reside in this microaerophilic region of the termite hindgut due to its isolation when *R.flavipes* hindgut contents were incubated in a 2% oxygen chamber [32]. Other studies strengthen this possibility because related species of *Verrucomicrobium* have been found in microaerophilic to anoxic habitats such as rice paddy fields [44, 37].

Numerous bacteria are classified as microaerophilic or anaerobic due to their cellular components being damaged or inhibited by oxygen. Many microorganism metabolisms cannot tolerate oxygen because they involve enzymes which react with oxidants [48]. Additionally,

cells which utilize low-potential substrates such as iron or sulphur have difficulty manipulating these elements once they have reacted with oxygen. For example, ferric iron is poorly soluble and difficult to use by microorganisms, but ferrous iron is widely used as a electron donor for cell metabolisms [48, 30]. Oxygen can also have a toxic effect on some microorganisms by inhibiting enzymatic functions such as monoxide (CO) dehydrogenase and nitrate reductase [30]. In fact obligate anaerobes cannot live in oxygen environments because many rely on low-potential flavoproteins for anaerobic respiration as a metabolism. The enzymes utilized react with oxidants and produce superoxides and hydrogen peroxide when they are exposed to aerobic environments. These chemicals in turn cause substantial damage to the cell membranes and DNA causing the cells to perish [48]. Information gathered on how TAV2 utilizes the oxygen in the microoxic periphery of the termite hindgut can allow further work to proceed on oxygen's influence on the termite hindgut, and particularly on microorganisms residing around the gut wall [28].

The *Verrucomicrobium* sp. strain TAV2 has been shown to possess a different transcription profile when grown under two contrasting oxygen conditions. Microarray information gathered shows conclusive evidence that the transcriptional regulation affecting genes controlling substrate metabolism to genes with unknown functions are affected by oxygen concentrations surrounding the cells.

Many affected genes have unknown functions or produce hypothetical proteins. Genes up-regulated in 20% oxygen were more present in COG group S whereas genes up-regulated in 2% were more prevalent in COG group R. This implies that a greater number of general function genes may help the everyday existence of TAV2 and more hypothetical genes aid in oxygen management. This information provides evidence for the need of future work to be completed on the nature of these genes and what metabolic systems they influence. These genes may provide a key in producing usable energy from byproducts of lignocelluloses degradation.

Although differences between COG groups gives an idea into cellular systems that are influenced by oxygen conditions, specific genes such as ObacDRAFT1002, which controls ATP synthase F1 alpha subunit, gives a much more detailed picture. This gene has been classified in the energy and conversion system. By analyzing its specific function one may infer that ATP synthesis may be up-regulated to account for additional energy needed for controlling oxygen present. F-type ATP synthases are found as a membrane bound proton channel and composed of two subunits. The F1 subunit is composed of 5 parts (alpha, beta, gamma, delta, and epsilon). The complexes form two rotors that counter rotate in order to drive ATP synthesis to be used within the cell [49]. By up-regulating components such as this, TAV2 cells incubated in 20% oxygen may produce more ATP for compensating with added oxidative stress. Other ATP related genes such as ObacDRAFT3995 are affected by higher oxygen concentrations as well. This ATP-dependent DNA helicase RecQ is involved with genome maintenance [50]. RecQ genes have been found to stabilize and repair damaged DNA replication forks and DNA damage checkpoint signaling. These could be up-regulated to maintain TAV2's genome when under additional environmental stress such as higher oxygen concentrations [50].

Additional regulatory genes such as ones that control regulatory protein GntR HTH are affected when cells were grown in 20% oxygen. This family of proteins generally binds DNA through a helix-turn-helix motif and regulates transcriptional control [51]. The helix-turn-helix domain is well conserved and binds tightly to dsDNA to regulate transcription [52]. Originally the GntR gene protein was found to be a transcriptional repressor of the gluconate operon, but as of 2009 the GntR superfamily consist of more than 6200 members with a helix-turn-helix domain [51, 52]. Since this gene is up-regulated in TAV2 cells grown under 20% oxygen perhaps greater control over gluconate metabolism is required so energy may be expended elsewhere.

Oxygen has the potential to destroy cells not accustomed to aerobic environments, and up-regulation of genes that protect a cell from environmental stress should be up-regulated.

ObacDRAFT4060 codes for heat shock protein Hsp20 that can act as a chaperone protecting other proteins against heat or other environmental stresses [53]. Heat shock proteins protect the cell through protein folding as well as preventing protein denaturation [53]. One study has shown that eukaryotic mesenchymal cells transformed with Hsp20 gene had increased resistance to oxidative stress [54]. Therefore up-regulation of this gene is one manner in which TAV2 cells protect themselves against the destructive properties of higher than normal oxygen conditions.

When compared to TAV2 cells grown under 20% oxygen, cells incubated under 2% oxygen had certain genes expressed in higher concentration across multiple cellular systems. ObacDRAFT0630, a gene that produces CRISPR-associated cas1 protein, was expressed at a greater rate in cells incubated under 2% oxygen. CRISPR proteins are widespread in bacteria and are suggested to provide some resistance to foreign DNA from things such as bacteriophages and transposons [55]. Thought to inhibit the actions of invasive DNA elements, the stronger presence of these genes suggest that when cells are put under 20% they lose some ability to protect themselves from foreign DNA. Whether this ability is down-regulated so energy may be expended elsewhere or lost completely is unknown at this time.

Up-regulation of ObacDRAFT2549 helps establish the basis that TAV2 cells natural environment is at 2% oxygen concentration because its products, DNA polymerase III beta subunit, aid in cell replication. The beta subunit on DNA polymerase III acts as a sliding clamp that maintains the polymerase on the template strand [56**,** 57]. By having this gene expressed at a greater rate, cells would have supplementary machinery to replicate at a quicker doubling time.

Along with replication machinery, substrate metabolism is affected by the oxygen surrounding the TAV2 cells. ObacDRAFT2443 is a gene that controls regulatory protein LacI. This gene acts as a transcriptional regulator for the lac repressor and generally changes the DNA binding activity of the repressor domain once a sugar binds [58]. Both this gene and

ObacDRAFT1722, which controls malate/L-lactate dehydrogenase, are up-regulated in 2% oxygen when compared to cells grown under 20% oxygen. Since both genes are up-regulated it can be inferred that lactate plays a substantial role in transcriptional regulation when cells are grown under 2% oxygen. ObacDRAFT1722 plays a role in the utilization of malate/L-lactate sugars for carbon and energy needs. Interestingly, when this enzyme is absent, cells cannot take up oxygen in the presence of L-lactate [59]. By up-regulating this enzyme, the cells are accustomed to metabolizing the substrate lactate in a microaerophilic environment. This is in balance to the regulatory LacI being up-regulated to repress downstream lactate metabolism by enzymes such as β-galactosidase [58, 30]. But when the oxygen concentration is raised to 20%, the balance of lactate metabolism and oxygen usage must be recalibrated. Lactate dehydrogenase production decreases as does the LacI repressor protein so L-lactate may then proceed to be metabolized in another manner. Shuffling genes that metabolize L-lactate may be one manner in which TAV2 copes with the added stress of extracellular oxygen concentrations higher than its normal habitat.

RT-PCR genes were selected to verify microarray data and also provide information into what genes are affected by the oxygen concentration surrounding the TAV2 cells. Cells grown in 20% oxygen had two genes selected, ObacDRAFT0946, which is a gene for xylose isomerase domain protein TIM barrel and ObacDRAFT1002, which codes for ATP synthase F1, alpha subunit. By choosing a gene in the COG group G, ObacDRAFT0946, I could examine a cellular system that solely had genes up-regulated by cells grown under 20% oxygen. Real-time PCR results were consistent with microarray analysis by demonstrating that the gene was in greater concentration in cells grown under 20% oxygen when compared to cells grown under 2% oxygen (Figure 2.6). The gene ObacDRAFT1002 was chosen as well so a comparison between energy production and conversion could be made. Results were once again consistent with microarray results in that cells incubated under 20% oxygen expressed a greater amount of this ATP synthase gene.

To verify microarray results concerning up-regulated genes of TAV2 cells grown under 2% oxygen the genes ObacDRAFT1722 and ObacDRAFT2549 were selected. DNA polymerase III confirmed microarray analysis values in that it was in greater concentration and gave a lower Ct value when RT-PCR was performed. ObacDRAFT1722 did not have statistically significant differences in expression when analyzing the 1µL concentration, but when observing 2 µL and 0.5 µL concentrations conclusions can be drawn as to a slight up-regulation by cells grown under 2% oxygen. By choosing ObacDRAFT1722 and ObacDRAFT1002, which both fall in the COG group C, a comparison could be made across oxygen conditions for energy production and conversion. TAV2 cells grown under 20% and 2% oxygen each had genes that were up-regulated regarding this cellular system, suggesting that certain parts of the cellular machinery that manipulates ATP production for energy usage are affected by oxygen present.

A house-keeping gene was selected as a RT-PCR control. It was not found to be significantly expressed in either oxygen condition on the microarray platform. ObacDRAFT1087, which controls DNA primase, was selected as a positive control. DNA primase adds a RNA primer to a strand of template DNA and is necessary for replication to begin [30]. Because this gene is needed for replication it is likely to be found in equal concentrations within identical cells and this was confirmed by RT-PCR analysis across three biological replicates.

The hindgut of the termite *R. flavipes* contains a complex food web of nutrients which multiple bacteria utilize for survival. From lignocellulosic material being broken down by protists, to hydrogen being combined with carbon dioxide to form acetate and methane, microorganisms are an integral part in degrading plant biomass. By studying *Verrucomicrobium* sp. strain TAV2 information can be applied to other metabolisms and the effect of oxygen concentrations.

Future work completed on metabolites and microorganisms isolated from the termite hindgut can provide information for industrial application in degrading plant biomass for commercial energy needs.

APPENDIX A

HYBRIDIZATION PROTOCOL
(ADAPTED FROM DR. WOO-SUK CHANG)

Bj110 Oligonucleotide Microarray Hybridization Protocol by Woo-Suk Chang (April 2009)

Adapted by Austin Willis for *Verrucomicrobium* sp. strain TAV2

Prehybridization Treatment:

-Turn on water baths to 50℃ and 95℃
-Get Ice

1. Preheat prehyb soln to 50℃
2. Incubate slides for 45 min in prehyb soln (in plastic Coplin jar at 50℃)
3. Wash slides in sterile MilliQ H_2O
    a. Wash 1 – fill slide straining dish with water, grasp slide at numbered end with forceps, wash slide(array side down) 30-40 times by rocking side-to-side
    b. Wash 2 – repeat Wash 1 in a second staining dish with fresh water
    c. Wash 3 – place slides in a Coplin jar filled with water, agitate with a circular motion for about 20 revolutions (you should be going just fast enough to hear the slides rocking in the jar)
    d. Wash 4 – repeat Wash 3 in a new Coplin jar
    e. Dip the slide in isopropanol (in Coplin jar)
    f. Dry immediately by centrifugation (700 rpm for 2 min at room temp)

Hybridization:

1. Resuspend aRNA in 65µL 1X Hybridization solution (preheated to 50℃)
2. Mix well by pipetting
3. Mix, Vortex and Spin
4. Incubate 3 min at 95°
5. Snap cool 30 s on ice (important to put directly on ice)
6. Spin 1 min at 13,000 rpm at room temp
7. Dust slide, cover slip and hybridization chamber
8. Place filter paper soaked with ~300 µL of hybridization soln on bottom of hybrid chamber below where the slide will be placed
9. Place slide in hybridization chamber
10. Place lifter slip over array
11. Apply 60 µL aRNA to array (don't take the last 5 µL – particulates)
12. Add 500 µL nuclease free water to wells in hybridization chamber
13. Seal the chamber and wrap (all slides you are working with) with aluminum foil
14. Incubate 16-18 hours at 50℃ waterbath (be sure the slide is level)

Post-hybridization Washing:

1. Preheat Wash 1 to 50℃ – in staining dish
2. Unwrap slides and gently dip in dish of Wash 1 to remove the lifter slip
3. Place slide in slide rack in Wash 1 staining dish
    a. Wash 6 min with gentle agitation on a shaker
    b. Every 1 min lift the rack up and down six times
    *Watch the temperature – keep it close to 50℃ by p lacing it on a hot plate
4. Transfer rack to Wash 2 (room temp) – wash 6 min as in step 3
5. Transfer slides to a Coplin jar with wash 3 (room temp) - wash by gently moving Coplin jar in circular motion being careful not to let slide rattle around in jar or spots at edges will be destroyed
6. Transfer slides to a fresh slide rack in a staining dish with wash 3
    a. Wash 3 min as in step3

b. Transfer to a new Coplin jar with wash 3 and wash an additional 3 min

7. Dry slide by centrifugation (700 rpm for 2 min at room temp) and scan

Solutions:

Prehybridization Solution:

20X SSC	50mL
10% SDS	2mL
BSA	200µL
MilliQ H_2O	148mL

2X Hybridization Buffer

Formamide	500 µL
20X SSC	200 µL
10%SDS	20 µL
Tween	0.1 µL

*Make just before use, preheat to 50℃, add 500 µL to 500 µL nuclease free water (at 50℃) when ready to use

Hybridization Wash Solutions:

	Wash 1	Wash 2	Wash 3	Wash 3 Coplin
20X SSC	25mL	2.5mL	1mL	280
10% SDS	10mL	10mL	---	---
MilliQ H_2O	465mL	487.5mL	497.5	55.28µL

APPENDIX B

RT-PCR PROTOCOL FOR TAV2

Real-Time Polymerase Chain Reaction Protocol for TAV2 (June 2010)

Adapted by Austin Willis for *Verrucomicrobium* sp. strain TAV2

-Set up 96 well plate to accommodate a comparison between cells grown under the two oxygen conditions. Perform triplicates of each condition as well as a dilution gradient.

- Dilution gradient – 3 wells with 2 μL of isolated aRNA at concentration of 50 ng/μL
  3 wells with 1 μL of isolated aRNA at concentration of 50 ng/μL
  3 wells with 0.5 μL of isolated aRNA at concentration of 50 ng/μL

  -each dilution should have 3 replicates and this should all be repeated per oxygen condition

- Reactants – combine 10 μL of reaction mixture (iTaq Fast SYBR Green Supermix With ROX – Bio-RAD, Hercules, CA Cat #172-5100) with amount of forward and reverse primers optimized from primer optimization reactions (generally 1.2 μL of forward and reverse primers at a concentration of 5μM.)
  add appropriate amount of aRNA and add nuclease free water to a final volume of 20 μL.

-RT-PCR set-up
  Make sure volume for machine is set at 20μL and data collection occurs at annealing temperature part of cycle
  1 cycle of 48°C for 30 min
  40 cycles of 95°C for 15 seconds and annealing temperature for 1 min
  Conclude with disassociation cycle

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BIOGRAPHICAL INFORMATION

Austin Willis graduated Baylor University with a Bachelor of Arts in Biology and a minor in Chemistry in 2006. In the summer of 2010 he graduated from the University of Texas at Arlington with a Master's of Science in Biology with a focus in Microbiology. His work consisted of working on *Verrucomicrobium* sp. strain TAV2 when grown under different oxygen concentrations as well as attempting to insert a plasmid vector into the same organism. Following this work he will enter medical school at the Texas College of Osteopathic Medicine to pursue his interest in becoming a medical doctor to treat disease in the local population.