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MOLECULAR AND BIOCHEMICAL STUDIES OF *TRICHOMONAS VAGINALIS*

**A Thesis Submitted to the
School of Graduate Studies
University of Ottawa**

**In Partial Fulfillment of the Requirements for the Degree of
Master of Science
Department of Microbiology and Immunology
Faculty of Medicine**

By

Dino P. Petrin

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ABSTRACT

Trichomonas vaginalis is known to produce a 60 kDa extracellular cysteine proteinase (ECP) that is believed to be important in the initial stages of *T. vaginalis* infection. Upon further purification, the 60 kDa protein breaks down into a 43 and 23 kDa subunits. Anti-serum raised against a purified preparation of the 23 kDa subunit was used to screen a λ gt11 *T. vaginalis* cDNA library. The strongest reactive clone of the four was partially sequenced and found to have homology to an exoantigen called ABRA found in *Plasmodium falciparum*. It was decided to sequence the other three clones to determine if they contained sequences that are indicative of cysteine proteinases (CP). Molecular analysis of the remaining clones indicated that they were distinct and did not represent cysteine proteinases. The anti-serum used in the initial screening most likely had multiple specificities, resulting in the isolation of these other genes. Clone 23-4-1 did not show significant homology to anything in the Genbank database. Clone 23-2-1 was homologous to phosphoenolpyruvate carboxykinase and clone 23-4-2 was found to be homologous to an NADPH-dependent butanol dehydrogenase (ADH1) found in *Clostridium acetobutylicum*. The deduced amino acid sequence had 75% identity and 80% overall similarity to the *adh1* gene product. It was decided at this time that the 23-4-2 clone be further characterized.

By using the cDNA sequence of 23-4-2, gene specific primers (GSPs) were used in RACE PCR in an effort to obtain a translational start codon and to determine if the putative gene designated *bdh* was polyadenylated. The *T. vaginalis bdh* gene was found to have an 1163 bp open reading frame (ORF) and a poly (A) tail that started 25 nucleotides

after the stop codon. However, no translational start was found at the expected position based on the homology to the *adh1* gene of *C. acetobutylicum*. There is an ATG codon located 96 bp downstream from the 5' end of the sequence. The possibility that this ATG is the translational start seems unlikely in that the "5' UTR" that is upstream of the codon is homologous to the *adh1* gene and other members of the *adh* family.

To determine if *T. vaginalis* did produce butanol as a byproduct of metabolism, *T. vaginalis* cultures were incubated under anaerobic conditions and subsequently under aerobic and anaerobic conditions and the culture supernatants were extracted with ether. The ether layer was analyzed using gas chromatography and mass spectrometry (GC-MS). It was found that *T. vaginalis* produces butanol under both culture conditions.

The discovery of this gene in *T. vaginalis* is intriguing. Though eucaryotic, this urogenital pathogen shares many facets with regards to metabolism to the anaerobic bacteria. This is the first time that an alcohol dehydrogenase has been discovered in *T. vaginalis*, elucidating that a novel metabolic pathway exists in this parasitic protozoon. Using drugs to target enzymes involved in other metabolic pathways holds promise in eradicating metronidazole-resistant strains of *T. vaginalis*.

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LIST OF ABBREVIATIONS

A	adenosine
<i>adhI</i>	NADPH-dependent butanol dehydrogenase gene of <i>C. acetobutylicum</i>
ADH	NADPH-dependent butanol dehydrogenase enzyme of <i>C. acetobutylicum</i>
Amp	ampicillin
AP	adhesion protein
A _{xxx}	absorbance at xxx nm
<i>bdh</i>	NADPH-dependent butanol dehydrogenase gene of <i>T. vaginalis</i>
BDH	NADPH-dependent butanol dehydrogenase enzyme of <i>T. vaginalis</i>
BSA	bovine serum albumin
C	cytosine
CDF	cell detaching factor
CP	cysteine proteinase
Da	dalton
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double stranded
DP-BDH GSP1 & 2	<i>bdh</i> gene specific primers for 5' (GSP1) and 3' (GSP2) RACE PCR
ECP	extracellular cysteine proteinase
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
G	guanine
GC	gas chromatography
IPTG	isopropyl β -D-thiogalactopyranoside

kb	kilobase
kDa	kilodalton
LB	Luria-Bertani broth
MS	mass spectrometry
NADH	Nicotinamide adenine dinucleotide reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NCBI	National Centre for Biological Information
NIH	National Institutes of Health
ORF	open-reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
STD	sexually transmitted disease
TAE	Tris-acetate buffer
TB	terrific broth
TBE	Tris-borate buffer
TE	Tris-EDTA buffer
TLCK	N- α -p-tosyl-L-lysine- chloromethyl ketone
T	thymidine
TYI	Diamond's TYI-S-33 medium
VECs	vaginal epithelial cells

X-gal 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

CHAPTER 1: INTRODUCTION

Taxonomy and Structure of *T. vaginalis*

Trichomonas vaginalis (phylum Parabasalidae; order Trichomonadida (Kirby 1947); family Trichomonadidae; sub-family Trichomonadinae (Chalmers and Pekkola 1918)) was first described by Donné in 1836. *T. vaginalis* is an anaerobic protozoan and is said to be aero-tolerant. Though eucaryotic, *T. vaginalis* lacks two distinguishing features found in higher eucaryotes: peroxisomes and mitochondria. Gene sequence data of the rRNA 23S and 16S-like subunits of *T. vaginalis* suggest that trichomonads evolved prior to the advent of mitochondria, placing *T. vaginalis* among the early branches of the eucaryotic tree (Baroin *et al.* 1988; 1989; Gunderson *et al.* 1995; Katiyar *et al.* 1995; Viscogliosi *et al.* 1993). Other investigators have further supported this concept by finding *T. vaginalis* homologues to the CDC2/28 protein kinase genes (Riley *et al.* 1993). These genes which are essential to eucaryotic cell division, belong to a family of genes which are believed to have arisen at the same time that the nuclear envelope appeared but prior to the endosymbiosis of mitochondria. These findings along with the rRNA evidence show that *T. vaginalis* is indeed one of the most ancient eucaryotes.

T. vaginalis is a flagellated protozoan, possessing 5 flagella, 4 of which are located at the anterior end. The fifth flagellum is incorporated into the parasite's undulating membrane, also termed the recurrent flagellum. The undulating membrane is supported by a slender rib-like structure called a costa. The undulating membrane and the flagella give *T. vaginalis* its characteristic quivering motility. *T. vaginalis* has an anteriorly located nucleus and a characteristic axostyle that bisects the parasite longitudinally terminating at

the posterior end in a sharp point (Honigberg and King 1964). This structure is thought to anchor the parasite to vaginal epithelial cells (VECs).

Though *T. vaginalis* lacks some characteristic eucaryotic organelles, it does contain typical organelles such as Golgi bodies and endoplasmic reticula (Honigberg *et al.* 1984). A unique structure of *T. vaginalis* is the hydrogenosome (Müller 1980). Because hydrogenosomes possess a double membrane, it is likely that they also arose via endosymbiosis. Hydrogenosomes lack genetic material and possess hydrogenase and pyruvate:ferredoxin reductase making these structures distinct from mitochondria. Many enzymes that are present in the hydrogenosomes have been characterized in the attempt to determine the origin of this organelle. Two hypotheses have been put forward as to their origin: (a) that they arose by conversion of mitochondria or (b) through the independent endosymbiosis of an anaerobic bacterium (Johnson *et al.* 1993; Lahti and Johnson 1991).

Epidemiology, Clinical Manifestation, Complications

Of the three species found in humans, *T. vaginalis* is the only trichomonad which is truly pathogenic, infecting the urogenital tract of humans. *Trichomonas tenax* and *Pentatrichomonas hominis*, the other two members of Trichomonadidae, are considered colonizers rather than pathogens and are found in the oral and lower GI tracts, respectively. *T. vaginalis* is the causative agent of trichomoniasis, the world's most common non-viral sexually transmitted disease (STD) (Brown 1972; Catterall 1972). *T. vaginalis* is said to have a cosmopolitan distribution, affecting individuals in all racial groups and socioeconomic strata. The estimated number of cases in 1995 was reported to be 170 million worldwide with over 8 million cases in North America alone (World Health

Organization 1995). The primary mode of transmission is via sexual intercourse. Non-venereal modes of transmission have been reported but are rarely seen (Whittington 1957). Newborn infants of mothers infected by *T. vaginalis* have, on occasion, acquired a *T. vaginalis* urinary tract or vaginal infection (Hiemstra 1984). Parasites were found in 2%-17% of the female neonates of infected women (al-Salihi 1974).

Trichomoniasis has a broad clinical presentation in women ranging from an asymptomatic carrier state to flagrant vaginitis (Wisdom *et al.* 1965). Once a woman is infected, the parasite can persist for extended periods of time, while in males the infection is usually of short duration. The highest incidence of the disease occurs primarily during the reproductive years, though cases have been reported in post menopausal women (Brown 1972; Wolner-Hanssen *et al.* 1989). The incubation period varies between 4-28 days. The disease can be classified as either asymptomatic, acute or chronic, depending on the severity.

Asymptomatic women have a normal vaginal pH (4.5) with normal vaginal flora and this state of infection is termed the carrier stage. Acute trichomoniasis shows diffuse vulvitis, a frothy, purulent, yellow or green discharge, and small punctuate hemorrhagic spots on the vaginal and cervical mucosa (Rein 1990). This speckled appearance has been termed the "strawberry cervix," which is only found in 2% of cases (Fouts and Kraus 1980). These clinical signs are exacerbated at the time of menses. The chronic stage is characterized by mild symptoms with pruritus and dyspareunia predominating and the secretion is usually scanty (Nicoletti 1961).

Though the clinically apparent disease predominates in women, men are also infected by this parasite. Men are said to be the carriers of the disease since they are usually asymptomatic. In some, acute purulent urethritis occurs. In mild cases, the disease can be clinically indistinguishable from other forms of non-gonococcal urethritis. Krieger (1995) has shown that 11% of all cases of non-gonococcal urethritis in males are caused by *T. vaginalis*. Duration of the infection appears to be 10 days or less in most cases. Other symptoms found in men include scanty, clear to mucopurulent discharge, dysuria, mild puritis or burning following intercourse (Krieger 1990).

Complications arising from trichomoniasis in women include post-partum endometritis (Rein and Chapel 1975), infertility (Grys 1973), low birth weight (Hardy *et al.* 1984) as well as pre-term infants, adnexitis, pyosalpinx (Sebek 1972), cervical erosion (McLellan *et al.* 1982), and an enhanced predisposition to HIV infection (Cameron and Padian 1990; Laga *et al.* 1994; Laga *et al.* 1993). In men, complications associated with *T. vaginalis* include prostatitis, balanoposthitis, epididymitis, chronic urethritis and infertility in rare cases (Holmes *et al.* 1975; Krieger 1984; Mardh and Colleen 1975; Tuttle *et al.* 1977).

Diagnosis and Treatment of Disease

Diagnosis of *T. vaginalis* infection based solely on clinical presentation is not reliable since symptoms of the disease can be similar to other STDs. Thus in the past, the major diagnostic criterion for *T. vaginalis* has been microscopic wet-mount (Lisi *et al.* 1988) examined in conjunction with a culturing method, which has proven to be as sensitive or better than culture techniques on their own. However, shortcomings of both

techniques are that the number of trichomonads needed for detection under a microscope is quite high and culture based methods may not be readily available to physicians and cultures require a 2-7 day incubation period. This window period is unacceptable if treatment is to be administered immediately and help prevent the patient from potentially spreading the infection.

The use of monoclonal antibodies (MAb) holds promise of rapid detection of *T. vaginalis*. Kreiger *et al.* (1985) used two broadly reactive MAbs that identified all 88 isolated strains from across North America. Furthermore, MAbs raised against a 62 kDa and 65 kDa antigen specific to *T. vaginalis*, gave results that had a sensitivity observed to be comparable to the wet mount preparation (Lisi *et al.* 1988). The use of peroxidase- and fluorochrome-labeled monoclonal antibodies to *T. vaginalis* (Trichomonas Direct Enzyme Immunoassay and Fluorescent Direct Immunoassay) has also been shown to be sensitive and specific in comparison to the broth culture technique (Thomason and Gelbert 1989). An added advantage is that this test may be completed within an hour, allowing diagnosis and initiation of treatment in a single visit.

Molecular techniques show promise in providing the most accurate and quickest methods in detecting *T. vaginalis*. The Affirm VP system (Briselden *et al.* 1994), PCR (Riley *et al.* 1992), dot blots (Rubino *et al.* 1991) and other molecular techniques (Chapin-Robertson 1993) have been used in clinical settings. They have shown to be moderately to very sensitive in detection (Affirm VP and PCR, respectively) and quite rapid. The Affirm VP tests uses a synthetic oligonucleotide that is specific for *T. vaginalis*. The test does not cross react with DNA from humans or other causative agents of STDs.

Some tests can be completed under 1 hour and are specific. This is advantageous since diagnosis and treatment may be carried out in a single visit.

Treatment

Prior to the advent of α - β hydroxyethyl-2-methyl-5 nitro-imidazole (metronidazole) topical preparations were available and provided symptomatic relief, but did not eradicate the parasite (Lossick and Kent 1991). Furthermore, treatment for the male partners was not available and thus re-infection would occur quickly. Today, standard protocol involves the treatment of both partners simultaneously to prevent the recurrence of infection.

The byproducts of metronidazole are cytotoxic for anaerobic organisms including *T. vaginalis* (Heine and McGregor 1993). Briefly, the drug is taken up by the hydrogenosomes of the parasite and the nitro group of the drug is reduced by pyruvate:ferredoxin oxidoreductase resulting in the generation of nitro radical ion intermediates which cleave the parasite DNA between thymidine and adenosine residues (Müller and Lindmark 1976; Müller 1986; Tocher and Edwards 1994). This causes cell division to cease and cell death occurs within 8 hours (Nielson 1976). The standard regimen for treatment is a 250 mg oral dose taken 3 times daily for 7 days or a single 2 g dose (Underhill and Peck 1974; Hager *et al.* 1980). The latter method is preferred since patient compliance is better, there are fewer side effects and the total drug dose is less (Lossick 1982).

Metronidazole resistant strains of *T. vaginalis* have been on the rise (Narcisi and Secor 1996). *T. vaginalis* has aerobic and anaerobic mechanisms resulting in resistance to

metronidazole. In strains that are resistant when grown aerobically the oxidase enzymes have a lower affinity for oxygen and subsequently allow increased levels of oxygen in the cell, thereby interfering with drug activation (Quon *et al.* 1992). In parasites that are resistant when grown anaerobically, the activities of pyruvate:ferredoxin oxidoreductase and hydrogenase are decreased, or not present (Kulda *et al.* 1993). The decreased level of ferredoxin is a result of the decreased transcription of the ferredoxin gene.

New antitrichomonal agents are needed for cases of resistant *T. vaginalis* and in cases of allergy to metronidazole, the only chemotherapeutic agent available in North America. There has been a renewed interest in furazolidone (Trichofuran) in dealing with metronidazole resistant strains (Narcisi and Secor 1996). Other drugs that are being tested include gynalgine (Sikorski *et al.* 1992), butoconazole (Bendova *et al.* 1992) and mebendazole (Juliano *et al.* 1987).

Pathogenesis

T. vaginalis is the most intensely studied trichomonad to date, yet its pathogenic mechanisms have not been clearly elucidated. Research has focused on factors important in the initial stages of infection and on the secretion of cytotoxic factors, with particular emphasis on cysteine proteinases which *T. vaginalis* produces in higher numbers than any other parasitic protozoan.

Cytoadherence, Contact Dependent Cytotoxicity and Hemolysis

A prerequisite to infection by *T. vaginalis* is the adherence to VECs. Attachment to the cells is time, temperature and pH dependent (Alderete and Garza 1985, 1988; Alderete *et al.* 1988; Arroyo *et al.* 1992). *T. vaginalis* has a preference *in vitro* to parasitize epithelial cell lines (Alderete and Pearlman 1984; Alderete and Garza 1985; Rasmussen *et al.* 1986). This is not surprising since VECs are the principle cell type that *T. vaginalis* interacts with *in vivo*. *T. vaginalis* has been observed to kill VECs without phagocytosis (Krieger *et al.* 1985b).

Adhesion of the parasite to epithelial cells is mediated by four proteins termed adhesins. These proteins range in size from 23 to 65 kDa and have been designated AP23, AP33, AP51 and AP65 (Arroyo *et al.* 1992). During infection, adhesin synthesis is upregulated when the parasite comes in contact with a cell and *T. vaginalis* takes on a more amoeboid morphology (Arroyo *et al.* 1993; Heath 1981; Mirhaghani and Warton 1996). The means of attachment to VECs suggests that a specific receptor-ligand interaction is involved since trypsinized parasites failed to adhere to VECs *in vitro* (Alderete *et al.* 1988). Parasites are able to attach to dead cells implying that *de novo* protein biosynthesis is not essential for cell parasitism (Alderete *et al.* 1988). It has been reported that the side opposite the undulating membrane attaches itself to the epithelial cell and microfilaments become concentrated in the parasite on the side in contact with the parasitized cell (Alderete and Garza 1985). A glycoprotein, P270, expressed on the surface of the parasite has been shown to undergo phenotypic variation in a variety of strains, in tandem with adhesion of *T. vaginalis* to cells via the adhesin proteins (Alderete

et al. 1986). Alderete has demonstrated that adherent minus (adh-) organisms express the P270 glycoprotein whereas adherent positive (adh+) parasites lack expression of P270 (Alderete and Garza 1988). Strains that are capable of expressing both types revert to a heterogeneous population when grown *in vitro* even if the starting population was either adh+ or adh-. Adherence, however, does not correlate directly with virulence (Krieger *et al.* 1983).

Glycoproteins have also been implicated in the binding of *T. vaginalis* to epithelial cells. A 118 kDa laminin-binding protein is ubiquitous on the surface of the parasite, even on the undulating membrane (Costa e Silva Filho *et al.* 1988). No correlation has been shown to date regarding the degree of pathogenicity of *T. vaginalis* strains and the expression of laminin binding proteins. Laminin can aid the parasite in attachment to the cells of the vaginal mucosa and the receptors may play a role in pathogenesis.

T. vaginalis has the ability to lyse erythrocytes (Dailey *et al.* 1990; Fiori *et al.* 1993; Potamianos *et al.* 1992). β -hemolytic activity is greatest at pH 4.5 suggesting that hemolysis occurs *in vivo* (Krieger *et al.* 1983). Since *T. vaginalis* lacks the ability to synthesize lipids, erythrocytes may be an ideal source of fatty acids for *T. vaginalis* (Lehker *et al.* 1990). Furthermore, iron is an important mineral for *T. vaginalis* and may be acquired upon lysis of red blood cells. Lysis of the cells is mediated by a receptor-ligand interaction and evidence implies that perforin-like proteins may be the molecules involved. Interestingly, 5 adhesin proteins have been identified to adhere to erythrocytes, 3 of which are identical to the ones involved in adhering to VECs (Arroyo *et al.* 1992). Hemolysis appears to occur in three stages. A specific ligand-receptor interaction allows

the trichomonad to attach itself to the red blood cell. This is followed by the release of perforin-like proteins (possibly cysteine proteinases). Finally *T. vaginalis* detaches itself from the cell and lysis immediately follows. Unlike with epithelial cells, phagocytosis of erythrocytes by *T. vaginalis* has been observed *in vitro* (Fiori *et al.* 1993).

Contact Independent Cytotoxicity

There exists a complex host-parasite interrelationship between *T. vaginalis* and humans. It has been reported that *T. vaginalis* releases soluble factors that may contribute to a cytopathic effect (Garber *et al.* 1989; Pindak *et al.* 1986). One of the key factors studied in detail is Cell Detaching Factor (CDF) which correlates with clinical presentation of the disease (Garber and Lemchuk-Favel 1990). In cell culture monolayers, *T. vaginalis* cell-free filtrates caused the cells of the monolayer to detach and clump together, but cells remained viable (Pindak *et al.* 1986). In acute *T. vaginalis* infections, the shedding of epithelial cells from the vaginal mucosa is observed, suggesting that CDF may play a role in pathogenesis. CDF was collected by allowing *T. vaginalis* to grow on McCoy cell monolayers. The supernatant was collected, adjusted to pH 6.8 and filter-sterilized to yield a CDF-containing filtrate. This filtrate caused the detachment and clumping of McCoy cell monolayers and was able to inhibit freshly added cells from forming monolayers (Pindak *et al.* 1986).

Of clinical relevance is that estrogen levels affect the production of CDF. It was noted that the severity of symptoms increase during the time of menses, a time when estrogen levels are lowest (Garber *et al.* 1991). The application of estradiol pellets

intravaginally lessens the clinical symptoms without eradicating *T. vaginalis* (Liriosi and Guarascio 1972).

Though CDF is not cytotoxic, investigators have reported that cell-free filtrates of *T. vaginalis* were able to elicit hemolysis of erythrocytes to the same degree as reported for the contact-dependent model (Pindak *et al.* 1993). The molecule(s) that cause this contact-independent CPE have not been defined. It has been hypothesized that metabolic products of *T. vaginalis*, such as free acids, may be the cause of this pathogenic effect. Recently, Fiori *et al.* (1996) demonstrated that as pH rose above 5.0, *T. vaginalis* was able to lyse erythrocytes without coming into direct contact with the target cells. The principle molecule responsible for cytolysis appears to be a protein of 30 kDa. Whether other products are responsible for erythrocyte lysis remains to be investigated.

Though two hypotheses have been proposed, it is more likely that both contact-dependent and contact-independent mechanisms play a role in the pathogenesis of *T. vaginalis*.

Immune Evasion

T. vaginalis is known to secrete large quantities of soluble antigens that induce a host humoral response. It is proposed that the release of these antigens is advantageous for *T. vaginalis* since they may prevent antibodies or lymphocytes from attacking the parasite directly (Mason and Patterson 1985). *T. vaginalis* is also reported to coat itself with plasma proteins (Peterson and Alderete 1982). This may allow escape from detection by secretory antibodies from the vaginal mucosa since the body would not recognize the host protein coated parasites as foreign.

Phenotypic variation of P270 not only plays a role in adherence of the parasite to VECs, but may play a role in immune evasion as well. Parasites may or may not express this immunogenic glycoprotein on the cell surface and thus the phenotypic designation P270+ for those parasites expressing the immunogen and P270- for those lacking the immunogen, respectively (Alderete *et al.* 1986). A monoclonal antibody has been raised against this immunogen and P270+ parasites are recognized by this antibody and are killed while those which do not express the immunogen live (Alderete and Kasmala 1986). *In vitro*, there is a shift from a heterogeneous population to parasites that do not express the antigen, and are not detected by this monoclonal antibody. At the same time, P270- parasites are able to adhere to VECs to a greater degree (Alderete *et al.* 1986b). This could be an important step in evading the host immune response.

A dsRNA virus has also been found in a number of *T. vaginalis* strains (Wang and Wang 1986). It was initially thought that the dsRNA virus was nonsegmented. However, upon reevaluation of the total amount of dsRNA extracted from strains infected with the virus, showed the virus was composed of three segments ranging from 4.3 to 4.8 kbp (Khoshan and Alderete 1993; 1995). The virus appears to be icosahedral in shape (Khoshan and Alderete 1995). The double stranded viral RNA is synthesized by viral RNA-dependent RNA polymerase (Khoshan *et al.* 1994). An interesting finding is that P270 phenotypic variation only occurs among trichomonads that are infected with the virus (Wang *et al.* 1987; Khoshan and Alderete 1994). Since the virus seems to play a role in gene regulation of some *T. vaginalis* proteins, it was thought that it may confer

resistance to metronidazole treatment. Flegr *et al.* (1987), however, showed that three resistant and five susceptible strains were found to contain the dsRNA virus.

Activation of complement by either the classical or alternative pathway is another host defense against infection. The classical complement cascade requires antibody, Mg^{2+} , Ca^{2+} , and the C1-4 early complement factors (Abbas *et al.* 1991). The alternative pathway does not require antibodies or Ca^{2+} and requires factors B, D, properdin and C3. Both pathways lead to the cleavage of C5 and the resulting C5-C9 complex, causing the chemotaxis of polymorphonuclear leukocytes (PMNs) which ultimately lead to cell lysis. *T. vaginalis* has been shown to activate the alternative complement cascade (Gillin and Sher 1981). *T. vaginalis* grown *in vitro* in iron-enriched medium have been shown to be resistant to cell lysis by the alternative complement pathway when compared with parasites grown in iron-depleted medium (Alderete *et al.* 1995). More specifically lactoferrin, a principle source of iron for *T. vaginalis in vivo*, but not transferrin, rendered the trichomonads resistant to cell lysis. During menses, the microenvironment becomes saturated with red blood cells, proteins, immunoglobulins as well as complement. However, at this time, symptoms of trichomoniasis are exacerbated. *T. vaginalis* has been shown to degrade the C3 complement complex on its cell surface thus evading cell lysis by complement. The erythrocytes provide *T. vaginalis* with an abundant source of iron, especially during menses. It has been suggested that the gene encoding the enzymes that degrade the C3 complex are upregulated by lactoferrin (Alderete *et al.* 1995). These mechanisms by which *T. vaginalis* can utilize iron and modify the effect of complement are examples of the diverse pathogenic strategies of this parasite.

Cysteine Proteinases

Various types of proteinases are synthesized by *T. vaginalis* including cysteine and metallo proteinases. (Bóznér and Demeš 1991; Lockwood *et al.* 1987; North *et al.* 1990). Cysteine proteinases (CPs) play a crucial role in the pathogenesis of *T. vaginalis*. *T. vaginalis* has been reported to have between 11 to 23 distinct cysteine proteinases, most of which are lysosomal (Coombs and North 1983; Neale and Alderete 1990). However, there are proteinases that have been found to be excreted by *T. vaginalis* and others that are found on the cell surface (Neale and Alderete 1990; Arroyo and Alderete 1989).

IgG is the antibody that is primarily found in the vaginal mucosa. Supernatants of logarithmically growing cultures contained cysteine proteinases that degraded all classes of human antibodies (Provenzano and Alderete 1995). Lysates of *T. vaginalis* have also been shown to degrade antibodies, and this is thought to be due to cysteine proteolytic activity.

Adherence is also mediated by CPs found on the surface of the parasite. TLCK, a potent proteinase inhibitor greatly diminished the ability of *T. vaginalis* to adhere to epithelial cells (Arroyo and Alderete 1989). Incubating TLCK-treated parasites with other cysteine proteinases restored adherent capabilities of the cells indicating that CP activity is required for attachment to VECs. Moreover, the parasites were shown not to kill host cells after being treated with TLCK thus demonstrating that CP activity is necessary in contact dependent cytotoxicity.

It has also been suggested that CPs inhibit lysis via complement by degrading C3 on the parasite surface (Alderete *et al.* 1995). Moreover, investigators have reported that

CP inhibitors greatly reduced the erythrocyte hemolytic activity of live parasites (Dailey *et al.* 1990).

One particular extracellular cysteine proteinase (ECP) that is under study is believed to be important in the initial stages of infection. Found to have a molecular weight of 60 kDa, this proteinase is present in all strains of *T. vaginalis* and is most active at a pH of 4.5 (normal vaginal pH) (Garber and Lemchuk-Favel 1994). Upon subsequent purification by ion exchange chromatography and gel filtration, the ECP of *T. vaginalis* breaks down into a 43 and 23 kDa subunits. The 23 kDa was found to cross react with antiserum reactive to the 60 kDa protein. This 60 kDa ECP has also been found to be immunogenic and the serum of mice immunized with a purified 60 kDa fraction reacts with ECP in cell-free filtrates of *T. vaginalis* using Western blots. The 60 kDa ECP has been identified in the vaginal washes of women with active *T. vaginalis* infection but not in uninfected women or in women that have been cured of the infection.

The 23 kDa specific rabbit antiserum was used to screen a λ gt11 library containing cDNA from *T. vaginalis* strain DG. A total of 4 clones were identified and all were subcloned into pGEM 3Z vector (Promega). Out of the 4 clones, the most strongly reactive clone, designated 23-1-1, was partially characterized and was found to be 60% homologous to a malarial exoantigen called ABRA found in *Plasmodium falciparum* (Garber *et al.* 1993).

The cysteine proteinases of *T. vaginalis* are certainly one of the more important factors in the parasite's ability to survive in this ever-changing environment, the vagina.

Parasite Metabolism

Metabolism of *T. vaginalis* has been studied since the 1960's (Danforth 1967; Fulton 1969; Ryley 1967; Shorb 1964). Though many enzymes of a number of metabolic pathways in *T. vaginalis* have been discovered (Arese and Cappuccinelli 1974), others remain to be discovered. Being one of the most primitive eucaryotes known, *T. vaginalis* shares many features including some metabolic pathways common to the anaerobic bacteria. However, pre-formed metabolites make up a large part of a trichomonad's diet which indicates that major biosynthetic pathways are not present (Müller 1990). Most of the data on metabolism have been collected through the use of large volume broth cultures to provide enough material to study the physiological and biochemical attributes of *T. vaginalis* (ter Kuile and Müller 1993). Chemostats have proven to be invaluable instruments in metabolic studies. Thus it should be noted that there may be marked differences to *T. vaginalis in vivo*.

The only amino acid that it synthesized in detectable quantities by *T. vaginalis* is alanine (Chapman *et al.* 1985; North *et al.* 1986) and evidence shows that greater amino acid uptake occurs when parasites are grown in the absence of maltose (Zuo *et al.* 1995). Beach *et al.* (1990, 1991) have demonstrated that lipid biosynthesis is absent in *T. vaginalis*. These nutrients are acquired by the trichomonads from the lysis of erythrocytes and the parasitizing of the VECs in the vaginal mucosa (Lehker *et al.* 1990).

Carbohydrates are the main sources of energy for *T. vaginalis*. Many of the intermediates of carbohydrate catabolism and the enzymes involved have been documented (Steinbuchel and Müller 1986a, 1986b). Glycolysis takes place in the cell

cytoplasm, resulting in the production of pyruvate and the release of CO₂. In addition to glycolysis, some enzymes of the pentose phosphate shunt have also been isolated from the cytosol. (Arese and Cappuccinelli 1974).

Enzymes localized in the hydrogenosome (hydrogenase, pyruvate:ferredoxin oxidoreductase, acetate:succinate CoA transferase and succinate thiokinase) are involved in metabolic pathways that are markedly different from those found in typical eucaryotic cells and are more closely related to enzymes found in anaerobic bacteria (Johnson *et al.* 1990).

Many of these biochemical pathways have not been fully characterized. Understanding the parasite's biochemical pathways may allow researchers to design alternative chemotherapeutic agents, targeting the metabolic machinery that will in particular be effective against metronidazole resistant parasites.

Alcohol Dehydrogenases

Dehydrogenase enzymes are important catalysts in the production of alcohols in a number of bacteria and primitive eucaryotes. It is known that these enzymes have a specificity for either NAD or NADP as co-factors in the reduction of aldehyde groups to alcohols (Voet and Voet 1990). Butanol, and in general, alcohol dehydrogenases, have been found in both procaryotes and in eucaryotes. Three types of alcohol dehydrogenases (ADHs) have been identified. Jörnvall *et al.* (1987) have described the relationship between the different enzymes. There are long chain Zn-containing ADH enzymes that are widely distributed among eucaryotes which can be further subdivided into the dimeric enzymes such as those found in horse liver and the tetrameric enzymes that are

predominantly found in yeast (Jörnvall 1970; Williamson *et al.* 1980). Secondly, there is an ADH that is a short chain, dimeric, non-metallo containing protein that is known to be produced in *Drosophila melanogaster* (Benyajati *et al.* 1981). The final category of enzymes are tetrameric Fe-containing ADHs such as the ones found in *Zymomonas mobilis*, *Saccharomyces cerevisiae* and in most bacteria such as *E. coli* and clostridial species (Scopes 1983; Neale *et al.* 1986; Conway *et al.* 1987). No significant homology exists between the three families of dehydrogenases.

Alcohol dehydrogenases have been studied in other parasitic protozoa, however, the most intensely studied protozoa to date has been the anaerobic protozoan, *Entamoeba histolytica*. *E. histolytica* is found in the lumen of the colon and has numerous dehydrogenase enzymes which include alcohol dehydrogenases (EhADH I and III) (Samuelson *et al.* 1992; Rodriguez *et al.* 1996) and an acetaldehyde dehydrogenase (EhALDH I) (Samuelson *et al.* 1992). All three of the enzymes are NADP⁺-dependent. EhADH I shows homology to the Fe-containing alcohol dehydrogenase family where as EhALDH I has 36% amino acid identity over a 451 amino acid overlap with ALDH3, a human stomach dehydrogenase. EhADH III showed 26% amino acid identity to the Fe-containing alcohol dehydrogenases. EhADH II (Bruchhaus and Tannich 1994) presents an interesting finding since it is a bifunctional 95 kDa molecule that harbours both acetaldehyde and alcohol dehydrogenase activities. Unlike the above mentioned enzymes, EhADH II uses NAD⁺ as a co-factor.

Tritrichomonas foetus is a protozoan parasite that causes bovine abortion. It is the only other parasitic trichomonad that has been found to produce a secondary alcohol

dehydrogenase, with a molecular weight of 115 kDa composed of 6-8 subunits of identical size (~17 kDa). The enzyme converts 2-propanol to acetone using NADP⁺ as a cofactor. Primary alcohols, such as ethanol, are oxidized at a rate of about 5% of that observed for 2-propanol (Kleiner and Johnston 1985).

Characterization of the adh1 Gene

In *C. acetobutylicum* butyraldehyde is reduced by the Fe-containing NADPH dependent butanol dehydrogenase (ADH I) to form butanol (Petersen *et al.* 1991). It is the only bacterium known to produce 1-butanol as a major fermentation product (Gottschalk 1979). The acetate-butanol fermentation process is discerned by an initial acid-producing growth phase followed by a solvent-producing phase (Jones and Woods 1986). The shift to alcohol production correlates with the synthesis of solventogenic enzymes and a decrease in the activity of acidogenic enzymes (Andersch *et al.* 1983; Hartmanis and Gatenbeck 1984). Butanol synthesis from butyryl-CoA is catalyzed by butyraldehyde and butanol dehydrogenase enzymes. Other genes known to produce alcohols in *C. acetobutylicum* are butanol dehydrogenase I and II (BDH I and II) (Welch *et al.* 1989) and aldehyde/alcohol dehydrogenase (AAD) (Nair *et al.* 1994).

NADPH Binding Domains

As mentioned above, the alcohol dehydrogenases use either NADH or NADPH as a co-factor in the synthesis of alcohols. NADH utilizes the free energy generated from metabolite oxidation to synthesize ATP whereas NADPH uses the free energy of metabolite oxidation for endergonic reactive biosynthesis. Dehydrogenase enzymes have a high degree of specificity for their co-factors. The [NAD⁺/NADH] ratio in cells is around

1000, which favours metabolite oxidation while the $[NADP^+/NADPH]$ ratio is close to 0.01 which favours metabolite reduction (Voet and Voet 1990).

All dehydrogenases contain two major domains, one binds the co-factor (NADH or NADPH) and the other binds the substrate (Rossman *et al.* 1975; Eventoff and Rossmann 1975)). In the Zn-containing ADH enzymes, the NADH binding domain has been found to contain a conserved glycine rich region with a consensus sequence of GxGxxG or GxxGxxG within the β - α - β Rossmann fold (Wooten 1974; Rossmann *et al.* 1974; Jörmvall *et al.* 1987). In the Fe-containing ADH proteins, this consensus sequence could not be found. Since ADH I of *C. acetobutylicum* is specific for NADPH, this could account for the absence of an NADH binding domain. NADPH enzymes have been compared at the structural and amino acid level and have been shown to differ in their NADPH binding domains, suggesting that consensus sequences in the binding region are lacking (Krauth-Siegel *et al.* 1982)

Alcohol Metabolism in *T. vaginalis*

Though *T. vaginalis* is the most intensely studied trichomonad to date, little is known concerning the ability of *T. vaginalis* to produce alcohols as a byproduct of anaerobic respiration. *Trichomonas foetus* does produce an alcohol dehydrogenase (Kleiner and Johnston 1985) that is much larger (115 kDa) than the Fe-containing ADHs that are more commonly found in bacteria, and uses secondary alcohols as substrates rather than primary alcohols. This evidence suggests that this ADH differs significantly from the short chain Fe-containing ADHs found in procaryotes. No evidence has been put forward indicating whether or not *T. vaginalis* has ADH activity.

Experimental Rationale / Objectives

T. vaginalis is the most intensely studied trichomonad to date, yet the mechanisms of its pathogenesis are not clearly understood. All pathogenic isolates have been shown to produce a 60 kDa extracellular cysteine proteinase which has been detected in trichomonal supernatants. It is most active at normal vaginal pH, but is broadly active between pH 4.0 and 7.0 even though *T. vaginalis* proliferates at pH 6.5. This implies that this CP may play a key role in the initial stages of infection. Recently, work in our lab has shown that this extracellular CP has the ability to inhibit the growth of *Lactobacillus acidophilus*. The acidic pH (4.5) of the vagina is maintained due to *L. acidophilus*. Inhibiting the growth of this bacterium may make the vagina more susceptible to infection by *T. vaginalis* or other STDs. The subsequent rise in pH would allow *T. vaginalis* to proliferate at a more neutral pH (6.5). Once this is achieved, an increase in CDF activity occurs since CDF is inactive at a pH of less than 5.0 *in vitro*. This hypothetical model illustrates the potential complexity of the host-parasite inter-relationship. Central to this model is the hypothesis that the 60 kDa extracellular CP that is secreted by *T. vaginalis* is critical in the early stages of infection. Characterization of the gene and its regulatory elements may provide clues as to how to control the spread of infection. The original objectives of this study therefore were the sequencing and molecular characterization of 4 cDNA clones that were reactive to the 60 kDa polyclonal antisera to the extracellular CP. After obtaining the cDNA fragments, the clones that most resembled CPs would be used to isolate a genomic copy of the extracellular CP and to determine the start codon of the gene and any regulatory sequences present.

However, as it turns out, none of the clones contained sequences that are universally present among all cysteine proteinases. Therefore, it was decided to work on the clone that was most complete. This cDNA showed significant similarity to an NADPH-dependent butanol dehydrogenase gene found in *C. acetobutylicum*. The gene in *T. vaginalis* did not appear to have a translational start or a poly-(A) tract, but it did contain a stop codon.

The presence of alcohol dehydrogenases in *T. vaginalis* an interesting yet unexpected finding. Other organic end products are known to be produced by this urogenital parasite, primarily because it lacks mitochondria and is thus unable to completely oxidize glucose to water and CO₂. Little is known about dehydrogenase enzymes in *T. vaginalis*, except that they are most likely located in the hydrogenosome (Bui and Johnson 1996).

Initially, the objective of this project was to characterize a gene encoding for a 60kDa ECP found in the common urogenital pathogen, *T. vaginalis*. After finding that the sequences obtained did not contain sequences that were representative of cysteine proteinases, it was decided that the clone with homology to an NADPH-dependent butanol dehydrogenase gene (*adh1*) found in *C. acetobutylicum*, would be studied. Thus, the objectives became (a) to find the translational start codon of this *adh1* clone, and (b) to determine if *T. vaginalis* did produce butanol as a byproduct of fermentative metabolism.

CHAPTER 2: MATERIALS AND METHODS

Strains

***Trichomonas* Species**

Isolates of *T. vaginalis* were obtained from vaginal secretions of woman suffering from trichomonal vaginitis. The principle strain used was *T. vaginalis* strain DG. *T. vaginalis* 202 and 263 were also used. Organisms were grown in screw cap tubes in Diamonds TYI-S-33 medium (TYI) at pH 6.2 in the presence of 10% heat-inactivated fetal bovine serum (FBS) (Gibco/BRL, Bethesda, Maryland), 100U/mL penicillin, 100µg/mL streptomycin (Pen/Strep. solution Gibco/BRL) and 2.5µg/mL Amphotericin B (Fungizone, Gibco/BRL). Cultures were incubated at 37°C in a 5% CO₂ atmosphere as well as in anaerobic glass jars using the GasPak Anaerobic system from BBL. The cultures were passaged every two to three days. Frozen axenic stocks were prepared by adding 10% FBS and 10% dimethyl sulfoxide (DMSO) to log phase growth cultures which, after mixing, were dispensed into 1.5mL cryovials (Sarstedt, Lachine, Quebec) and frozen at -70°C or in liquid N₂.

Plasmid Methodology

Small Scale Plasmid DNA Isolation from E. coli (Mini-prep)

Plasmid DNA isolation was carried out by a modified alkaline extraction procedure that was originally described by Birnboim and Doly (1979). A single colony of *Escherichia coli* from a Luria-Bertani (LB) ampicillin plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 50 µg/mL ampicillin) was inoculated to a 5 mL volume of LB-ampicillin broth. Cells were pelleted by centrifugation at 13 000 X g in a 1.5 mL

microfuge tube for 1 minute at room temperature. The bacterial cells were resuspended in GET medium (50 mM glucose, 10 mM EDTA 25 mM Tris-HCl, pH 8.0, with 4 mg/mL egg white lysozyme) and incubated at room temperature for 5 minutes. Cells were lysed by the addition of freshly prepared NaOH/SDS solution (0.2 N NaOH, 1% SDS) and allowed to incubate for 5 minutes at room temperature. The suspension was neutralized by adding 300 μ L of 7.5 M prechilled ammonium acetate to the suspension, mixed thoroughly and the sample was incubated on ice for 10 minutes. The samples were then centrifuged at approximately 13 000 X g for 10 minutes, the supernatant transferred to a new tube, and the plasmid DNA was precipitated by the addition of 500 μ L of isopropanol. The nucleic acid was collected by centrifugation at 13 000 X g for 20 minutes, the pellet was washed with 70% ethanol and then resuspended in 40 μ L of TE buffer. The solution was stored at -20°C.

Large Scale Plasmid DNA Isolation from E. coli (Midi-prep)

Plasmid DNA was isolated using the Circle Prep Spin Midi isolation procedure as described by the manufacturer (Bio/Can Scientific, Mississauga, Ontario). The Circle Prep procedure is based on the alkaline lysis procedure of Birnboim and Doly (1979). Briefly, a single fresh colony was inoculated to 30 mL of LB-ampicillin broth and shaken overnight at 37°C. The bacterial cells were pelleted, resuspended in water, and transferred to a 1.5 mL microfuge tube. Following alkaline lysis and neutralization, chromosomal and cellular debris were removed by centrifugation. The supernatant was then transferred to a new tube and Glassmilk buffer (a crystalline silica matrix) was added. The Glassmilk was used to bind the DNA and the Glassmik/DNA sample was transferred to a spin filter. The

Glassmilk/DNA complex was washed with NEW wash buffer and then the DNA was eluted from the Glassmilk with pyrogen-free water by centrifugation. The spin filter was discarded and the plasmid DNA was stored at -20°C.

DNA Sequencing

Sequencing of 5 cDNA clones from *T. vaginalis* was carried out using the dideoxy chain terminator method developed by Sanger *et al.* (1977). Previously, these genes were cloned into a λ gt 11 cDNA library using EcoRI linkers. One of the genes was found to contain a single EcoRI site and the two fragments, a and b, were subcloned into the pGEM 3Z vector. The other cDNA clones were cloned into the same vector. Upon my arrival in the lab, I proceeded to manually sequence all of the 5 clones including the clone having the internal EcoRI site. Primers and their respective sequences are listed in Appendix 1.

Oligonucleotides

Oligonucleotide primers were synthesized either at the University of Ottawa Biotechnology Institute by Mr. André Bergeron or Mrs. Nancy Delcellier, or by Genosys corporation. Sequences are listed in Appendix 1.

Plasmid DNA Preparation for Sequencing

Preparation of Template DNA for Manual Sequencing

The preparation of plasmid DNA for manual sequencing is similar to the small scale isolation procedure described earlier. A 5 mL LB-ampicillin broth culture was inoculated with a single colony and allowed to grow overnight with shaking at 37°C. Cells were centrifuged at 13 000 X g in a 1.5 mL microfuge tube. Cells were lysed in 100 μ L of

GET medium containing 100 µg/mL of RNase A. Tubes were incubated at room temperature for 15 minutes. A 200 µL volume of NaOH/SDS solution was added, the tube was gently mixed and was incubated at room temperature for 5 minutes. The suspension was neutralized by the addition of ice-cold potassium acetate solution (60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL distilled water) and was incubated on ice for 15 minutes. The chromosomal DNA was pelleted by centrifugation for 10 minutes at 13 000 X g. The supernatant was transferred to a new tube and was extracted with 400 µL of chloroform. One mL of absolute ethanol was added to the tube and plasmid DNA was allowed to precipitate overnight. DNA was centrifuged for 20 minutes at 13 000 X g. The DNA pellet was washed with 1 mL of 70% ethanol, centrifuged at 13 000 X g for 20 minutes, allowed to air dry, and was then resuspended in 40 µL of TE buffer.

Preparation of Template DNA for Automated Sequencing

A similar procedure was used in the preparation of plasmid DNA for automated sequencing. Terrific Broth with 50 µg/mL ampicillin was used in place of LB medium in order to achieve a higher cell density. Once the cells were pelleted the bacterial pellet was resuspended in 200 µL of GET medium, a 300 µL volume of NaOH/SDS solution was added, mixed by inversion, and incubated for 5 minutes on ice. The lysate was neutralized by the addition of 300 µL of 3.0 M ice-cold potassium acetate, pH 4.8, mixed by inversion and incubated on ice for 5 minutes. Cellular debris and chromosomal DNA were removed by centrifugation at room temperature for 5 minutes. The supernatant was transferred to a new tube. RNase A was added to a final concentration of 20 µg/mL and the sample was

incubated at 37°C for 20 minutes. The RNase treated solution containing plasmid DNA was extracted twice with 400 µL of chloroform. Each time the layers were vortexed briefly followed by centrifugation for 2 minutes, and the aqueous phase (top layer) was recovered. Total DNA was precipitated by the addition of an equal volume of 100% isopropanol, followed by centrifugation at room temperature for 10 minutes. The DNA pellet was washed with 500 µL of 70% ethanol and allowed to air dry for 10 minutes. The pellet was dissolved in 32 µL of double distilled water. Plasmid DNA was precipitated by adding 8 µL of 4 M NaCl and then adding 40 µL of autoclaved 13% PEG 8000 (polyethylene glycol MW 8000) solution. After thorough mixing, the sample was allowed to incubate on ice for 20 minutes and was pelleted by centrifugation at 13 000 X g for 15 minutes at 4°C in a microcentrifuge. The supernatant was carefully removed and the pellet was rinsed with 500 µL of 70% ethanol. The plasmid DNA was allowed to air dry for 10 minutes, resuspended in 20 µL of deionized water, and stored at -20°C.

Sequencing Reactions and Denaturing Polyacrylamide Gel Electrophoresis

Sequencing reactions were either performed manually using the dideoxy-chain terminator method developed by Sanger *et al.* (1977) using Sequenase version 2.0 enzyme (USB/Amersham, Oakville, Ontario) and the procedure as outlined by the manufacturer, or given to André Bergeron to be sequenced using the automated sequencer from Applied Biosystems (Foster City, California). Manual sequencing reaction products were separated by electrophoresis on 6% denaturing polyacrylamide gels containing 7 M urea using the IBI model STS-45 sequencing apparatus. A 1 X TBE buffer (10 X TBE buffer: 0.89 M Tris-base, pH 8.0, 0.89 M boric acid, 20 mM EDTA) was used in the upper chamber and

1.5 X TBE buffer was used in the lower chamber for electrophoresis. Gels were pre-run for at least 1 hour prior to loading of samples. Electrophoresis was carried out using 40 watts constant power and maximal setting of 40 milliamps and 2 500 V (Pharmacia LKB ECPS 3000/1500 power supply, Baie d'Hurffé, Quebec). After electrophoresis, plates were separated and the gels were lifted onto 3 MM Whatman paper, dried under vacuum for 1 hour and 15 minutes at 80°C (Bio-Rad, Model 583 Gel Dryer, Mississauga, Ontario) and exposed to X-ray film (Cronex 4, Picker International, Brampton, Ontario) for 48 hours.

Sequence Analysis and Primer Design

Sequence analysis was carried out using the IBI Pustell sequence analysis program in order to generate a restriction map of the sequence of interest, and to determine potential amino acid sequences from the DNA fragments. Database searches were carried out using the National Institutes of Health (NIH) NCBI BLAST programs. NCBI was accessed via e-mail at blast@ncbi.nlm.nih.gov. Primers were designed using nearest neighbour analysis as well as the Primer Designer program.

Isolation of *T.vaginalis* Nucleic Acids

Total Parasite RNA

Total RNA was isolated from 2×10^8 log phase organisms grown under aerobic and anaerobic conditions using TriPure Reagent (Boehringer Mannheim, Laval, Quebec) as described by the manufacturer with the following exception. To ensure that the RNA was free of any DNA, DNase 1 was added to a final concentration of 10 µg/mL and the sample was incubated at 37°C for 20 minutes and at 55°C for 10 minutes. The RNA was

quantitated by absorbance at A_{260} using the Genequant Spectrophotometer (Pharmacia).

The remaining sample was stored at -70°C .

Isolation of mRNA

Polyadenylated RNA was purified from total RNA using the Oligotex Spin Column protocol as described by the manufacturer (Qiagen). Briefly, 1 mg of total RNA was mixed with DEPC-treated water, 2 X binding buffer and the Oligotex matrix. The RNA was heat-denatured for 3 minutes at 65°C followed by an incubation at room temperature for 10 minutes. This allowed for hybridization between the Oligotex beads and polyadenylated RNA. The mixture was centrifuged at $13\,000 \times g$ for 2 minutes in a microfuge tube and the supernatant was aspirated. The oligotex-RNA complex was resuspended in 400 μL of wash buffer, transferred to a spin column, centrifuged for 30 seconds and the flow-through was discarded. This step was then repeated using a new microfuge tube. The mRNA was eluted with 20 μL of preheated (70°C) elution buffer. Using the same eluate, the procedure was repeated to ensure maximal yield. Typical yields of mRNA were in the range of 1 to 5 μg as determined by A_{260} using the Genequant Spectrophotometer (Pharmacia).

RNA Analysis

Denaturing Glyoxal Agarose Gel Electrophoresis

The method described was originally outlined by McMaster and Carmichael (1977). Briefly, total RNA, mRNA and non-polyadenylated RNA suspended in DEPC treated water were added to 15 μL of sample buffer (0.01 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.0, 0.1% SDS, 50% DMSO, 6% deionized glyoxal). Samples were denatured at 60°C for 15

minutes, cooled on ice for 2 minutes and then loaded onto a 1.2% agarose gel containing 10mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0. Gels were run with re-circulation of the running buffer in a LKB Miniphor electrophoresis unit (LKB/Pharmacia) at 40 volts for 2-2.5 hours. After electrophoresis, gels were stained two ways: for 30 minutes in the running buffer with acridine orange at a concentration of 30 $\mu\text{g}/\text{mL}$ and destaining for 30 minutes in sodium phosphate buffer, pH 7.0. In the second method, the gel was stained for 10 minutes in the running buffer with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide with subsequent destaining in sodium phosphate buffer, pH 7.0 for 20 minutes. RNA was visualized and gels were photographed under short-wave ultraviolet (UV) light.

Isolation of Genomic DNA

Isolation of DNA was carried out according to the method described by Chou and Tai (1996). Cells were harvested from 13 mL of logarithmic culture at 900 X g for 10 minutes in a Heraeus OmnifugeRT Centrifuge at 4°C. Cells (ca. 2×10^7) were transferred to a 1.5 mL microfuge tube and washed with 1 mL of cold, sterile PBS, pH 7.4. After centrifugation for 1 minute at 4°C, the cell mass was lysed by adding 50 μL of lysis buffer containing 1% Triton X-100 in sterile PBS, pH 7.4, vortexed vigorously for 30 seconds and immediately centrifuged in a microfuge tube for 1 minute at 4°C. The Triton X-100 soluble fraction was transferred to a fresh tube and the insoluble fraction was resuspended in another 50 μL of lysis buffer. SDS was added to both fractions to give a final concentration of 1% and the nucleic acids were immediately extracted with water saturated phenol, pH 7.9 at 65°C. 10 μL of the nucleic acids in each fraction were examined by electrophoresis in a 1% agarose gel. RNA was found in the Triton soluble

fraction and DNA was predominantly found in the Triton insoluble fraction. RNA was removed from the Triton insoluble fraction by RNase digestion to obtain pure DNA. The quality and size of DNA was evaluated by overnight electrophoresis at 4°C on a 0.5% agarose gel. Nucleic acids were precipitated a final time in 90% ethanol at -80°C for 20 minutes, followed by washing with 70% ethanol. DNA was resuspended in Tris-EDTA (TE) buffer.

DNA Analysis

DNA Agarose Gel Electrophoresis

Samples of DNA were mixed with loading buffer containing 5% glycerol, 0.05% bromophenol blue and 0.05% xylene cyanol. Electrophoresis was carried out in 0.5-2% agarose gels using Tris-borate buffer or Tris-acetate buffer. Both the LKB 2013 (Miniphor unit) and the LKB 2012 (Maxiphor unit) were utilized. Electrophoresis of mini gels was at 40 volts for 2- 2.5 hours and/or 100 volts for 1 hour. For maxi gels the same voltage settings were used for 3-4 hours or for 2 hours. High molecular weight DNA was electrophoresed at 25 volts overnight at 4°C in a 0.5% agarose gel. Ethidium bromide (0.5 µg/mL) was used to stain the gels for 10-30 minutes in the original running buffer. Destaining took place in double distilled water for 15-30 minutes.

Isolation and Purification of DNA Fragments From Agarose Gels

Low melting point (LMP) agarose gels in TAE buffer were used whenever DNA fragments were to be purified. After staining, DNA was isolated and excised out of the LMP/TAE gel using long wave UV light, and placed in a pre-weighed microfuge tube. The DNA fragment was purified by either using the Wizard PCR Preps DNA Purification

System (Promega) or the GeneClean II Kit with or without Spin Modules (Bio/Can Scientific) as described in the protocols supplied.

Southern Blotting and Hybridization

Transfer Using Nylon Membranes

Two methods were employed for Southern transfers. After agarose gel electrophoresis, DNA was depurinated by soaking the gel in 0.25 M HCl, for 10 minutes followed by several rinses with distilled water. DNA was transferred to a Pall Biodyne nylon membrane B using 0.4 M NaOH and the capillary transfer procedure outlined by the manufacturer. The membrane was rinsed in 2 X SSC for 5 minutes and then allowed to air dry. The membrane was either used immediately or stored overnight at 4°C. Membranes were soaked in 2 X SSC (0.3 M NaCl, 0.03 M sodium citrate dihydrate) and prehybridized for at least 2 hours at 65°C in 5 X Denhardt's solution, 5 X SSC, 0.1% (w/v) SDS and 100 µg/mL (final concentration) of sheared salmon sperm DNA. Hybridizations were performed overnight at 65°C in the same pre-hybridization buffer supplemented with probe solution (0.1 mL of 5 mg/mL sheared salmon sperm carrier DNA, 0.9 mL of water and approximately 1×10^6 cpm/mL of [α - 32 P]dCTP labeled DNA/10 mL of hybridization buffer). The probe solution was heated for 10 minutes at 100°C and chilled for 15 minutes in an ice-bath prior to being added to the hybridization solution. Membranes were rinsed with 2 X SSC for 10 minutes at room temperature to remove excess probe. Membranes were washed twice for 45 minutes in 2 X SSC, 0.1% SDS. Prehybridization and hybridization steps were carried out in glass bottles using either the Autoblot Micro Hybridization Oven (Bellco Glass Inc., Vineland, NJ) or the Model

2000 Micro Hybridization Incubator (Robbins Scientific, Sunnyvale, CA). Washed membranes were wrapped in plastic wrap and exposed to X-ray film (Cronex 4 or Amersham Hyperfilm-MP, Amersham Canada Ltd., Oakville Ontario) at -70°C using film cassettes.

Hybridizations in the Agarose Gel Matrix

The second method involved performing a Southern hybridization directly on a dried agarose gel. Gel Bond Film (FMC Bioproducts) is a flexible support consisting of agarose-coated polyester. An appropriate size of the matrix is cut to fit the agarose casting tray snugly with none of the edges overlapping the casting tray. The matrix was placed hydrophilic side up and was held down by taping the edges with sequencing tape. A 1.2% TBE agarose solution was allowed to cool to 55°C and was then poured over the agarose matrix. Once the gel had solidified, it was placed in the LKB 2012 or 2013 and electrophoresis was carried out at 100 volts for 1 hour and 2 hours respectively. After the DNA had migrated a sufficient distance, the gel was placed on two pieces of 3MM Whatman paper, each piece larger than the one above it. The gel was dried under vacuum at 70°C for 1 hour (Maxi Gels) or for 45 minutes (Mini Gels) using a Bio-Rad Gel Dryer, Model 583. The dried gel was then placed into a hybridization bottle and allowed to prehybridize 16 hours at 40°C in a solution containing a final concentration of 6 X SSC, 5 X Denhardt's solution, 0.05 M Na_2HPO_4 and 0.05 mg/mL of sonicated, salmon sperm DNA. Probe solution was prepared as above and the prehybridization solution was decanted. The probe solution was added to a new volume of prehybridization solution and hybridization was allowed to proceed for 7 to 8 hours at 37°C . Membranes were washed 5

times with 3 X SSC at 50°C for 15 minutes. Membranes were removed from the bottles, the hydrophobic side of the matrix was wiped off with a Kimwipe (Kimberly-Clark, Roswell, GA) and the gel was washed twice with 3 X SSC, 1% SDS for 15 minutes at 50°C. The gel was wrapped in plastic wrap and exposed to X-Ray film (Amersham Hyperfilm-MP) overnight at -70°C.

Restriction Digestion and Modification of DNA

Enzymes

All restriction endonucleases and modifying enzymes were supplied by the following manufacturers: Perkin-Elmer, Pharmacia, New England Biolabs, Gibco/BRL, Clontech, Promega and Stratagene.

Restriction Digests

Generally, for every microgram of DNA 1-2.5 units of an enzyme was used in a total reaction volume of 10 µL and 2-5 units were used in a 20 µL reaction. All digestions were performed for 1 hour in the recommended buffers and temperatures as outlined by the manufacturer. Restriction endonucleases were inactivated by heating.

Ligations

In ligating restriction fragments into a Multiple Cloning Site, normally a 1:1 molar ratio of vector to insert was used. Ligations took place in 30 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT and 5 mM ATP with 2 Weiss units of T4 DNA ligase per 20 µL reaction. The reaction was allowed to proceed for 6 hours at 15°C. In ligating PCR products into pCR-Script (SK+) vector, a blunt-ended vector, a 100:1 insert to vector ratio was carried out under similar reaction conditions with the addition of 5 units of *Srf* I

in a 10 μ L volume. The reaction was incubated at room temperature for 1 hour. PCR products were also ligated into the T-tailed vector pCR 2.1 (Invitrogen, Carlsbad, California) in a 1:1 vector to insert molar ratio. A 10 μ L ligation reaction was set up as follows: 0.5 - 1.5 μ L of fresh PCR product, 1 μ L of 10X ligation buffer (60 mM Tris-HCl, pH 7.5, 60 mM MgCl₂, 50 mM NaCl, 1 mg/mL BSA, 70 mM β -mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, 10 mM spermidine), 25 ng/ μ L pCR 2.1 vector, sterile water to a volume of 9 μ L and 1 μ L of T4 DNA ligase (4.0 Weiss units). The reaction was allowed to incubate overnight at 14°C.

Double Stranded (ds) cDNA Synthesis and Adaptor Ligation

Polyadenylated RNA isolated from both aerobic and anaerobic cultures was used as a template in the Marathon cDNA Amplification System (Clontech, Palo Alto California). Briefly, the first strand of cDNA is synthesized using a modified lock-docking oligo (dT) primer which contains two degenerate nucleotide positions at the 3' end. These nucleotides place the primer at the start of the poly-A tail. Using Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) the cDNA is synthesized. Second strand synthesis is carried out according to the method of Gubler and Hoffman (1983) in the presence of *E. coli* DNA polymerase I, RNase H, and *E. coli* DNA ligase. Following the generation of ds cDNA, the DNA is blunt-ended with T4 DNA polymerase. Using DNA ligase, the blunt-ended DNA fragments are ligated to the Marathon cDNA Adaptors. The adaptors are partially double stranded and are phosphorylated at the 5' end to favour blunt-ended ligation to both ends of the cDNA. The result of the ligations is an uncloned library of adaptor ligated ds cDNA. Gene Specific Primers (GSPs) (Appendix 1)

were designed in areas of high sequence similarity to the NADPH-dependent butanol dehydrogenase gene (*adh1*) found in *C. acetobutylicum*. One primer was designed from the 5' end, the other from the 3' end of the sequence. The 5' and 3' RACE reactions were carried out with KlenTaq-1 DNA Polymerase (Clontech). In brief, a 5 µL aliquot of the ds cDNA generated above was diluted in 245 µL of Tricine-EDTA buffer, pH 8.3. For 5' RACE, the primer DP-BDH-GSP1 (GSP1) and the Adaptor Primer 1 (AP1) supplied with the kit were used to generate the 5' RACE fragment. Similarly, DP-BDH-GSP2 (GSP2) and AP1 were used to generate the 3' RACE fragment. The following components were combined in Perkin-Elmer Gene-Amp 0.5 mL reaction tubes (Applied Biosystems); 36 µL of sterile water, 5 µL of 10X KlenTaq PCR reaction buffer, 1 µL of dNTP (10 mM), and 1 µL of Advantage KlenTaq Polymerase Mix (50X), 5 µL of the diluted ds cDNA, 1 µL of GSP1 or 2 and 1 µL of AP1. The following controls were also performed: 5' and 3' RACE controls with DNA supplied with the system, positive internal control using DNA supplied with the system and the GSPs for the TFR gene, 5' and 3' RACE controls with ds cDNA generated from placental mRNA, positive internal control using the GSPs for the TFR gene and ds cDNA that was generated from placental mRNA. The negative controls entailed using only one of the primers in a reaction. The cycling parameters were as follows: 94°C for 1 minute, 94°C for 30 seconds followed by 72°C for 4 minutes (5 cycles), 94°C for 30 seconds followed by 70°C for 4 minutes (5 cycles), 94°C for 20 seconds followed by 68°C for 4 minutes (25 cycles). All primers had a T_m of at least 70°C. This allowed for utilization of the Touchdown PCR protocol outlined above.

Transformation of *E. coli* Competent Cells

Electroporation of E. Coli

DH5 F' *E. coli* bacteria was used for all experiments involving electroporation. The BRL Cell-Porator Electroporation System with microelectroporation chambers (Gibco/BRL) were used. The required number of microelectroporation chambers and autoclaved microfuge tubes were placed on ice. The electroporator chamber safe was filled with ice-water. 1 mL of room temperature S.O.C. medium (2 g tryptone, 0.5 g yeast extract, 1 mL of 1 M NaCl, 0.25 mL of 1 M KCl, 1mL of 2 M Mg^{2+} stock solution, 1 mL of 2 M filter sterilized glucose to 100 mL) was placed in a 17 x 100 mm culture tube (Falcon). 1-50 ng of ligated plasmid DNA was added to each microfuge tube. A 100 μ L aliquot of DH5 F' *E. coli* cells was removed from -70°C and allowed to thaw on wet ice. Thawed cells were mixed by gentle tapping and 20-25 μ L of the cells were added to the each chilled microfuge tube containing plasmid DNA. A 20 μ L volume of the cell/DNA mixture was pipetted into each microelectroporation chamber. Electroporation was carried out using the following settings: 400V, 330 μ F, low ohms, and a fast charge rate 4000 Ω resistance on the voltage booster. After electrotransformation, cells were removed from the electroporation chamber and immediately added to the culture tubes containing 1 mL of room temperature S.O.C. medium. Tubes were placed in a shaking incubator set at 225 rpm for 1 hour at 37°C. Various aliquots of *E. coli* were spread on LB plates containing 50 μ g/mL ampicillin, 0.5 mM IPTG and 40 μ g/mL of X-Gal. Plates were incubated overnight at 37°C.

Chemical Transformation of Epicurian Coli XL1-Blue MRF' Kan Supercompetent Cells

The supercompetent cells were used in the transformation of PCR amplicons ligated into the pCR-Script Amp SK(+) cloning vector (Stratagene, PDI BioScience). The transformation protocol was as described by the manufacturer. Briefly, the Epicurian cells were thawed on ice and 40 μ L of cells were transferred to a prechilled 15 mL Falcon 2059 polypropylene tube for every transformation. β -mercaptoethanol was added to the cells to a final concentration of 25 mM, cells were mixed gently every 2 minutes on ice for 10 minutes. 2 μ L of the cloning reaction were mixed with the cells. The transformation reaction was incubated on ice for 30 minutes. The reaction was heat pulsed for 45 seconds at 42°C, and subsequently chilled on ice for 2 minutes. A 450 μ L volume of 42°C S.O.C. medium was added to the transformation reaction and the sample was incubated at 37°C for 1 hour with shaking at 225 rpm. Using a sterile spreader, 50, 100, 150 and 190 μ L of the transformation reactions were plated on LB-ampicillin plates containing X-Gal, and IPTG. The control reaction was plated on LB-chloramphenicol agar plates. This control was carried out to show that ligation into the cloning site was successful. Plates were incubated overnight at 37°C. Colonies were lifted onto nylon membranes and screened using a cDNA probe that contained the fragment of interest.

Transformation of pCR 2.1 Plasmid Into INV α F' Cells

All reagents and tubes were placed on ice prior to carrying out the procedure. A 50 μ L vial of frozen INV α F' cells was thawed for each ligation/transformation reaction on ice along with a vial of 0.5 M β -mercaptoethanol. A 2 μ L volume of the 0.5 M β -

mercaptoethanol was pipetted into each vial of competent cells and the cells were mixed by gentle stirring with the pipette tip. A 2 μ L aliquot of the ligation reaction was added directly to the competent cells and the mixture was stirred gently with the pipette tip. The vials were incubated on ice for 30 minutes. The cells were then heat shocked for 30 seconds in a 42°C water bath and subsequently chilled on ice for 2 minutes. A 250 μ L volume of room temperature S.O.C. was added to each tube and the vials were shaken horizontally at 37°C for 1 hour at 225 rpm. The mixtures were then placed on ice. From each transformation reaction, a 50 and 200 μ L aliquot were spread onto separate labeled LB agar plates containing ampicillin, X-Gal, and IPTG. Plates were incubated at 37°C for at least 18 hours. Plates were shifted to 4°C for 2-3 hours to allow for proper colour development. 10 white colonies from each transformation reaction were picked and grown in 2-5 mL of LB broth containing 50 μ g/mL of ampicillin. The plasmids were isolated and restriction digests were carried out to determine if the PCR product was in fact incorporated into the vector.

Preparation of Radiolabeled Probes and Bacterial Colony Lifts

Randomly Primed Labeling

DNA was labeled using the Prime-A-Gene System from Promega. This system is based on the method developed by Feinberg and Vogelstein (1983) which uses random hexanucleotides to prime DNA synthesis. 10-25 ng of DNA were denatured by heating for 2 minutes at 100°C, followed immediately by chilling the DNA in an ice-bath. The DNA was added to a reaction tube containing 1 X labeling buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 2 mM DTT, 200 mM HEPES, pH 6.6, 5.2 A₂₆₀ units/mL random

hexanucleotides), 20 μ M of dATP, dGTP, dTTP, 400 μ g BSA, 333 nM (50 μ Ci,) [α - 32 P]dCTP (3000 Ci/mmol). 5 units of DNA polymerase I, Large Klenow Fragment, were added and the sample was incubated at room temperature for 1 hour (25 ng of template) or overnight (10 ng of template) and then heated for 2 minutes at 100°C. EDTA was added to a final concentration of 20 mM and the reaction was passed through a Sephadex G-50 column (Pharmacia) in order to remove unincorporated nucleotides. 18 fractions were collected. Fractions containing the probe were pooled, counted using the LKB 1214 RackBeta Liquid Scintillation counter and either used immediately in the hybridization procedures or stored at -20°C.

Colony Lifts and Hybridizations

Bacterial colonies were lifted onto Colony/Plaque hybridization transfer membranes as described by the manufacturer (NEN Life Science Products, Dupont). The aqueous prehybridization/hybridization buffer consisted of the following ingredients in a 10 mL volume: 8.46 mL water, 1 g Dextran Sulphate, sodium salt (MW ~ 500 000), 1 mL of 10% SDS. After heating for 30 minutes 0.58 g of NaCl was added and the solution was heated for an additional 15 minutes. The buffer was stored at -20°C until needed. The membranes were prehybridized from 2 to 16 hours at 65°C. The solution was decanted, and fresh solution with [α - 32 P] dCTP labeled probe was added to the bottles. Hybridization was allowed to occur for 7 to 16 hours. Membranes were washed twice with 2 X SSC for 5-10 minutes at room temperature. Two washes were carried out at 60°C for 30 minutes with 2 X SSC/1.0% SDS. Two washes were performed at room temperature with 0.1 X SSC for 30 minutes. If background was still high, an additional

wash was carried out at 65°C for 20 minutes with 0.1 X SSC/0.1% SDS. The membranes were wrapped in plastic wrap and exposed to X-Ray Film (Cronex 4, or Amersham Hyperfilm-HP) at -70°C. The formamide protocol used the following reagents in the prehybridization/hybridization buffer. 1% SDS, 2 X SSC, 10% Dextran Sulphate, 50% deionized formamide. Prehybridization was carried out at 45°C for 2 to 16 hours. Hybridization was carried out for 7 to 16 hours at 45°C. The membranes were washed with 2 X SSC for 10 minutes. The discs were then washed twice at 45°C for 20 minutes with 2 X SSC/1.0% SDS. The third and fourth washes were carried out at 45°C for 20 minutes with 0.2 X SSC/1.0% SDS. The membranes were wrapped in plastic and exposed to X-ray film as above.

Detection of Butanol in Supernatants of *T. vaginalis*

T. vaginalis was grown under aerobic and anaerobic conditions in 15 mL screw-cap culture tubes as described previously. Cultures were passaged at least 3 times before being passaged into a 30 mL volume in 50 mL polypropylene tubes (Corning, Nepean, Ontario). Mid log phase cultures were counted (2×10^6 Tv/mL) and centrifuged at 900 X g for 10 minutes at 4°C. Supernatants were transferred to new polypropylene tubes. NaCl was added to the supernatants until the solutions were supersaturated. The foam was aspirated from the supernatant and 3 mL of diethyl ether was added to the supernatant. Samples were vortexed and centrifuged at room temperature for 10 minutes at 900 X g. The organic layer (top) was collected and placed into 1.5 mL cryovials (Sarstedt). A series of dilutions (1:2, 1:5, 1:10, 1:100) were carried out using ether as the diluent. Samples were analyzed at the Mass Spectrophotometry Centre by Dr. Clem Kazakoff, Department

of Chemistry, University of Ottawa. 1-butanol was used as a positive control, followed by an ether blank (negative control). This was then followed by the organic sample from the supernatant of *T. vaginalis*. Prior to analyzing the samples in the gas chromatograph, ether, the solvent in which the samples are suspended, was loaded into the column to ensure that column was free of any other organic material that may interfere with organics present in the experimental samples. Each time the column was to be loaded with a new sample, the blank was run before to ensure the column was clean.

CHAPTER 3: CHARACTERIZATION OF *T. VAGINALIS* cDNA CLONES

INTRODUCTION

The original goal of this project was to fully characterize the ECP gene, since its proteolytic activity is believed to be important in the initial stages of *T. vaginalis* infection. Four cDNA clones had been identified because of their reactivity to an anti-serum raised against ECP. Clone 23-1-1 gave the strongest signal, was partially sequenced, and was found to be homologous to a malarial exoantigen called ABRA found in *Plasmodium falciparum*. Since 3 of the four potential proteinase clones isolated from the cDNA library had not been sequenced, it was thought that sequence analysis would reveal one of the other clones would contain sequences encoding for a cysteine proteinase.

This chapter outlines the analysis of these clones.

RESULTS

Sequence Analysis of Potential *T. vaginalis* ECP cDNA Clones

By using synthetic oligonucleotide primers, the cDNA clones 23-2-1, 23-4-1, 23-4-3a and 23-4-2b were sequenced in their entirety in both directions via the dideoxy-chain terminator method (Sanger *et al.* 1977). Primers are listed in Appendix 1. Clone 23-4-2 was found to have an internal EcoR I site and the two fragments were cloned into the EcoR I cloning site of the pGEM 3Z vector. The cDNA fragments were termed 23-4-3a and 23-4-2b, 3a being the smaller of the two fragments. The other clones were called 23-2-1 and 23-4-1. Sequences were analyzed by using a non-redundant database search.

Clone 23-2-1 was 368 nucleotides long and had an ORF spanning its entire length with no translational start or stop (Figure 1). This clone showed similarity at the amino acid level to phosphoenol pyruvate carboxykinase (Figure 2). Clone 23-4-1 was found to be 240 nucleotides in length and was not found to be homologous to anything in the Genbank database. The two fragments of clone 23-4-3 were found to have ORFs spanning 217 bases (23-4-3a), and 946 bp (23-4-2b), respectively. (Figure 3). The entire insert was found to have an ORF of 1163 bp which was terminated by a stop codon. A poly (A) tract was not observed (Figure 3). The sequence was found to have 70% amino acid identity and over 80% similarity to an NADPH-dependent butanol dehydrogenase protein (ADH1) found in *C. acetobutylicum* (Youngleson *et al.* 1989) (Figure 4).

Figure 1. Sequence analysis of clone 23-2-1 showing both the nucleic acid and amino acid sequences.

5' CTACACATGGTGACTACTTCAAGCACTACCTCGAGATGGGCCAAGCTCGCCAAGACACCACTTGTCTTC⁶⁹
L T M V T T S S T T S R W A K L A K T P L V F²³

CACGTCAACTGGTTCCGCAAGTCCCCAGCTGGCAAGTTCCTTTGGCCAGGCTACGGTCAGAACGCTCGT¹³⁸
H V N W F R K S P A G K F L W P G Y G Q N A R⁴⁶

GTCCTTGGCTGGATGGTCAACAGAACTCTACGCAAGGCCCCAGAGCACCTTTCTCCACTCGGCTACGTC²⁰⁷
V L G W M V N R T L R K A P E H L S P L G Y V⁶⁹

CCAGAGTACGAGGATATCGATTGGGAAGGCCTCAACTTCACAAAGGAGCAGTTCGAGGAAGTTATGCAC²⁷⁶
P E Y E D I D W E G L N F T K E Q F E E V M H⁹²

CAGGATAAGGAGAAGATCAAGGCCCAAGTTGCTGCTAACGATGACTACCTCATCAACAAGATCGGCCAC³⁴⁵
Q D K E K I K A Q V A A N D D Y L I N K I G H¹¹⁵

GTTTCCTGGGAAACGTCGAGGAGT 3'³⁶⁹
V S W E T S R S¹²³

Figure 2. Comparison of the protein coding sequence of 23-2-1 to human phosphoenol pyruvate carboxykinase.

TV_PPCK LTMVTTSSTSRWAKLAKTPLVFHVNWFRKSPAGKFLWPYGGQNAR
:: : . :::::::::: . :::::::::::::::
PPCK_HUMAN MHDPFAMRPFFGYNFGKYLAHWLSMAQHPAALKPKIFHVNWFNRDKEGKFLLWPGFGENSR
480 490 500 510 520 530

50 60 70 80 90
TV_PPCK **VLGWMVNR**TLRKAPEHLSPLGYVDKEKIKAQVAANDDYI-INKIGHVSWETSRS
 :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
PPCK_HUMAN **VLEWMFN**RIDGKASTKLTPIGYIPKEDALNLKGLGHINMMELFSISKEFWEKEVEDIEKY
 540 550 560 570 580 590

Figure 3. The complete DNA sequence of clone 23-4-2 that is homologous to *adh1*. The EcoRI site is indicated by a dotted line. Various restriction sites have been underlined. The termination codon is indicated by a double underline.

Xho I
↓

5' GATCTCTATCATGGCAAGGGCGCTCTCGAGAATCTCAAGACACTCAAGGGCAAGAGAGCCTTCG⁶³
D L Y H G K G A L E N L K T L K G K R A F₂₁

Hpa I
↓

TCGTACACAGGTTCCACGCTATGCGCAAATTCGGATTCTTGACAAGGTTGTTAACTACCTCAAGGAAGCCAAGT¹³⁸
V V T G S H A M R K F G F L D K V V N Y L K E A K₄₆

EcoR I
↓

TCGAGGTTTCATGTCTTCGATGGTGTGCGAGCAAGACCCATCTGTGCAACAGTCCTCAAGGGCGCTAAGACCATGG²¹³
F E V H V F D G V E Q D P S V E T V L K G A K T M₇₁

Bgl II
↓

AAGAATTCAAGCCAGACTGGATTGTTGCTCTCGGTGGTGGCTCTCCAATCGATGCTGCCAAGGCCATGTGGGCTG²⁸⁸
E E F K P D W I V A L G G G S P I D A A K A M W A₉₆

Bgl II
↓

TTTACGAATACCCAGAGATGACCTTCGAAAAGCTCATCGTCCCATTCATTCCCAAGAACTCCGCCAAAAGGCC³⁶³
V Y E Y P E M T F E K L I V P F S F P E L R Q K A₁₂₁

Alw I
↓

ACTTCTGCGCCATCACATCAACATCTGGCACAGCTACAGAAGTCACAGCCTTCTCTGTTCATCACAGACTACCAGA⁴³⁸
H F C A I T S T S G T A T E V T A F S V I T D Y Q₁₄₆

Alw I
↓

AGGGCATCAAGTACCCACTTGCTGATTCAACATCACACCAGATGTTGCCATTGTGATCCAGATCTTGCTGAGA⁵¹³
K G I K Y P L A D F N I T P D V A I V D P D L A E₁₇₁

Hind III
↓

CAATGCCAAAGAAGCTCGTCGCTTTACAGGCATGGATGCCCTCACACAGCCTTTCGAAGCCTACGTTTCTACAC⁵⁸⁸
T M P K K L V A F T G M D A L T H A F E A Y V S T₁₉₆

Hind III
↓

TTCACTGCGATTACACTGATGGCTTGCCCTCCACTCCATCAAGCTCATCTTCGAAACACTCGAAGCTTCCTTCA⁶⁶³
L H C D Y T D G L A L H S I K L I F E T L E A S F₂₂₁

Sal I
↓

ACGGCTGCATGAAGGCCGCGCTACAATGCACAACGCTCAGTGCCTTGCTGGTATGGCCTTCTCCAACAGCCCTC⁷³⁸
N G C M K A R A T M H N A Q C L A G M A F S N S P₂₄₆

BstX I
↓

CTCGCATTGTCCACTCAATGGCCCAAGACAGGCGCTGCTTACACAAGTGGCCACATCGTCCACGGCTGCGTA⁸¹³
P R H C P L N G P Q D R R C L H K W P H R P R L R₂₇₁

Sal I
↓

ACGCCATGTACCTTCCAAAGGTTATCAAGTACAACCTCCAAGAACGAAGAGGCCCTCCACCGCTATGGTGACATCG⁸⁸⁸
N A M Y L P K V I K Y N S K N E E A L H R Y G D I₃₉₆

Sal I
↓

CTCGCTTCCTTGGAATGCAAGGCAAGGATGATGTCGAAGCTGTCGAGGCTATCTGCCAGAGAATCCGTGACCTCT⁹⁶³
A R F L G M Q G K D D V E L V D A I C Q R I R D L₃₂₁

Mva I
↓

GCGTCAAGATCGAAGTCCCACTCTGCATCAAGGACTACGAGAACGGAATGATCAACGAACAAGAGTACCTTGCCA¹⁰³⁸
C V K I E V P L C I K D Y E N G M I N E Q E Y L A₃₄₆

Mva I
↓

AGCTCAAGGATGTTGCTACAAACGCTATCGGCGATGCTTGACAGGCTCTAACCCACGACAGCCAACCCAGGAGG¹¹¹³
K L K D V A T N A I G D A C T G S N P R Q P T Q E₃₇₁

Xho I
↓

AAATGGAGAAGCTCCTCCTCGCTGTCTTCTATGACAAGCCAATCGATTTC¹¹⁸⁸
E M E K L L L A V F Y D K P I N F₃₈₈

Xho I
↓

AAGATTACCC 3'¹¹⁹⁹

Figure 4. Comparison of the putative BDH protein found in *T. vaginalis* and the ADH1 protein of *C. acetobutylicum*.

	10	20	30	40	50
BDH	DLYHGKGALENL	KTLLGKRA	VFVTGSHAMR	KFGFLDKVV	NYLKAEAKFEVHVF
ADH1_C	MMRFTLPRDI	YYGKSLEQL	KNLKGKKA	MLVLGGGSM	KRFGFVDKVLGYLKEAGIEVKLI
	10	20	30	40	50
	60	70	80	90	100
BDH	DGVEQDPSVET	VLKGAKTMEEF	KPDWIVALGGG	SPIDAAKAMW	AVYEYPEMTFEKLI
ADH1_C	EGVEPDPSVET	VFKGAEELMRQ	FEPDWIIAMGGG	SPIDAAKAMW	IFYEHPEKTFDDIKDPF
	70	80	90	100	110
	120	130	140	150	160
BDH	SFPELRQKAHF	CAITSTSGTATE	VTAFSVITDYQ	KGIKYPLADFN	ITPDVAIVDPDLAET
ADH1_C	TVPELRNKAFL	AIPSTSGTATE	VTAFSVITDYK	TEIKYPLADFN	ITPDVAVVDSLAET
	130	140	150	160	170
	180	190	200	210	220
BDH	MPKKLVAFTG	MDALTHAFEAY	VSTLHCDYTD	GLALHSIKLI	FETLEASFNGCMKARATMH
ADH1_C	MPPKLTATHT	GMDALTHAIEAY	VATLHSPFTD	PLAMQAIEMINE	HLFKSYEGDKEAREQMH
	190	200	210	220	230
	240	250	260	270	280
BDH	NAQCLAGMAF	SNSPPRHCP	LNQPDRLCLH	KWPHRPLRNAM	YLPKVIKYNSKNEEALHR
ADH1_C	YAQCLAGMAF	SNALLGICH	SMAHKTGAVF	HI-PHG--CANAI	YLPYVIKFNSKT--SLER
	250	260	270	280	290
	300	310	320	330	340
BDH	YGDIAFLG	MQGKDDVEL	VDAICQIRID	LCVKIEVPLCI	KDYENGMINQEY
ADH1_C	YAKIAQ	QISLAGNTNEEL	VDSLINLVKEL	NKKMQIPTTL	KEY--G-IHEQE
	300	310	320	330	340
	360	370	380		
BDH	TNAIGDACTGS	NPRQPTQEEME	KLLLVFYDKP	INF	
ADH1_C	ERAIGDACTGS	NPRQLNKDEM	KKIFECVYYG	TEVDF	
	360	370	380		

DISCUSSION

The most comprehensive classification scheme of proteinases is based on their catalytic mechanism and they are also distinguished from each other based on their sensitivity to various inhibitors (Hartley 1960). Cysteine proteinases are specifically inhibited by iodoacetamide, iodoacetate and N-ethyl maleimide. TLCK, L-tosyl-amido-2-phenylethyl chloromethyl ketone (TPCK), antipain, leupeptin and chymostatin are other potent inhibitors, TLCK being used extensively in *T. vaginalis* research. To further prove that a particular proteinase is a CP, reducing agents such as cysteine and DTT can be used to enhance activity. Azocasein hydrolysis is also a strong indicator that the enzyme being studied is a CP. Work in our lab using TLCK and azocasein has demonstrated that the preparation containing the 60 kDa protein has CP activity (North 1982).

Whenever they have been looked for, CPs have been found in a variety of parasitic protozoa, such as various *Trypanosoma* spp. (Steiger *et al.* 1979; 1980), *Leishmania mexicana mexicana* (Coombs 1982), *Crithidia* spp. (Eckout 1972) as well as *Tritrichomonas foetus* (Coombs and North 1982). *T. vaginalis*, however, has by far the largest number of cysteine proteinases present by comparison with all other parasitic protozoa. Between 11-23 distinct CP activities have been detected in *T. vaginalis*, most of which are lysosomal (Neale and Alderete 1990).

The original objective of this study was to sequence and characterize clones that had been selected from a λ gt11 cDNA library with anti-serum to the 23 kDa subunit of the 60 kDa ECP. To achieve this objective, 3 clones (23-2-1, 23-4-1 and 23-4-3a,2b) were sequenced. Since the ECP is a 60 kDa protein, the expected size of the transcript

would be approximately 1160 bp in length. Prior to my arrival in the lab, clone 23-1-1 had been sequenced and showed homology to ABRA. Clone 23-1-1 did not contain either of the consensus sequences that correspond to the active sites found in all cysteine proteinases. These sequences, QQGCGCW and WSNKVTWY have a cysteine residue and an asparagine residue as the active sites, respectively (Eakin *et al.* 1990). Furthermore, clone 23-1-1 contained 5 hexapeptide repeats most of which are located at the 3' end of the sequence. These repeat elements had similarity with the ABRA protein. The role that these repeat elements may play in the protein is not clear. Cysteine proteinases do not contain repeat elements and in looking at the ABRA protein, sequences characteristic of cysteine proteinases were not found. Paces *et al.* (1992), have reported a repetitive gene family called Tv-E650 which consists of a 650 bp A-T rich tandem repeat unique to *T. vaginalis* and which is present in all strains studied thus far. *T. vaginalis* has a genome that contains highly repetitive sequences that are likely to have coding potential (Wang and Wang 1985). Repeat elements have also been found in surface proteins of many parasitic protozoa such as *Plasmodium knowlesi* (Zavala *et al.* 1983) and in *Giardia lamblia* (Yang *et al.* 1994). These repetitive sequences are believed to be important in evading the host immune response. Other parasitic protozoa have repeat elements in genes related to the cytoskeleton (Lee *et al.* 1994; Hemphill *et al.* 1992).

Characterization of 23-4-3a and 2b demonstrated that these sequences also did not represent those of a cysteine proteinase. Although this sequence did possess a stop codon, no poly (A) tail was visible and the sequence did not possess a translational start codon. Furthermore, the predicted amino acid sequence showed significant sequence similarity to

the NADPH-dependent butanol dehydrogenase gene (*adh1*) product found in *C. acetobutylicum*. This suggested that this *T. vaginalis* sequence could encode an NADPH-dependent butanol dehydrogenase (BDH).

The reason for the broad spectrum of proteins isolated from the library could be due to multiple specificities of the anti-23 kDa rabbit serum. There are advantages to using polyclonal sera for screening λ libraries since they contain antibodies that would be expected to react with a number of epitopes on the protein that is sought. The disadvantage to this approach is that it also presents the potential for recognizing proteins totally unrelated to the one of interest which may be due to the impurity of the original antigen used to immunize the rabbits. Moreover, since the smaller fragment was chosen to screen the library, a number of other proteins of similar size could have reacted to the anti-sera.

At this time, the original objectives of the study were re-evaluated. It was decided not to pursue the characterization of the ABRA homologue, 23-1-1, since the sequence did not contain sequences characteristic of cysteine proteinases. The 23-2-1 clone, which showed homology to phosphoenol pyruvate carboxykinase was left aside since it seemed to encode an enzyme that was already known to be present in *T. vaginalis*. The *bdh* clone was chosen for further analysis since it was almost a complete coding sequence, the sequence encoded a protein with a high degree of similarity to a protein of known function, and finally, since the protein may be involved in a metabolic pathway previously undescribed for *T. vaginalis*. Hence the **new objectives** of the study were the molecular

analysis of the *bdh* gene of *T. vaginalis*, and to determine if *T. vaginalis* does in fact produce butanol under aerobic and in anaerobic conditions.

CHAPTER 4: MOLECULAR CHARACTERIZATION OF THE *bdh* GENE OF *T. VAGINALIS*

INTRODUCTION

Butanol dehydrogenases (BDHs) catalyze the synthesis of butanol from butyryl-CoA. These enzymes have been reported to be either NADPH-dependent (Andersch *et al.* 1983; Rogers 1986; Dürre *et al.* 1987; Hiu *et al.* 1987) or NADH-dependent (Andersch *et al.* 1983). In *C. acetobutylicum*, the conversion of glucose to pyruvate occurs via glycolysis. Pyruvate is then converted to acetyl-CoA by pyruvate:ferredoxin oxidoreductase, an enzyme which is also present in *T. vaginalis*' hydrogenosomes. Acetyl-CoA is then processed until it is converted to butyryl-CoA by butyryl-CoA dehydrogenase. This substrate is then converted to butyraldehyde by butyraldehyde dehydrogenase. The BDH enzymes come into play in a final metabolic step that catalyzes the conversion of butyraldehyde to butanol at the expense of oxidizing NADPH to NADP⁺ (Woods 1995).

Objectives

The protein sequence predicted for cDNA clone 23-4-2 exhibited significant similarity to the NADPH-dependent butanol dehydrogenase found in *C. acetobutylicum*. Therefore, the objective of this study became the characterization of the *T. vaginalis* gene which possibly encoded an NADPH-dependent butanol dehydrogenase. To complement the molecular analysis of this gene, it was decided that the capacity of *T. vaginalis* to synthesize butanol as a byproduct of a fermentation pathway, should also be examined. Since *C. acetobutylicum* is a gram positive anaerobe, and *T. vaginalis* exists in an

anaerobic environment *in vivo*, it was thought that *T. vaginalis* would produce butanol under anaerobic conditions. In order to fulfill the first objective, it was deemed important to find the 5' translational start codon. The use of gas chromatography and mass spectrometry (GC-MS) was used to detect the presence of organic byproducts, if any, that were present in *T. vaginalis* supernatants.

RESULTS

Detection of Butanol in the Supernatant of *T. vaginalis*.

Since the *bdh* gene in *T. vaginalis* was found to have significant homology to the *adhI* gene found in *C. acetobutylicum* and since *T. vaginalis* exists in a microenvironment that is primarily anaerobic, it was decided the *T. vaginalis* should be grown in anaerobic conditions and the resulting supernatants analyzed for the presence of butanol. The supernatant from 2×10^8 trichomonad cells was collected and was saturated with NaCl. An ether extraction was carried out to isolate the organic compounds present in the supernatant.

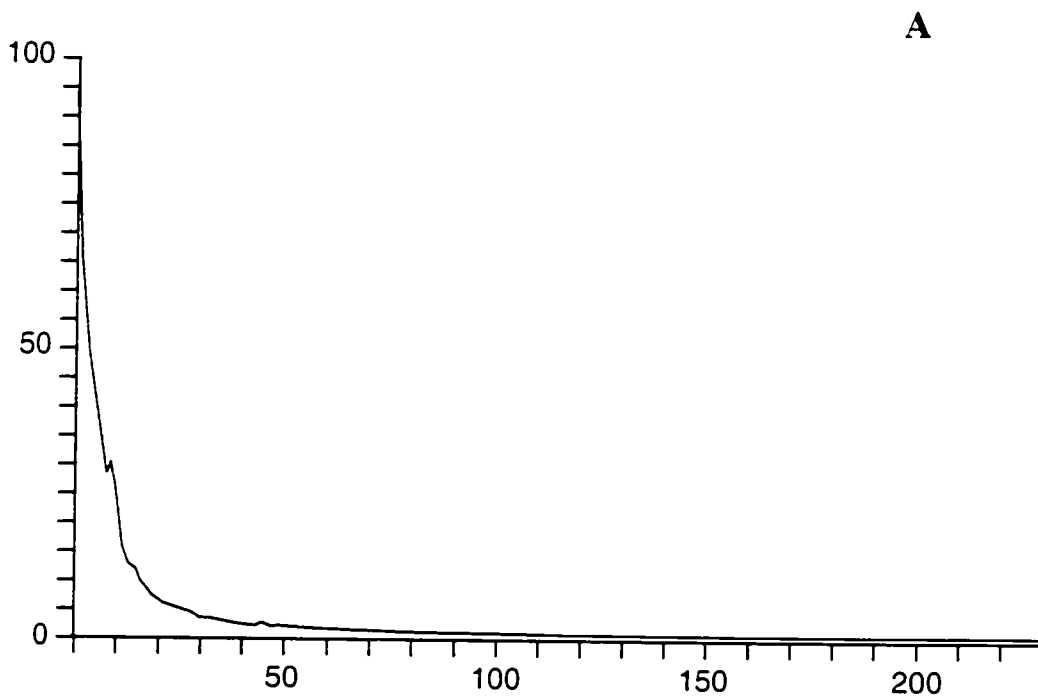
Since the samples were suspended in ether, an ether blank was loaded in to the column. This also served as the negative control (Figure 5). As a positive control, 1-butanol was used. A 1 μ L volume of 1-butanol was suspended in 1 mL of ether. 1 μ L of the mixture was loaded into the column and a single peak at approximately 50 scans was observed (Figure 5).

After running another ether blank, the anaerobic supernatant of *T. vaginalis* that was extracted with ether was loaded into the column. The gas chromatograph of the

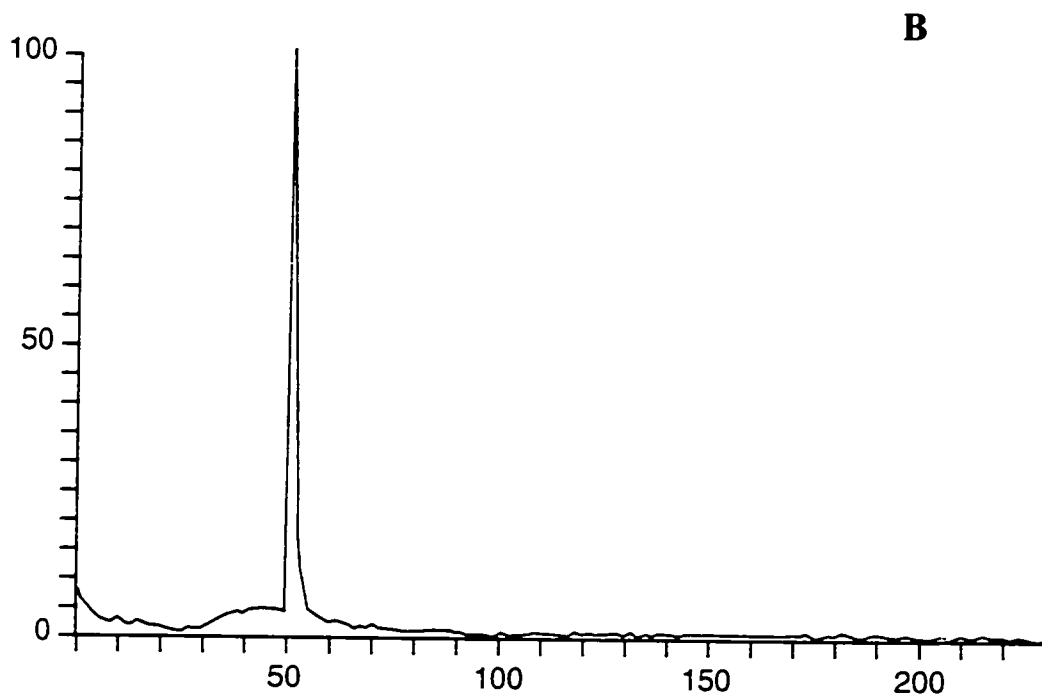
anaerobic supernatant showed various peaks (Figure 5). One distinct peak occurred around 50 scans. This peak, when analyzed by mass spectrometry showed that the species present was 1-butanol. Bars in the profile that indicate that 1-butanol is the species of interest are 41 which is propene, 56 which is water and 72 to 74 which are indicative of hydrogen (Figure 5) (Cornu and Massot 1975). The reason for the other bars present in the profile are a result of the sample being a mixture of various compounds.

Figure 5. Panel A. A gas chromatograph of the ether blank prior to loading of the column with various samples. It should be noted that an ether blank was run prior to the loading of each sample. Panel B. Gas chromatograph of 1-butanol. A 1 μ L volume of 1-butanol was mixed in a 1 mL volume of ether. 1 μ L of the mixture was loaded into the gas chromatograph. Panel C. Gas chromatograph of *T. vaginalis* anaerobic supernatant extracted with ether. Note the peak at approximately 50 scans which represents 1-butanol. Other peaks appearing later on are indicative of high molecular weight compounds leached from the plastic cryovial with an "O" ring into the ether solvent. Panel D. The mass spectrometry profile of the *T. vaginalis* supernatant indicating that 1-butanol is released by the parasite under anaerobic conditions. The top profile is pure 1-butanol, the others are samples of butanol from the *T. vaginalis* supernatant.

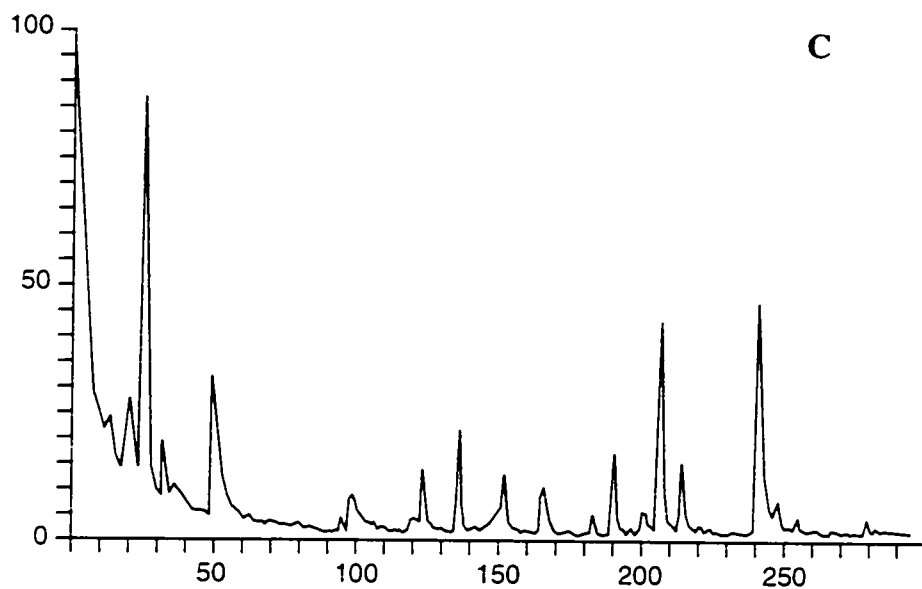
Runname : 9t 1680 Acquired Nominal data (Zero baseline).
8 Mar 96 5:58
Unsmoothed data only:
Ether Blank



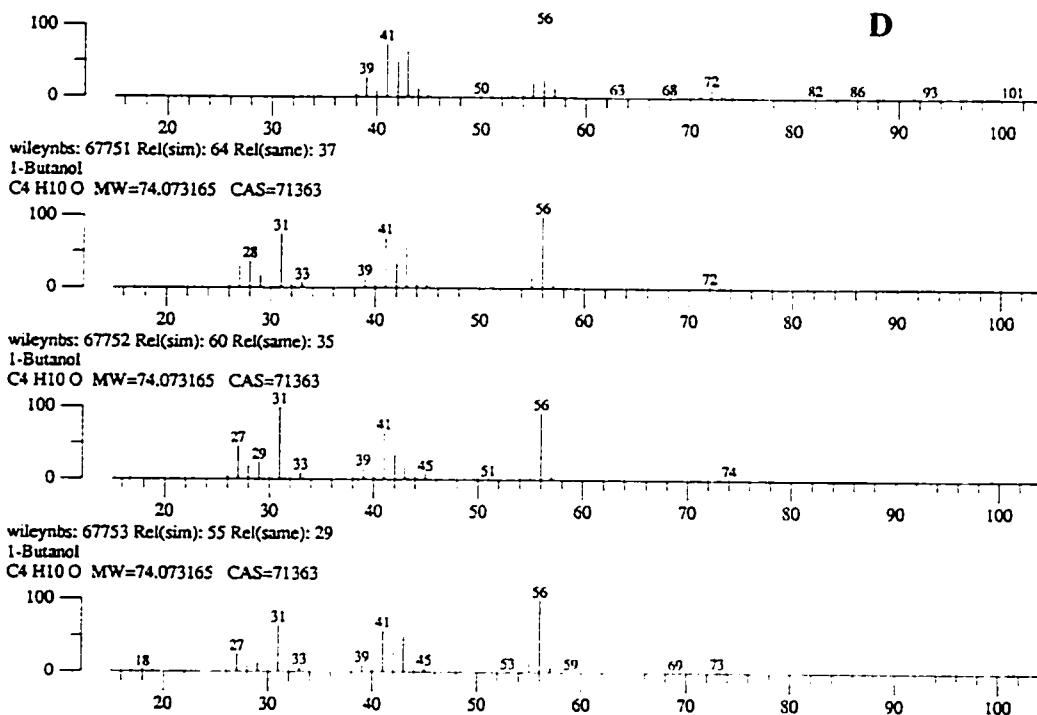
Runname : 9t 1656 Acquired Nominal data (Zero baseline).
29 Feb 96 11:21
Unsmoothed data only:
1-butanol



Runname : 9t 1681 Acquired Nominal data (Zero baseline).
 8 Mar 96 6:09
 Unsmoothed data only:
 T. vaginalis



Run=9t1681 Scan=50 RT=2:56 (Sub) 100%=45296971 ADC
 Mass Range=38-110
 8 Mar 96 6:09 LRP +EI T. vaginalis



Since *T. vaginalis* is described as aerotolerant and is known to grow under aerobic conditions, it was thought that butanol dehydrogenase may be expressed under anaerobic rather than aerobic conditions. Thus an experiment was designed to grow one isolate of *T. vaginalis* under both aerobic and anaerobic culture conditions in parallel, and to determine if butanol was produced only under certain culture conditions. The amount of *T. vaginalis* present in these sets of experiments was 2×10^6 cells.

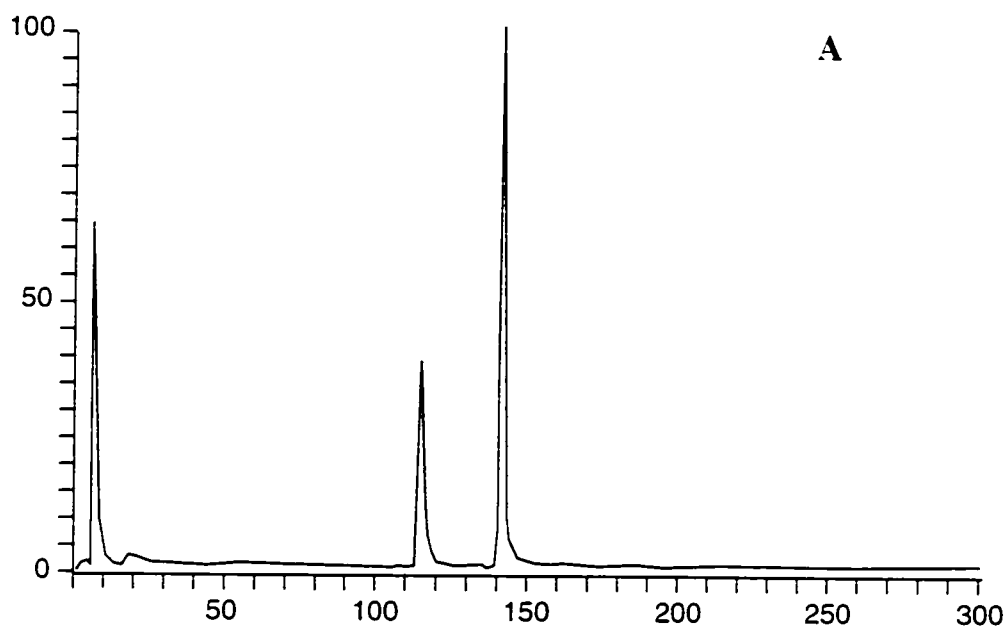
The following results proved a bit puzzling. Ether blanks were run as before. It should be noted that there are a number of peaks present in the blank. These peaks in order from left to right are ethyl acetate and high molecular weight sulfide compounds. These compounds were due to different solvents that were used prior to the analysis of *T. vaginalis* samples (Figure 6). A sample of 1-butanol was run as a positive control once again (Figure 6). Note that during this experiment, butanol comes out of the GC at 30 scans. In addition to the anaerobic and aerobic ether extracted supernatants, TYI medium was also ether extracted to see if 1-butanol is initially present in the medium itself, thereby contributing to the butanol found in cultured supernatants. Analysis of the TYI profile indicated that no butanol was present (Figure 7).

In both anaerobic and aerobic ether extracted supernatants, the results conveyed that in both culture conditions, butanol is produced and is detected by the GC at 30 scans (Figures 8 and 9). The results are not as readily apparent as the one obtained when only the anaerobic culture was analyzed (Figures 5). As seen in the first trial, the MS profile shows the following components of 1-butanol; propene at bar 41, water at bar 56 and

hydrogen at bar 74. As stated before, other bars are a result of dealing with a mixture of various compounds.

Figure 6. Panel A. This is a gas chromatograph of the ether blank prior to the running of the samples isolated from both aerobically and anaerobically grown cultures. Note the peaks that appear at 10, 115 and 140. These peaks correspond to the solvents used prior to the analysis of the organic compounds in the *T. vaginalis* supernatants. Panel B. The gas chromatograph of 1-butanol prior to the analysis of the *T. vaginalis* supernatants.

Runname : 1a0041 Acquired Nominal data (Zero baseline).
11 Feb 97 7:37
Unsmoothed data only:
ether blank



Runname : 1a0043 Acquired Nominal data (Zero baseline).
11 Feb 97 8:00
Unsmoothed data only:
butanol ref

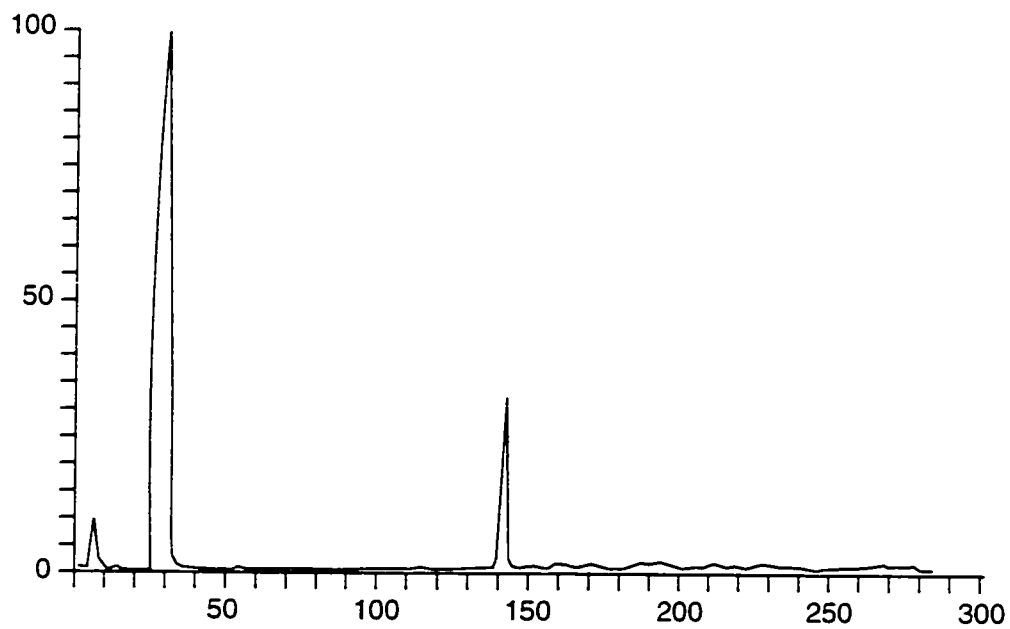


Figure 7. An analysis of TYI media using gas chromatography. Mass spectrometry profiles did not indicate that 1-butanol was present in the media itself.

Runname : 1a0048 Acquired Nominal data (Zero baseline).

11 Feb 97 9:05

Unsmoothed data only:

TYI

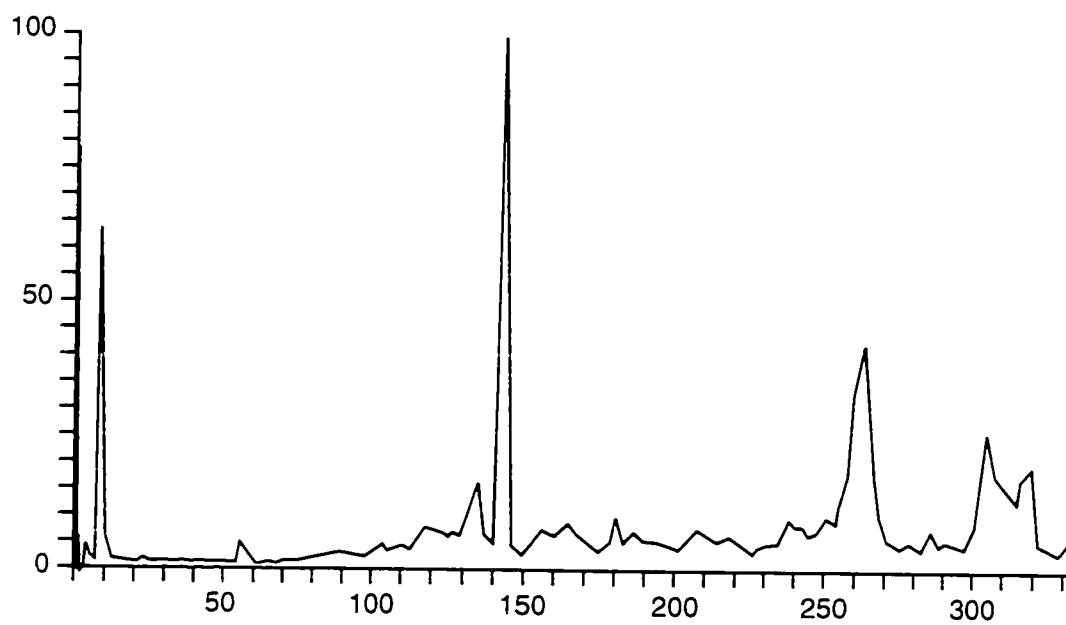
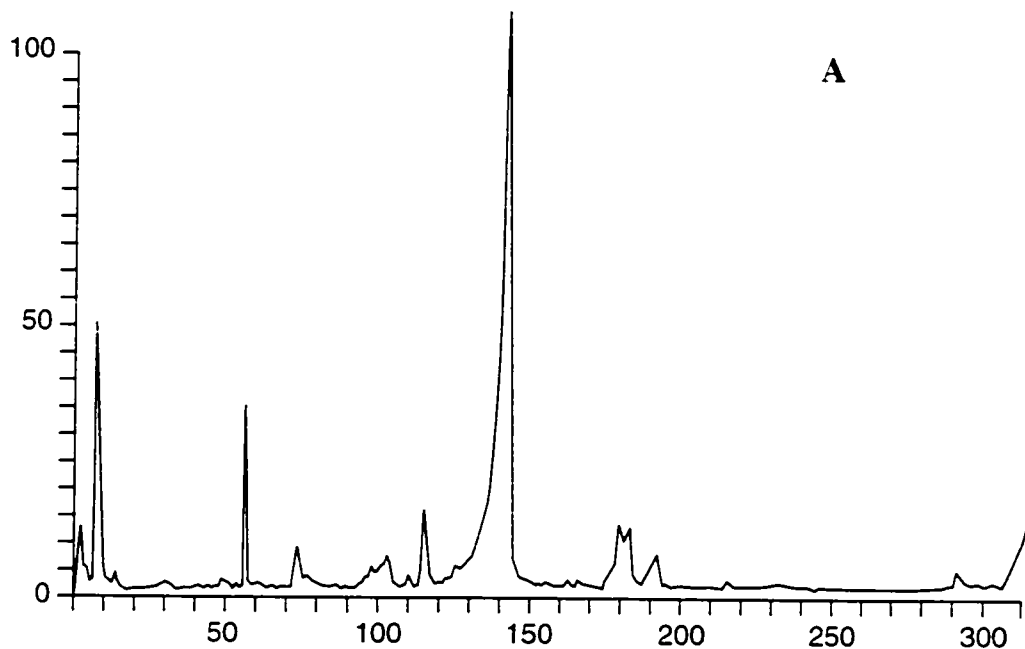


Figure 8. Panel A. A gas chromatograph of the supernatant of *T. vaginalis* grown anaerobically. 1-butanol was detected at 30 scans. Panel B. The mass spectrometry profile of the anaerobic supernatant indicating that 1-butanol is produced.

Runname : 1a0042 Acquired Nominal data (Zero baseline).
11 Feb 97 7:48
Unsmoothed data only:
anr1



1a0042 Scan 31 RT=2:25 100%=23392 mv 11 Feb 7:48
LPR +CI anr1

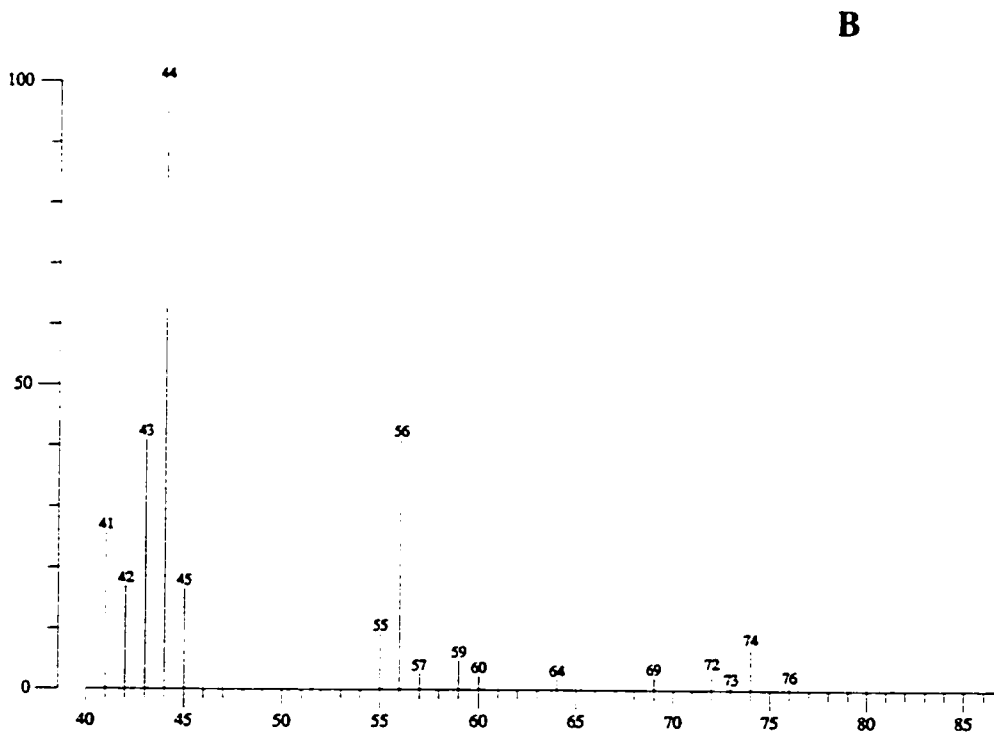
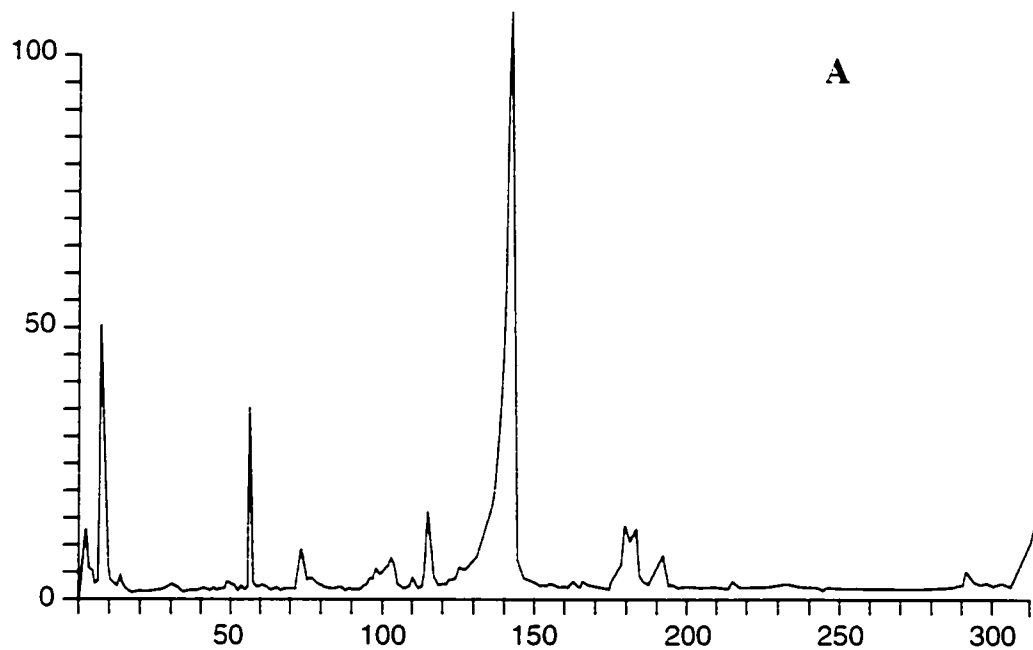
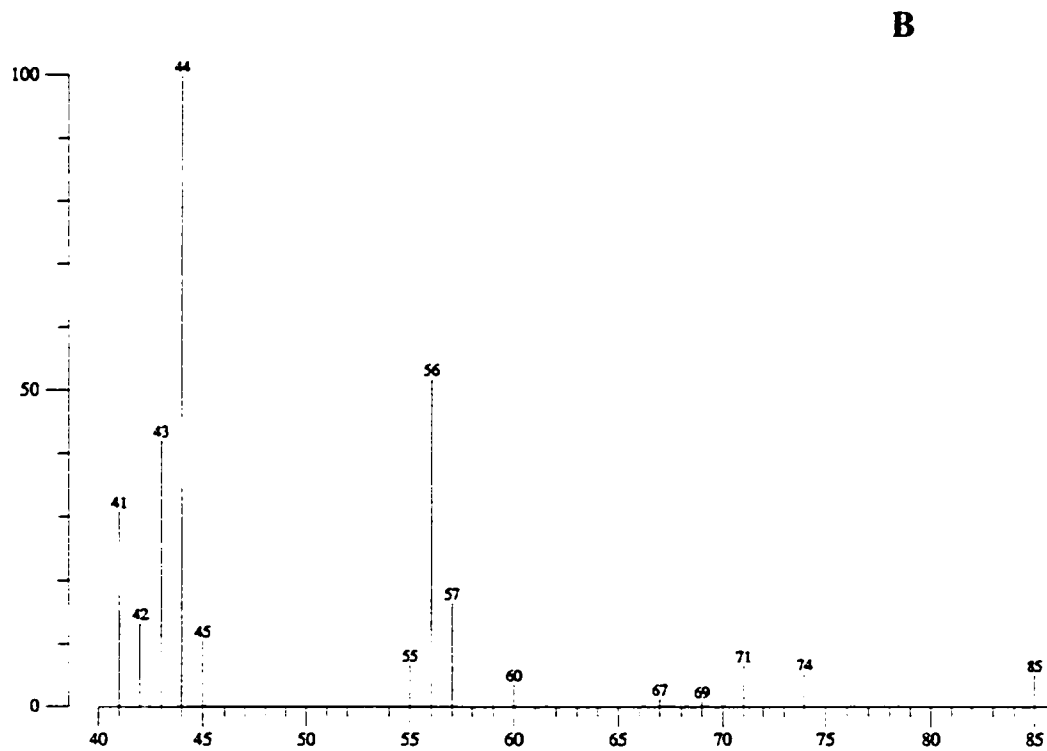


Figure 9. Panel A. The gas chromatograph of the *T. vaginalis* supernatant that was grown aerobically. 1-butanol was detected at 30 scans. Panel B. The mass spectrometry profile of the aerobic supernatant indicating the production of 1-butanol.

Runname : 1a0046 Acquired Nominal data (Zero baseline).
11 Feb 97 8:40
Unsmoothed data only:
a1



1a0046 Scan 31 RT=2:24 100%=23363 mv 11 Feb 8:40
LPR +CI a1



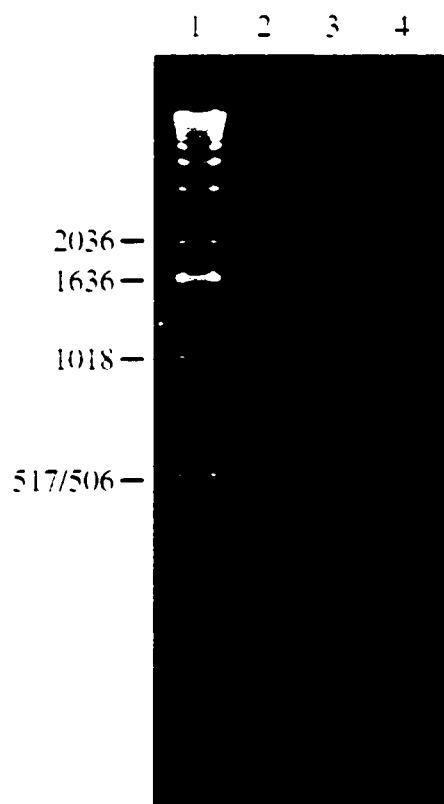
Characterization of the *bdh* Gene of *T. vaginalis*.

Initially all molecular experiments were carried out with genetic material isolated from *T. vaginalis* grown under aerobic conditions. Since the predicted amino acid sequence of clone 23-4-2 showed extensive similarity to the *adh1* gene product of *C. acetobutylicum* did not have a translational start codon (Figure 3), 5' RACE PCR was employed to obtain the initiation codon and possibly any initiator elements upstream from the ATG codon. Initiator elements have been found in a number of *T. vaginalis* mRNAs (Quon *et al.* 1994). In addition to this, though the putative gene contained a stop codon, a poly (A) tail was not in evidence. Since all *T. vaginalis* mRNAs described to date are polyadenylated (Lahti *et al.* 1992; Katiyar and Edlind 1993; Quon *et al.* 1992; Meysick *et al.* 1996), to determine if the BDH mRNA was in fact polyadenylated, 3' RACE was also used.

RACE PCR was carried out on both aerobically and anaerobically grown cultures of *T. vaginalis*. The integrity of the ds cDNA was checked by incorporating [α -³²P] dCTP into the reaction mix used in 1st strand synthesis from mRNA (Figure 10). The figure illustrates that the ds cDNA is of acceptable size.

The following primers were used in 5' RACE PCR; DP-BDH GSP1 and the AP1 primer that was supplied with the Marathon System. In 3' RACE PCR DP-BDH GSP2 was used in conjunction with AP1 as well. The 5' and 3' RACE reactions were expected to yield a products that were approximately 800 and 1300 bp in length, respectively based on the homology to the *adh1* gene found *C. acetobutylicum*. The RACE reactions clearly

Figure 10. ds cDNA synthesized from mRNA isolated from anaerobically and aerobically grown *T. vaginalis* cultures. Lane 1, 1 kb ladder; lane 2, ds cDNA from placental mRNA; lane 3, ds cDNA from anaerobically grown *T. vaginalis* cultures; lane 4, ds cDNA from aerobically grown *T. vaginalis* cultures.



indicated that under both aerobic and anaerobic culture conditions, the mRNA from the *bdh* gene in *T. vaginalis* was expressed and that they are of the same size (Figure 11). Moreover, the products are of the expected size. Though the positive 5' RACE control in lane 6 was not noticeable in this experiment, subsequent PCR trials did indicate that the control was working. The PCR products from the 5' and 3' RACE reactions were ligated into the pCR 2.1 vector from Invitrogen. Colony screening was carried out using the 669 bp amplicon as a probe. Various positive colonies were picked at random. Restriction analysis using EcoR I and BstX I and sequencing of the RACE PCR products confirmed that portions of the *bdh* gene of *T. vaginalis* had been amplified. (Figure 12).

Though the 5' RACE reaction appeared successful, upon sequencing plasmid 11, failed to yield a translational start codon at the expected location. Sequence analysis added the following bases to the 5' end of the gene: CACTCCACGC. These bases encode for the following amino acids: LPR. Though there is an ATG codon at 96 bp from the start of the ORF, no initiator element was readily apparent when compared to initiator elements of other *T. vaginalis* genes (Quon *et al.* 1994). 3' RACE confirmed the presence of a poly (A) tail starting 25 bases after the stop codon.

At this time, it was decided to perform 5' RACE PCR again using an anti-sense primer that was closer to the 5' terminus. The anti-sense primer called DP-BDH NGSP1, was 252 bp away from the 5' end of the gene. Agarose gel electrophoresis of the 5' RACE PCR reactions from both aerobic and anaerobic ds cDNA indicated a product of approximately 300 bp (Figure 13). It should be noted that the Adaptor primer 1 (AP1)

Figure 11. RACE PCR products from ds cDNA generated from the mRNA of both aerobic and anaerobic *T. vaginalis* cultures. Lane 1, 1 kb ladder; lane 2, 5' RACE control using TFR ds cDNA supplied from the Marathon System; lane 3, 3' RACE control using TFR ds cDNA supplied from the Marathon System, lane 4, positive internal control using ds cDNA supplied with the system and the GSP1 and GSP2 primers for the TFR gene, lane 5, 5' RACE positive control from ds cDNA generated from placental mRNA; lane 6, 3' RACE positive control from ds cDNA generated from placental mRNA; lane 6, lane 7, positive internal control using the ds cDNA that was generated from placental mRNA and the GSP 1 and 2 primers for the TFR gene; lane 8, 5' RACE PCR product from ds cDNA generated from mRNA isolated from aerobically grown cultures; lane 9, 3' RACE product from ds cDNA generated from mRNA isolated from aerobically grown cultures; lane 10, internal experimental control using aerobic ds cDNA and the primers DP-BDH GSP1 and 2; lane 11, 5' RACE PCR product from anaerobically grown cultures; lane 12, 3' RACE PCR product from anaerobically grown cultures; lane 13, internal experimental control for anaerobically isolated ds cDNA from mRNA using DP-BDH GSP1 and DP-BDH GSP2 primers; lane 14, 1 kb ladder.

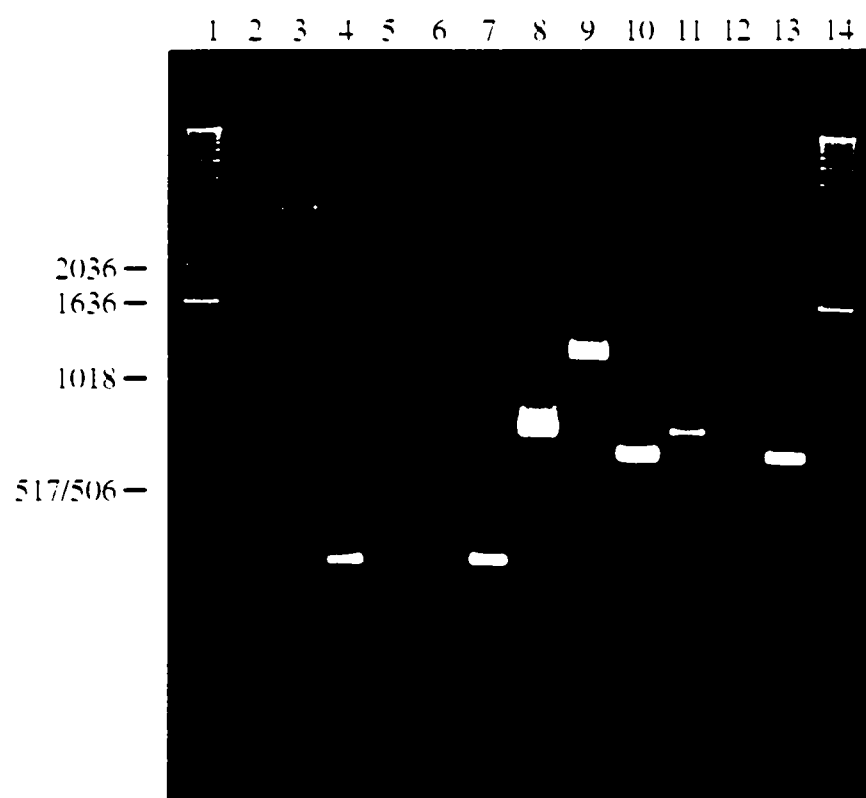


Figure 12. Restriction digests of the 5' and 3' RACE products of the *bdh* gene. Lane 1, 1 kb ladder; lanes 2-4, plasmids 1, 2 and 3 representing *Apa* I/*Sac* I digests of the 3' cloned RACE PCR products; lane 5, 1 kb ladder; lanes 6-8, *Eco*R I digests of plasmids 1, 2 and 3; lanes 9-11, *Eco*R I digests of 5' RACE cloned PCR products in plasmids 11, 12 and 13; lane 12; *Eco*R I digest of plasmid 5 illustrating no DNA was cloned into the vector (negative control); lane 13, 1 kb ladder; lane 14-16, *Bst*X I digests of the 5' cloned RACE PCR products in plasmids 11-13; lane 17-19, *Eco*R I digests of the 3' RACE PCR products in plasmids 1-3; lane 20, 100 bp ladder.

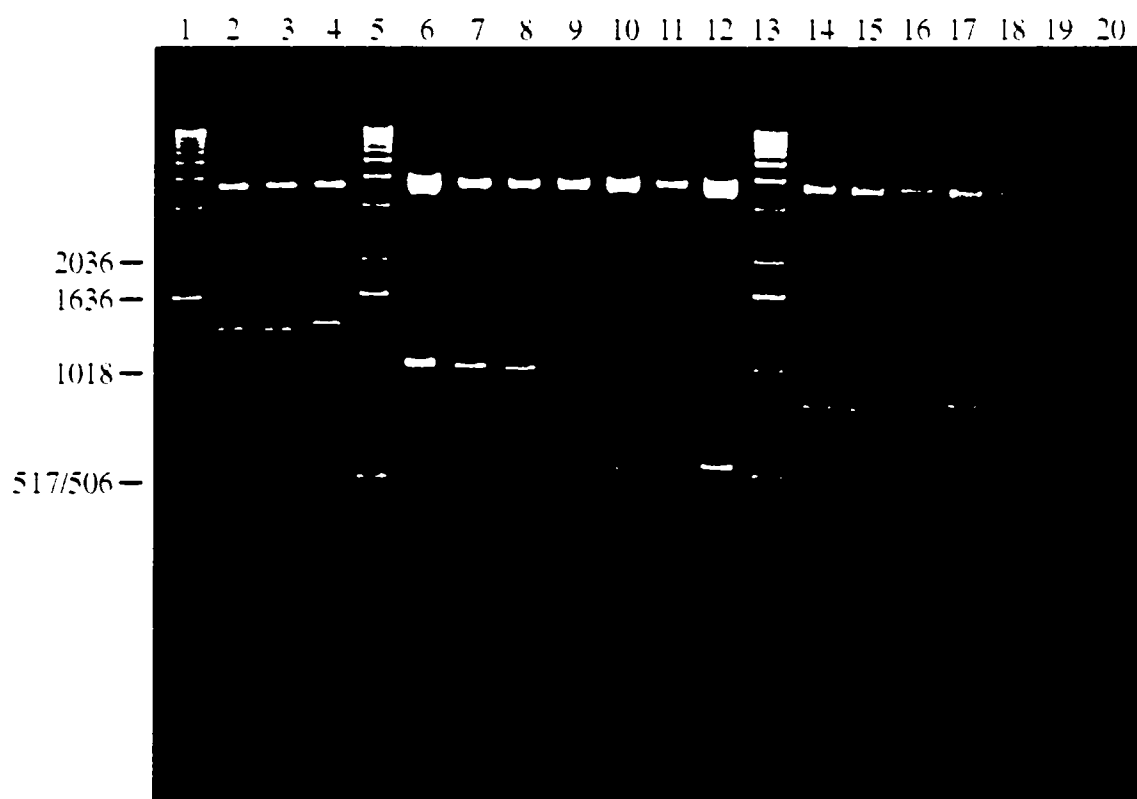
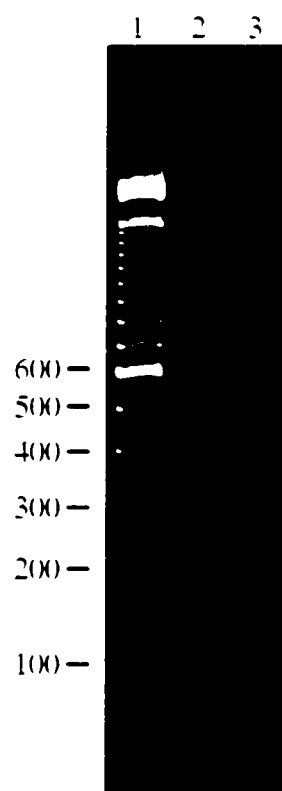


Figure 13. Agarose gel of 5' RACE PCR using both aerobic and anaerobic ds cDNA and the AP1 and DP-BDH NGSP1 primers. Lane 1, 100 bp DNA ladder; lane 2, 5' RACE PCR product using ds cDNA from aerobically grown *T. vaginalis*; lane 3, 5' RACE PCR amplicon using ds cDNA from anaerobically grown *T. vaginalis*.



supplied with the Marathon System is a 27 mer oligonucleotide and is in fact part of the Marathon cDNA adaptor. The entire length of this DNA fragment is 48 nucleotides long. Using the DP-BDH NGSP1 primer and the AP1 primer, in 5' RACE PCR, would thus generate an amplicon of 300 bp in length provided that no additional sequence information could be attained.

The *bdh* Gene in Other Strains of *T. vaginalis*.

In addition to the genomic DNA isolated from *T. vaginalis* strain DG (Figure 14), genomic DNA from 2 other pathogenic strains, 202 and 263, was also isolated to see if the gene was present in other isolates. The genomic DNA from the three strains was used in a PCR reaction involving the primers DP-BDH GSP1 and DP-BDH GSP2. Using this internal control showed that the putative *bdh* gene is present among other isolates of *T. vaginalis* (Figure 15). This amplicon was the same size as the one that was generated from ds cDNA of strain DG, indicating that no introns are present in the region that the gene was amplified (Figure 15). The genomic product generated by DP-BDH GSP1 and 2 was ligated into the Stratagene pCR-Script Amp SK(+) vector. A single EcoR I site is located in the multiple cloning site of the vector. Since the amplicon generated flanks a single EcoR I site in the gene, a restriction digest of the vector should liberate part of the fragment. Evidence of this is shown in figure 16. In addition to the EcoR I digest, a double digest using Apa I and Sac I was performed to liberate the insert from the vector. Sequencing of the amplicon demonstrated that the genomic copy of the gene was faithful to the cDNA that was generated in the 5' and 3' RACE reactions.

Figure 14. Genomic DNA isolated from *T. vaginalis* strain DG. Lane 1, undiluted genomic DNA; lane 2, 1 in 10 dilution of genomic DNA; lane 3, 1 in 25 dilution; lane 4, λ Hind III DNA marker; lane 5, 1 in 50 dilution; lane 6 1 in 75 dilution; lane 7, 1 in 100 dilution.

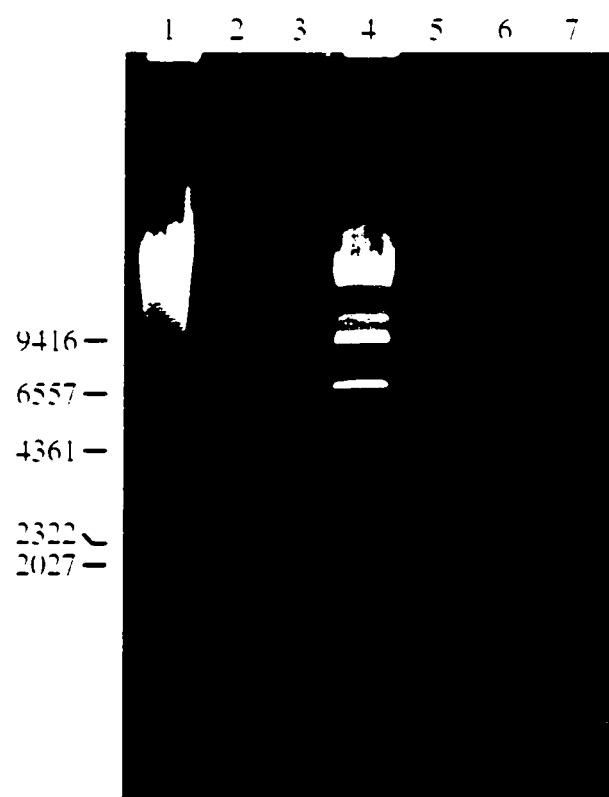


Figure 15. The PCR products from strains DG, 202 and 263 of *T. vaginalis* using DP-BDH GSP1 and 2 primers. Lane 1, 100 bp DNA ladder; lane 2-4, 669 bp PCR amplicon generated from genomic DNA of *T. vaginalis* DNA strains DG, 202 and 263 respectively. lane 5, 100 bp ladder.

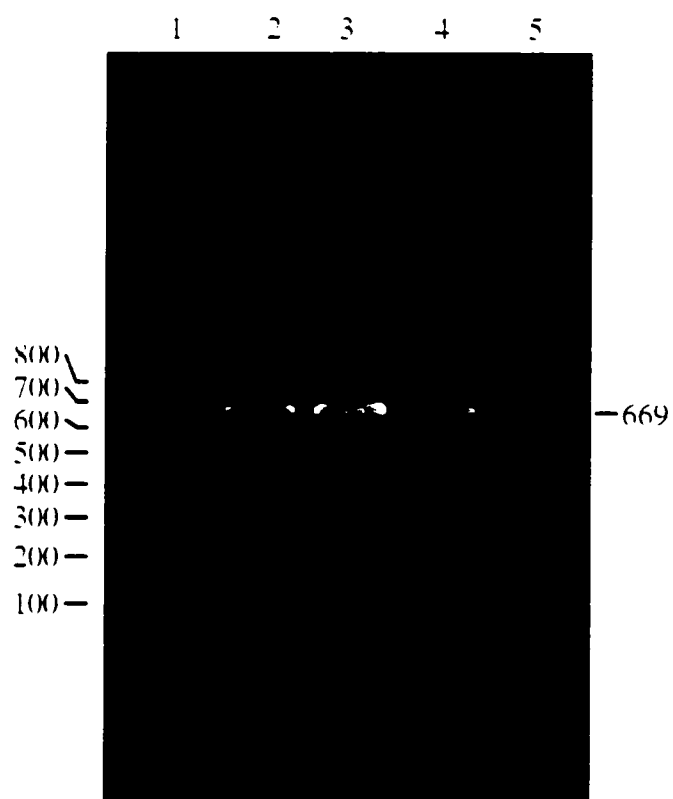
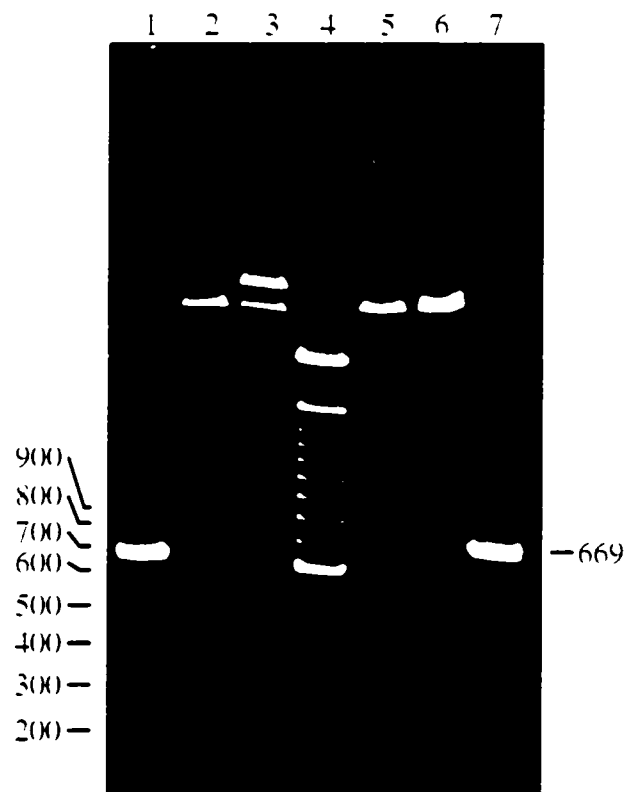


Figure 16. Restriction digests of the 669 bp amplicon that was subcloned into the Stratagene pCR-Script Amp SK(+) vector. Lane 1 and 7, 669 bp amplicon generated by PCR using DP-BDH GSP1 and 2 RACE PCR primers; lane 2 and 3 Apa I/Sac I digest; lane 4, 100 bp DNA ladder; lane 5 and 6, EcoR I digest of the vector.



DISCUSSION

Gas chromatography (GC) in conjunction with mass spectrometry (MS) is a very useful method in detecting organics that are present in extremely low quantities. Alugupalli *et al.* (1992) have applied GC-MS for rapid detection of secondary alcohols, mainly 2-docosanol (Larsson *et al.* 1989), released by *Mycobacterium xenopi* in drinking water.

Ether-extracted *T. vaginalis* supernatants indicated that 1-butanol was in fact produced by the parasite. The marked differences among the subsequent GC-MS profiles taken later may be attributed to two factors. Since 1-butanol can be found in both anaerobic as well as aerobic conditions, the culturing conditions do not seem to play a role in the production of butanol. The time that the cultures were harvested may contribute to the differing levels of 1-butanol found in *T. vaginalis*. In the GC-MS profile that was performed initially (Figure 2), the total amount of *T. vaginalis* was 2×10^8 organisms. When the same experiment was carried out with both anaerobically and aerobically grown cultures in parallel, the amount of the parasites in total was around 1×10^6 organisms. Thus, a possible explanation for the differences in the two sets of profiles is a difference in numbers. In the profiles that were performed with fewer organisms, these low peaks that came out at 30 scans could possibly be indicative of basal levels of butanol production. Three possibilities may arise if higher numbers of organisms are harvested; (i) no differences in the profiles would be observed in both aerobically and anaerobically grown cultures, (ii) in aerobically grown cultures, the levels may not be as high as in anaerobically grown cultures; and (iii) there may be an increase in butanol levels in aerobic versus

anaerobic cultures. It would be interesting to repeat the experiments with more organisms under both conditions to see if the results obtained are indicative of the relative amounts of butanol being produced in anaerobic versus aerobic conditions. Another explanation for the variance in gas chromatographs and the mass spectrometry profiles, is the collection method of the organics in the culture supernatants is prone to error. The vortexing of NaCl saturated supernatants generates a lot of foam. In order to extract the ether layer as fully as possible, the foam must be removed and in doing so, some of the ether layer may be removed. Moreover, in removing the ether layer from the aqueous layer, it is imperative that no aqueous material be allowed into the organic (ether) layer since this would hamper analysis in the GC and possibly damage the column.

In order to obtain consistent results and to determine when butanol begins to be excreted from *T. vaginalis*, cultures of the parasite would be set up with different starting inocula and grown under both anaerobic and aerobic conditions. A number of methods may be employed to trap 1-butanol from the parasitic supernatants. Analysis of the headspace above the supernatant seems to be the simplest method available, since analysis of the supernatant directly would involve filtering the supernatant followed by the passing of the supernatant into a pre-column to prevent particulates from reaching the GC column (Szafranski 1997).

Solid phase microextraction (SPME) would be an ideal alternative to the current method that has been employed. SPME is ideal in that it is used in the analysis of small analytes enabling detection at very low concentrations and does not use any solvents that are commonly used in GC. Polydimethylsiloxane (PDMS) is a compound that is used in

SPME, and can retain small analytes with concentrations as low as parts-per billion (ppb). The method is versatile in that samples can be analyzed by direct immersion or by using the head space of the sample. The sample can then be desorbed thermally into a GC directly (Shirey 1997).

Setting up standardized procedures with minimal manipulation would allow for more consistent and reproducible results.

C. acetobutylicum dehydrogenases have been studied in depth since this Gram positive bacterium is used extensively for the production of alcohols in industry. The *adhI* gene of *C. acetobutylicum* contains an ORF spanning 1163 bp which correspond to 388 amino acid residues, and has a molecular weight of approximately 43.3 kDa. A putative ribosome binding site is located -10 nucleotides from the ATG start codon. Upstream of the *adhI* ORF is another ORF that has been called ORF2. This second ORF is 843 bp in length, has a molecular weight of 31.4 kDa and is located 354 bp upstream of the *adhI* start codon. The function of this ORF is unknown (Youngleson *et al.* 1989)

The intergenic region between the two ORFs is A + T rich and does not seem to contain any consensus promoter sequences which have been identified for the various *E. coli* σ factors. Most *E. coli* operons have a Pribnow box (TATAAT) -10 nucleotides upstream from the translational start codon and a consensus sequence (TCTTGACAT) -35 nucleotides upstream from the initiation codon (Rosenburg and Court 1979; Hawley and McClure 1983). The intergenic region upstream of *adhI* contains inverted repeat sequences with dyad symmetry which may allow the mRNA to form stem-loop structures. The possibility of the mRNA derived from the intergenic region to form complex

secondary structures suggests that it may play a role in the stabilization or the regulation of the mRNA (Youngleson *et al.* 1989).

In sequencing the 5' RACE PCR product generated using the AP1 and DP-BDH GSP1 primers, it seemed that the reaction failed to generate a translational start codon at the expected position based on the homology to the *adh1* gene of *C. acetobutylicum*.

Consequently, it was decided to use a primer closer to the 5' terminus in conjunction with the AP1 primer to see if any additional sequence information could be obtained. As it turns out, using DP-BDH NGSP1 and AP1, a 300 bp product was generated. The size of the product would put the 5' end of the gene at about the same position as shown with the first set of primers. There is an ATG codon located 96 bp from the start of the sequence. Since the amino acid sequence upstream of this methionine residue showed homology to the *adh1* gene product of *C. acetobutylicum* as well as other Fe-containing ADHs, it was believed that the 5' RACE reaction failed. In comparing *T. vaginalis* genes that are homologous to genes found in other bacteria, either the 5' translational start codons are located at approximately the same location or are located further upstream in *T. vaginalis* (Katiyar and Edlind 1994; Lahti *et al.* 1992; Hrdý and Müller 1995; Meysick *et al.* 1996). It appears that the *bdh* gene product of *T. vaginalis* is a truncated protein differing in the N terminus. This would be a novel finding in contrast to the evidence that has been presented thus far in the literature. Many genes have not been fully characterized in *T. vaginalis*. There remains a possibility that some genes may in fact be truncated in comparison to their bacterial homologues. It is unclear as to why a 5' UTR in *T. vaginalis* would be homologous to a coding region in a similar protein found in

C. acetobutylicum, since the 3' UTR in *T. vaginalis* did not show any homology to the 3' UTR found in *C. acetobutylicum*.

Many *T. vaginalis* genes are known to have initiator elements present in their RNAs (Quon *et al.* 1994). These are regulatory elements are sites for transcription initiation and are located very close to the translational start codon. The nucleotide consensus sequence of the initiator element is TCAYTWYTCATTA. The boldfaced adenosine is the most frequently used transcription initiation site (Quon *et al.* 1994). The *bdh* gene of *T. vaginalis* does not seem to contain an initiator element per se, though at 17 bases upstream of the ATG codon there are 4 nucleotides that are present that are identical to the 1st 4 nucleotides of the consensus sequence. Considering that the initiation codon may be the ATG 96 bp downstream from the start of the sequence, primer extension analysis could be used in the presence of dideoxy nucleotides to confirm the translational start, as well as determining the transcriptional start site as well. Since other *T. vaginalis* genes have their translational and transcriptional starts close together, the same is expected for the *bdh* gene. In addition to this, primer extension analysis will also show if the mRNA is differentially expressed between anaerobic and aerobic cultures. Another method for locating the start codon would involve the isolation of genomic DNA from *T. vaginalis*, and performing restriction digest with various endonucleases that cut the *bdh* gene in a single location such as EcoR I. The 669 bp amplicon generated using the DP-BDH GSP1 and 2 primers would then be used to probe a Southern blot of the digested genomic DNA. It is hoped that this procedure would identify a fragment that could be cloned into a plasmid vector. Synthetic oligonucleotide primers would then be

designed to sequence the gene in the 5' direction. This method would identify regulatory elements, if any, in the DNA sequence upstream of the initiation codon.

Inverse PCR (iPCR) is another method that holds promise in finding the 5' end of the *bdh* gene. As in the procedure above, genomic DNA is digested and a Southern blot is performed using the same 669 bp amplicon as a probe. The fragment of the DNA should not be too large as this will diminish the efficiency of intramolecular ligation. If necessary, a double digest could be carried out to reduce the size of the DNA fragment. Instead of isolating and purifying the desired fragment from an agarose gel as in the preceding procedure, the digested DNA is ethanol precipitated and washed. The DNA is then used in an intramolecular ligation reaction with T4 DNA ligase. After ligation, the DNA is purified using phenol/chloroform extractions, and resuspended in sterile water. The DNA is then used in PCR reactions with primers designed so that they amplify the unknown regions of on either side of the *bdh* gene. The products can be sequenced directly or cloned into plasmid vectors. The advantage of iPCR is that ample product is generated with minimal manipulation. Primer extension analysis would confirm the *bdh* genes initiation codon whereas the Southern blotting technique described above as well as iPCR would locate regulatory elements, if present, in the DNA sequence.

Though a portion of the original objective was attained, a number of interesting findings were also made. Through the use of the DP-BDH GSP1 and 2 primers, it was shown that this *bdh* gene originally found in *T. vaginalis* strain DG is also present in other *T. vaginalis* strains, namely 202 and 263. It would be interesting to see if this gene is present in other trichomonal species using the same primers. The same primers used in a

PCR reaction with genomic DNA also demonstrated that this gene did in fact have a single EcoRI site which was confirmed by sequencing the 669 bp amplicon. In addition to this finding, the size of the cDNA amplified product was the same size as that of the genomic fragment indicating that no apparent introns in this area of the gene. This finding is in agreement with other investigators since all the other genes described so far in amitochondrial protozoans such as *E. histolytica*, *G. lamblia* as well as *T. vaginalis* appear devoid of intronic sequences (Bruchhaus *et al.* 1993; Holberton and Marshall 1995; Hrdy and Müller 1995a, 1995b; Johnson *et al.* 1990; Katiyar and Edlind 1994; Lahti *et al.* 1992; Lange *et al.* 1994; Meysick *et al.* 1996). The absence of introns in these protozoa, illustrate their early divergence in the eucaryotic realm.

Though much study has been devoted to *T. vaginalis*, little is known regarding the pathogenic mechanisms of this urogenital parasite. Some investigators (Pindak *et al.* 1993) have suggested that the byproducts of *T. vaginalis* metabolism may play a role in the pathogenesis of *T. vaginalis*. Though much has been written on the metabolism of *T. vaginalis*, much is still to be learned. The discovery of a putative *bdh* gene in this urogenital pathogen was surprising, however, should not have been unexpected. It is known that *T. vaginalis* is one of the most ancient eucaryotes studied to date (Quon *et al.* 1992) and it is known the parasite shares many features in common with anaerobic bacteria. *T. vaginalis*, from an evolutionary stand point, is an intriguing study. It possesses many organelles that are common to eucaryotic cells, yet it is amitochondrial. Looking at various genes from this transitive organism, illustrates the conservation of genes between anaerobic bacteria and *T. vaginalis*. The *bdh* gene of *T. vaginalis* may play a role in the

parasite's pathogenesis. This study shows for the first time that *T. vaginalis* produces butanol as a byproduct via a fermentation pathway and shows the presence of a putative NADPH-dependent butanol dehydrogenase gene that would encode such an enzyme. The relevance of this finding in vivo remains to be studied. A modified cell culture system is needed to study the effects that metabolites including butanol play in pathogenesis. A two chamber system could be designed to allow the metabolites to pass freely from one compartment to another without allowing the cells to come into contact with each other. The head space above the cultured cells from each compartment, one containing the monolayer, the other the parasites, can be analyzed at different time points to assay for the presence of butanol. The butanol would be analyzed via GC-MS. Positive controls would entail the addition of increasing amounts of 1-butanol to cell cultures and noting the effects that the various concentration have on the monolayer. Another variation would be to collect the butanol from the headspace above parasites grown on a monolayer of cells in a normal flask and release the butanol collected into another flask of cells growing in the absence of the parasite and noting the effects that the organics have on the monolayer.

Rodriguez *et al.* (1996) have proposed that Ehadh III, an alcohol dehydrogenase gene found in *E. histolytica*, may be a good target for the development of anti-parasitic drugs without adversely affecting the host. Alternative therapies for *T. vaginalis* could result from a better understanding of its metabolic pathways.

CHAPTER 5: CONCLUSIONS

The original objectives of this study were to characterize the gene that encoded for the 60 kDa ECP that is present in all pathogenic isolates of *T. vaginalis*. However, this ABRA homologue had no active sites in the cDNA fragment that was cloned into the plasmid pGEM 3Z vector. The presence of repeat elements in the gene and the size of the fragment that was cloned into the vector was approximately less than half that of the gene that would encode for the 60 kDa ECP and suggests that this was not the proteinase clone. The polyclonal sera that were used in the immunological screening picked out other clones that did not show homology any cysteine proteinases. An interesting finding was that the largest clone that had an internal EcoRI site showed significant homology to an NADPH-dependent butanol dehydrogenase that is found in *C. acetobutylicum*. This clone has been designated as the *bdh* gene of *T. vaginalis*. Due to the striking homology of *adh1* gene to that of the clone found in *T. vaginalis*, and the problems with the ABRA homologue, it was deemed prudent to investigate this clone. Indirect evidence via GC-MS has shown that this enzyme may be responsible for the production of 1-butanol in a newly described fermentative pathway in *T. vaginalis*. Whether the gene is upregulated under anaerobic conditions or if the gene is transcribed under conditions that favour fermentation in both aerobic and anaerobic remains to be seen. The use of primer extension analysis can be used to determine if the message is regulated differently under different conditions.

The *bdh* gene was found to lack introns in the region that was amplified with DP-BDH GSP1 and 2 and in addition to this, through restriction analysis, was found to have a single EcoRI site as was determined in the cutting of the 5' and 3' RACE products as well

as the internal PCR control using the primers DP-BDH GSP1 and 2. The 5' RACE PCR reaction failed to identify an initiation codon at the expected location. Since the sequence upstream of the first ATG codon was homologous to the *adh1* gene as well as other Fe-containing *adh* genes, it was thought that the initiation codon was further upstream from the 5' end of the sequence. In doing 5' RACE PCR with DP-BDH NGSP1 and AP1, the amplified product put the start of the sequence quite close to where it is now. Sequencing of the amplicon is needed to find out what the exact sequence is. Therefore, the ATG that is located 96 bp downstream of the 5' end of the gene may in fact be the initiation codon. This finding remains to be confirmed. Primer extension analysis could be used not only to determine if this holds true, but also to determine the start of transcription and any regulatory sequences that may be present upstream of the initiation codon. *T. vaginalis* has been known to possess what are known as initiator elements in other genes that have been characterized. It is possible that the *bdh* gene may have similar sequences. A poly (A) tract was found downstream of the termination codon using 3' RACE PCR. The gene has also been found to be present in at least 2 other isolates picked at random. The isolation of genomic DNA is a simple procedure and thus an interesting experiment would be to find out if the gene is present in nonpathogenic trichomonads.

In order to characterize the ECP gene of *T. vaginalis* various methods may be employed. The use of a panel of MAbs could be generated against the purified 60 kDa protein. The MAbs can then be used to screen the expression library in λ gt11. Using this approach means the use of a larger number of recombinants to be screened, however, the use of several MAbs to various areas of the protein would aid in identifying the clones that

are true to the ECP. Another approach would be to use an oligonucleotide probe corresponding to the active site of CPs to screen the cDNA library. Mallinson *et al.* (1994) have already used degenerate oligonucleotide primers corresponding to the 2 active sites of cysteine proteinases in RACE PCR to identify and partially characterize 4 cysteine proteinases. The designing of degenerate primers to the active sites and using them to screen the cDNA library holds promise in finding the ECP gene.

T. vaginalis is an important urogenital pathogen that affects millions of women every year. Knowing more about how the parasite lives will give investigators insight into how to better control the disease.

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APPENDIX 1**OLIGONUCLEOTIDE SEQUENCES****Sequencing Primers:**

T7	5' TAA TAC GAC TCA CTA TAG GG 3'
SP6	5' GAT TTA GGT GAC ACT ATA G 3'
M13 REV	5' CAG GAA ACA GCT ATG AC 3'
M13 FWD	5' GGT TTC CCA GTC ACG CA 3'
BDH SQRT	5' CGA CAC CAT CGA AGA CAT GAA CC 3'
BDH FWD	5' CGA TGC TTG CAC AGG CTC TAA 3'
BDH DP 1R	5' CAG CAT CGA TTG GAG AGC CAC C 3'
BDH DP 2R	5' CTG TGA CGA CGA AGG CTC TCT TG 3'
BDH DP 3R	5' GGC CAA GCC ATC AGT GTA AT 3'
BDH DP 1F	5' GCC TAC GTT TCT ACA CTT CAC 3'
BDH DP 2F	5' GCG TAA CGC CAT GTA CCT TCC AAA 3'
BDH REV	5' GAG AGC GCC CTT GCC ATG ATA 3'

PCR Primers used in 5' and 3' RACE

DP-BDH GSP1 5' CAT ACC AGC AAG GCA CTG AGC GTT 3'

DP-BDH GSP2 5' GCA AGA GAG CCT TCG TCG TCA CAG 3'

DP-BDH NGSP1 5' CAT ACC AGC AAG GCA CTG AGC GTT 3'

DP-BDH NGSP2 5' CAC CAC CGA GAG CAA CAA TCC AGT 3'

AP1 5' CCA TGG TAA TAC GAC TCA CTA TAG GGC 3'

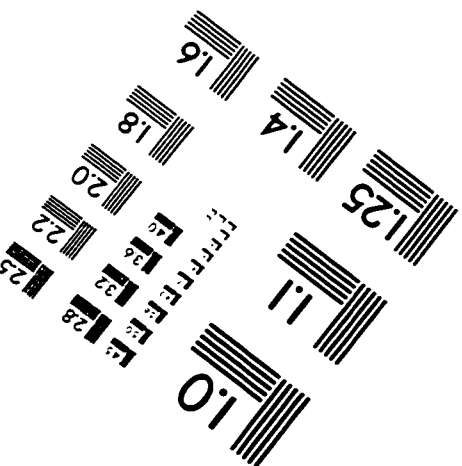
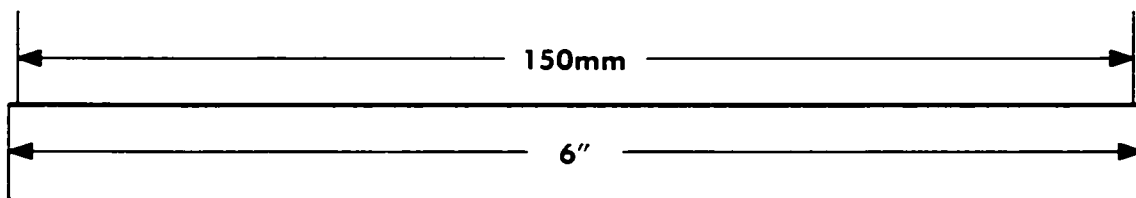
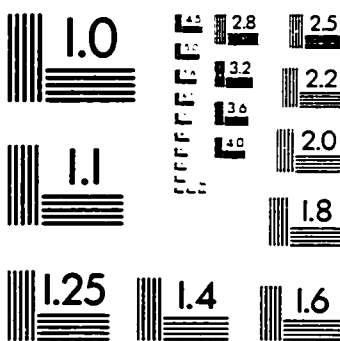
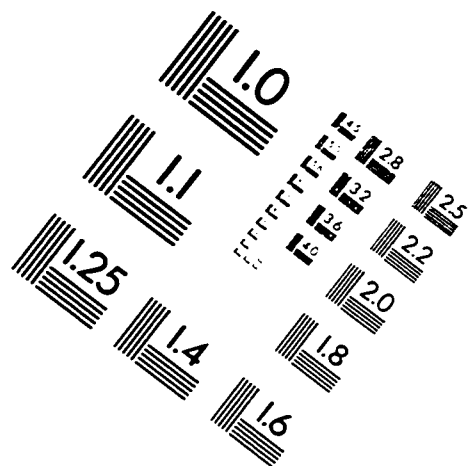
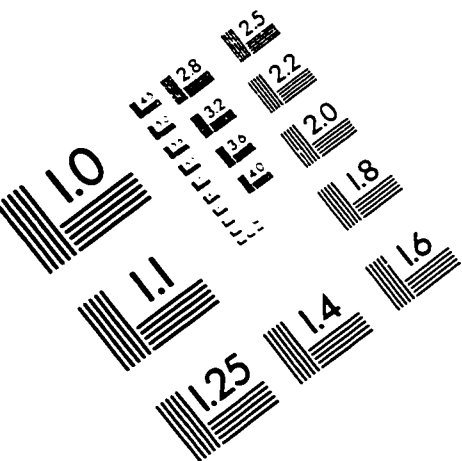
AP2 5' ACT CAC TAT AGG GCT CGA GCG GC 3'

Marathon cDNA Adaptor (Clontech)

5' CCATGGTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT 3'

3' H₂N-CCCGTCCA-PO₄ 5'

IMAGE EVALUATION TEST TARGET (QA-3)



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