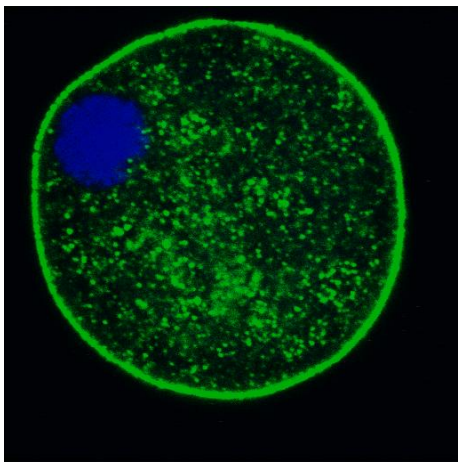
Regulation of antioxidant enzymes in *Entamoeba histolytica* (SCHAUDINN, 1903)



Dissertation

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List of abbreviations

A	Ampere
ALA	Amebic liver abscess
AP	alcaline Phosphatase
APS	Ammonium persulfate
AS	Antisense
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCA	Bicinchonin acid
BCIP	5-Brom-4-chlor-3-indoxylphosphate
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BSA	Bovine serum albumin
cDNA	complementary DNA
CIAP	Calf Intestinal Alcaline Phosphatase
C_T	Cycle of threshold
Cy3	Carbocyanin 3
Cy5	Carbocyanin 5
Da	Dalton
DAPI	4´,6-diamidino-2-phenylindol
DMF	Dimethyl formamide
DMNQ	2,3-Dimethoxy-1,4-naphthoquinone
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
dNTP	Desoxyribonucleotide-5-triphosphate
DTT	Dithiothreitol
E-64	L-transpoxysuccinyl-1-leucylamido-4-(guanidino)-butane
ECL	Electrochemi luminescence
ECM	Extracellular Matrix
EDTA	Ethylendiamintetraacetic acid
EGTA	Ethylenglycoltetraacetic acid
Eh	*Entamoeba histolytica*

et al.	*et alii* (and others)
EtBr	Ethidium bromide
EtOH	Ethanol
F	Farad
FCS	Fetal Calf Serum
Fig.	Figure
g	Gravity
Gal/GalNAc	Galactose/N-acetyl-D-galactosamine
gDNA	Genomic DNA
G418	Geniticin
GEA 5024	[1,2,3,4-oxatriazolium,5-amino-3-(3-chloro-2-methylphenyl)-,chloride]
GPI	Glycosylphosphatidylinositol
GSNO	[S-Nitroso-L-glutathion
h	Hour
HEPES	N-2-Hydroxyethylpiperazin-N'-2-ethansulfonic acid
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
IFA	Immunfluorescence analysis
IgG	Immunglobulin G
IPTG	Isopropyl-b-D-thiogalactopyranosid
k	Kilo
kb	Kilo base
L	Liter
LB	Luria-Bertani
M	Molar
m	milli
MALDI TOF MS	Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry
min	Minute
MOPS	3-(N-Morpholino)-Propansulfonic acid
mRNA	messenger RNA
NaAcetat	Sodium acetat
NADPH	Nicotinamide adenine dinucleotide phosphate

NaPBS	Sodium phosphate buffered saline
neo	Neomycin phosphotransferase
OD	Optical density
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygene Species
rpm	Round per minute
RT	Room temperature
s	Seconds
S	Sense
SDS	Sodium dodecyl sulfate
SIN-1 chloride	3-Morpholinosydnonimine
SOD	Superoxide dismutase
SSC	Saline Sodium Citrate
Taq	*Thermus aquaticus*
TBE	Tris borate acetate buffer
TBS	Tris Buffered Saline
TCA	Trichloro acetic acid
TEMED	N,N,N',N'-Tetramethylethylendiamin
TIGR	The Institute for Genomic Research (Data bank)
T_m	Melting temperature
Tris	Tris-Hydroxymethyl-Aminoethane
tRNA	Transfer RNA
TY-I-S-33	Trypticase yeast extract iron serum
U	Unit
UV	Ultraviolett
v/v	Volume per volume
Vol.	Volume

WHO	World health organization
w/v	Weight per volume
x-Gal	5-Brom-4-Chlor-3-indolyl-b-D-Galaktopyranosid

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Summary

Amoebiasis is the infection caused by the human parasitic protozoan *E. histolytica*. Although the parasite is distributed worldwide, it predominantly affects individuals of lower hygiene conditions especially in developing countries. Usually the infection with *E. histolytica* remains asymptomatic. In some cases the infection leads to intestinal colitis, and if the parasite succeeds to invade the intestinal mucosal barrier, the parasites migrate to different organs of the body especially the liver where an amoebic liver abscess (ALA) may develop. Whether or not the infection results in the formation of liver abscess may be influenced by the *E. histolytica* strain. Nowadays it is known that this parasite uses different mechanisms to invade the intestinal wall reaching the liver with the blood stream. One of the mechanisms, that help the microaerophilic parasite *E. histolytica* to overcome the elevated levels of Oxygen and its derivatives in blood and liver, is the use of antioxidant enzymes. This mechanism is considered as a defensive mechanism that contributes to the degree of virulence of the parasite.

In this work two syngenic amoeba clones (clone A and clone B), which are derived from the *E. histolytica* isolate HM-1: IMSS were investigated. The two clones differ in their virulence. Clone A is incapable of inducing liver abscess in rodents, while clone B is capable of producing large liver abscesses. Investigating the regulation of antioxidant enzymes in both clones may provide a clue for a better understanding of virulence in this parasite. With the help of different analytical methods difference between the two clones were investigated on the physiological, transcriptional and translational levels.

On the physiological level, there was no significant difference between the trophozoites of clone A and clone B upon the treatment with oxidative reagents that produce intracellular superoxide anions, hydrogen peroxide, and extracellular superoxide radicals. On the other hand, clone A was found to be significantly more sensitive to oxidative reagents that produce nitric oxide radicals. The overexpression of the antioxidant genes antioxidants *ehprx*, *ehtrxr*, *ehfesod*, *ehrbr*, and *ehfehyd* in both clones did not reflect always an increased level of protection against oxidative and nitrosative stress

Based on their differential behavior toward nitrosative stress, the differential gene expression of antioxidant genes in both clones A and B was investigated by means of real-time PCR (RT-PCR). Among the 15 investigated genes, 4 genes (*ehprx*, *ehfesod*, *ehfehyd*, and *ehtrx4*) were differentially higher expressed in the pathogenic clone B.

In order to correlate the transcriptional level of the four differentially expressed antioxidant genes with the translational level, the respective proteins were localized in both clones A and B. In addition, two more proteins that correspond to the genes thioredoxin reductase (*ehtrxr*) and rubrerythrin (*ehrbr)* were localized in both clones. These two genes are predicted to play a role in detoxifying hydrogen peroxide radicals. Due to the fact that there was no specific antibody for the gene *ehtrx4* availabe, localization studies could not be performed.

The localization analysis reflected that there is no significant difference between both clones in terms of the amount of the enzymes thioredoxin reductase and rubrerythrin. Both were found to be cytosolic localized. Rubrerythrin was found to be associated with the cell membrane. The amount of the enzymes peroxiredoxin, iron-superoxide dismutase, and iron-hydrogenase was found to be more in clone B than in clone A. All three enzymes were cytosolic localized. Peroxiredoxin and iron-superoxide dismutase were found to be associated with the cell membrane. The translational level of antioxidant enzymes correlated with the transcriptional level.

The overexpression of the antioxidant genes *ehprx, ehtrxr, ehfesod*, *ehrbr*, and *ehfehyd* in both clones was targeted to investigate whether their overexpression has an effect on the expression of other antioxidant genes. Surprisingly, the overexpression of the gene *ehtrxr* lead to the silencing of this gene in trophozoites of the clone A. Moreover, the overexpression of these genes had an influence on the expression of other antioxidant genes.

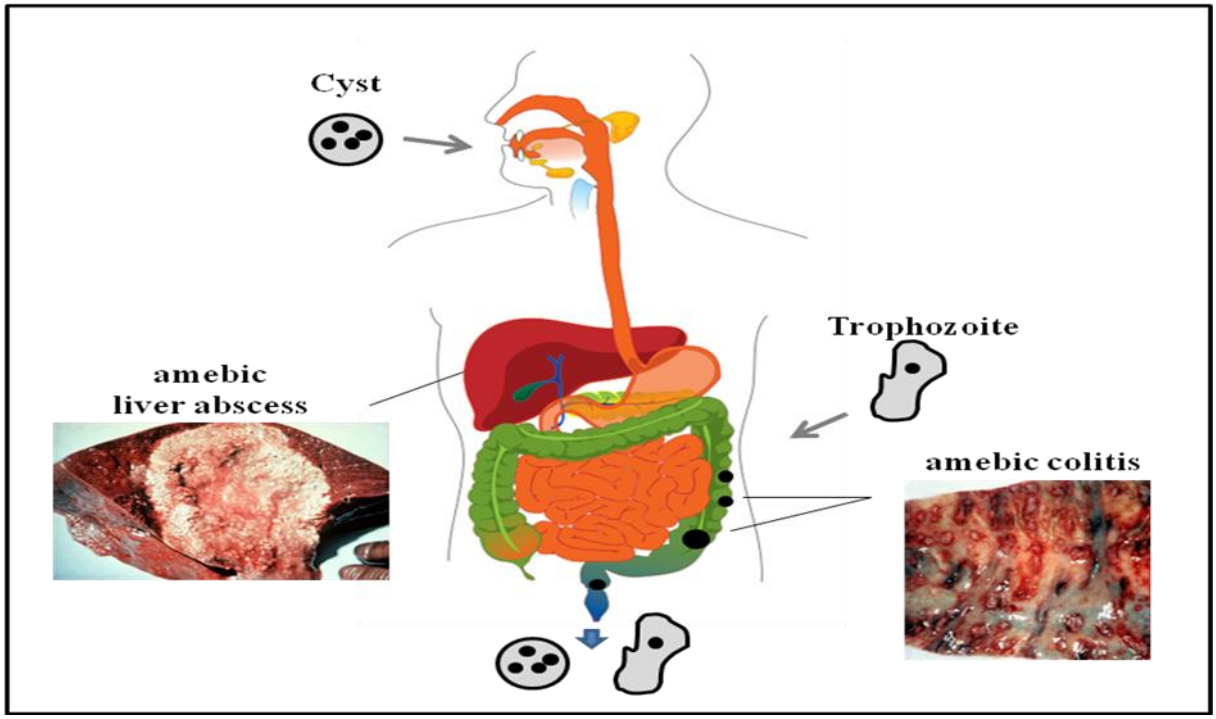
1. Introduction

1.1 The parasite *Entamoeba histolytica*

The enteric microaerophilic proprotozoan parasite *Entamoeba histolytica* is the causative agent of human amoebiasis (Akbar *et al.*, 2007). Although the parasite is distributed worldwide, it predominantly affects individuals of lower socioeconomic status who live in developing countries (Pinheiro *et al*., 2007). Amoebiasis leads to 50 million clinical cases, and 100.000 deaths per year. 90% of the infected individuals remain asymptomatic, whereas the remaining 10% develop colitis, diarrhea, dysentery, and in few cases, extra intestinal amoebic lesions, such as liver abscess (Bansal *et al.*, 2009). Consequently, amoebiasis is ranked as the third leading cause of death after malaria and schistosomiasis (William and Petri, 2008).

The life cycle of *E. histolytica* (Fig.1.1) is simple (does not require an intermediate host), and consists of two main stages, the trophozoite which is motile in the human intestine and the cyst which is dormant, quadri-nucleated, and usually found in the external environment (Lohia, 2003). The cysts which acquire their protection through their cell wall can survive up to some weeks in the external environment, and are responsible for the transmission. They can survive the acidic pH of the stomach and pass into the intestine. In the ileo-cecal region, cysts undergo excystation, and each cyst gives rise to eight trophozoites (Sehgal, *et al.*, 1996). On the other hand, trophozoites can be passed in diarrheal stools, but are rapidly destroyed once outside the body. If the trophozoites were ingested, they would not survive the exposure to the gastric environment. The trophozoites usually remain attached to the intestinal lumen of individuals. In some patients the trophozoites invade the intestinal mucosa, causing the flask-shaped ulcer that leads to amoebic dysentery.

In very few cases, the trophozoites travel in the bloodstream (extraintestinal invasion) through the portal vein into the liver where they develop abscesses that may lead to death. Rarely, other sites such as the brain and lungs could also be infected.



Firgure1.1: Life cycle of *Entamoeba histolytica*. Infection by *E. histolytica* occurs upon the ingestion of mature cysts in fecally contaminated food, water, or hands. Excystation occurs in the small intestine and trophozoites are released, which migrate to the large intestine. The trophozoites multiply by binary fission and produce cysts, which are passed in the feces

1.2 Cell biology and genetic content of *E. histolytica*

The trophozoites of *E. histolytia* are surrounded by a thin membrane. They metabolize actively and move by means of pseudopodia. According to Lucius and Loos-Frank 2008, the trophozoite diameter varies between 20 - 60 µm. The endoplasma contains a large number of vacuoles of different sizes that serve for the breakdown of nutritive materials. Nutrition uptake is done through a process called phagocytosis.

On the other hand, the cysts have a round or slightly oval form and measure 10 – 15 µm in diameter. They are dormant and environmentally resistant. The immature cysts contain one nucleus, while the mature ones contain 4 nuclei.

E. histolytica is an obligate fermenter. It lacks the proteins of the tricarboxylic acid cycle and the mitochondrial electron transport chain. A mitochondrion-derived organelle has been identified in *E. histolytica*, the mitosomes (Loftus *et al.*, 2005). Mitosomes are the simplest form of mitochondria. They have lost their capacity for ATP synthesis, lost all vestiges of a mitochondrial genome and so far only a limited set of proteins (such as molecular chaperones, rubrerythrin, and ATP sulfurylase) have been localized into these tiny double membrane-bound vesicles (Dolezal *et al.*, 2010). The morphology of *E. histolytica* mitosomes is

reflected in small organelles of around half a micron in diameter surrounded by double membranes (Aguilera *et al.,* 2008).

The genome of *E. histolytica* consists of 24 Mb. The 9,938 predicted genes average 1.17 kb in size and comprise 49% of the genome. One-quarter of the *E. histolytica* genes are predicted to contain introns, with 6% of genes containing multiple introns. The exact chromosome number is difficult to determine (Loftus *et al.,* 2005).

Approximately, 15% of annotated genes are potentially developmentally regulated. Genes enriched in cysts (672 in total) include cysteine proteases and transmembrane protein kinases. Genes enriched in trophozoites (767 in total) include the respective molecules involved in tissue invasion, putative regulators of differentiation, including possible G-protein coupled receptors, signal transduction proteins and transcription factors (Gomez *et al.,* 2010).

The molecular mechanisms participating in parasite invasiveness are not completely understood. Clones derived from particular strains display differential virulence phenotypes. In addition, long-time cultured trophozoites, which show poor virulence, recover their virulence after incubation with certain types of bacteria, or after their passage through hamster liver (Gomez *et al.*, 2010). Moreover, *E. histolytica* alters its transcriptional regulation in response to a number of stimuli including heat shock, drug resistance, oxidative stress and host invasion (Ehrenkaufer *et al.,* 2007).

1.3 Pathogenesis of *E. histolytica*

E. histolytica must respond to a wide variety of environmental stimuli as it excysts into a trophozoite in the intestinal lumen and enters the host by invasion of the intestinal mucosal epithelium (Beck *et al.,* 2005).

E. histolytica encounter natural barriers in both the intestine and systemic circulation after extra-intestinal invasion such as intestinal mucins, intestinal bacterial flora and the complement system. Gastrointestinal mucins are the first line of host defense against enteric pathogens since the binding sites of mucin compete with those of the underlying epithelium, preventing the attachment of pathogens to the intestinal wall. Intestinal bacterial flora, in turn, competes for the attachment to mucin and may prevent the amoebic lodgment. Vesicle

trafficking has a role in *E. histolytica* pathogenesis through phagocytosis and the delivery of secreted hydrolytic enzymes and amoebapores to the cell surface (Loftus *et al*. 2005).

Human complement-mediated cytolysis has an effective amoebicidal activity (William and Petri, 2008). However, the parasite manages to escape these barriers by using different mechanisms of pathogenesis. Offensive mechanisms are represented by Gal/GalNAc lectin, amoebapores, and cystein proteases. Defensive mechanisms are represented by the antioxidants used by the parasite, which play a significant role in its virulence.

1.3.1 Gal/GalNAc lectin

E. histolytica trophozoites express numerous cell surface and secreted molecules that assist with feeding and retention in the intestine and which contribute to the virulence of the parasite (Frederick and Petri, 2005). One of the most common features of *E.histolytica* pathogenicity is contact-dependent killing of host cells. It is capable of killing a variety of cell types including those of the human intestinal epithelium, erythrocytes, neutrophils and lymphocytes. Cytolysis occurs as a stepwise process that begins with the adhesion to target cells via galactose/N-acetyl $_{D-}$galactoseamine-inhibitable (Gal/GalNAc) lectin. Adhesion occurs when (Gal/GalNAc) lectin binds to exposed terminal Gal/GalNAc residues of target cell glycoproteins. Target cell death occurs within 5-15 min and is often followed by phagocytosis. The inhibition of Gal/GalNAc lectin with galactose blocks phagocytosis (Clark *et al.,* 2007; Petri, 2008).

1.3.2 Amoebapores

Once *E. histolytica* establishes contact with mammalian cells, a rapid cytolytic event takes place that result in swelling, surface blebbing and lysis of the target cell, leaving the parasite intact. In the lysosome-like granular vesicles of *E. histolytica* a family of small proteins is found called amoebapores. Amoebapores have the cytolytic affect towards human host cells (Clark *et al.,* 2007). They are capable of killing metabolically active eukaryotic cells and display antibacterial activity (Ankri, 2002).

Three amoebapore isoforms A, B, and C have been characterized. Trophozoites lacking the major isoform amoebapore A became avirulent demonstrating that this protein plays a key role in the pathogenesis of the parasite (Clark *et al.,* 2007).

1.3.3 Cysteine proteases

Cysteine proteases occur in a wide range of organisms including bacteria, plants, invertebrates, and vertebrates. In *E. histolytica*, the main lytic activity has been attributed to cysteine endopeptidases. Cyteine endopeptidases were found to be secreted and localized in lysosome-like vesicles or at the surface of the cell. The cytopathic effect is represented by the degradation of the components of the extracellular matrix, including fibronectin, laminin, and collagens as well as an extra-cellular matrix from vascular smooth muscles.

1.3.4 Antioxidants

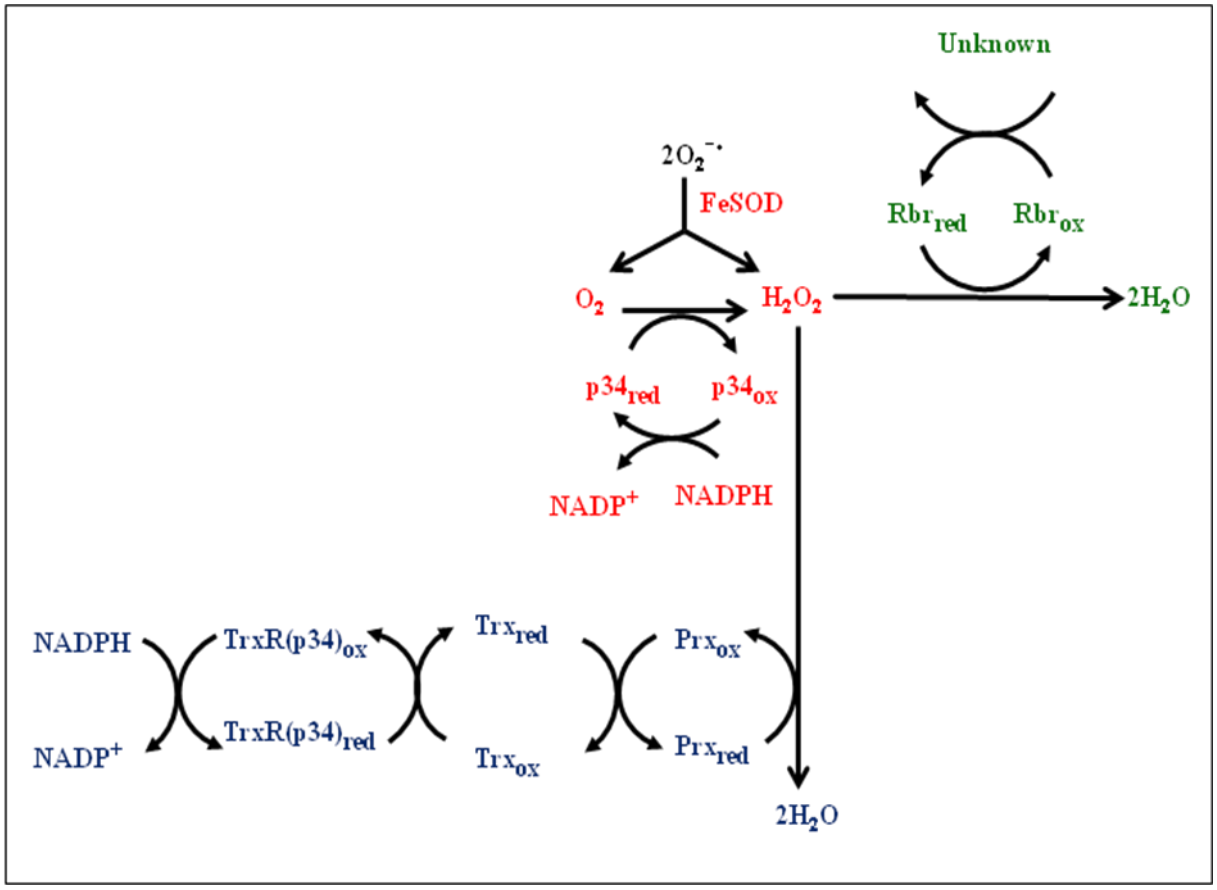
In aerobic organisms, molecular oxygen is a final acceptor of electrons in different electron transport systems, such as those present in the mitochondria. However, some parasites such as *Entamoeba* spp. and *Trichomonas* spp., which live in oxygen-poor environments, are particularly sensitive to oxidative stress (Docampo, 1995).

To fight against oxidative death caused by reactive oxygen species generated during inflammation or when phagocytosed by macrophages, many parasites have developed multilayered defense system, including DNA repair systems, scavenging substrates, and antioxidant enzyme systems (Choi *et al.*, 2000).

E. histolytica trophozoites usually reside and multiply within the human gut, which constitutes an anaerobic or microaerophilic (up to 5% oxygen in the gas phase) environment. However, during tissue invasion, the amoebae are exposed to an increased oxygen pressure and have to eliminate toxic metabolites such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) produced by activated phagocytes during respiratory burst. In addition, *E. histolytica* lacks a conventional respiratory electron transport chain that terminates in the reduction of O_2 to H_2O (Clark *et al.*, 2007). Accordingly, its endogenous toxic metabolites are not the only challenge it has to cope with.

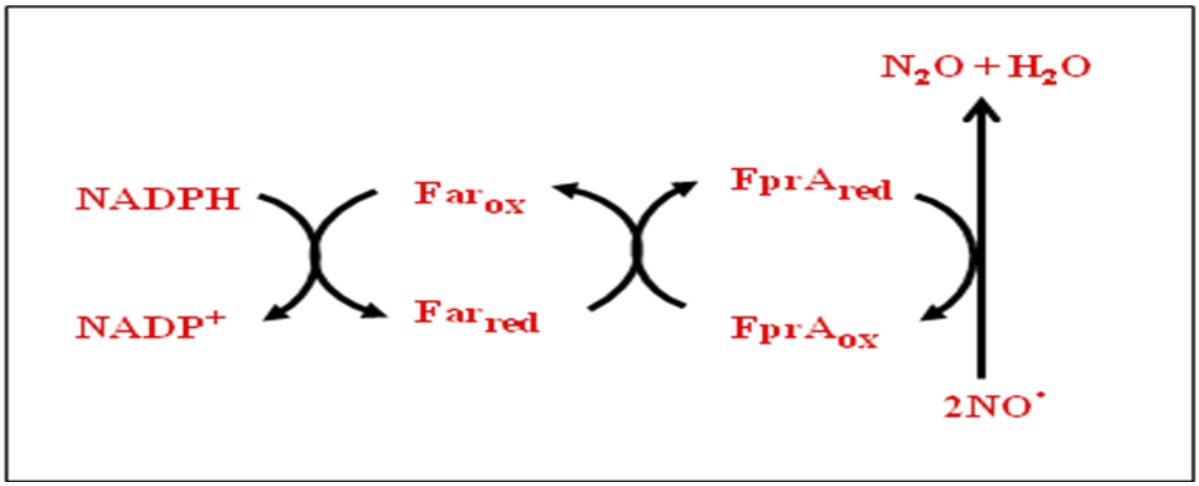
An oxidative (respiratory) burst of the immune system, which is defined as the sum of reactive oxygen species involved in the host defense against pathogens, imposes a considerable amount of oxidative stress. The optimal antimicrobial activity of neutrophils and other phagocytes is based on the burst production of superoxide anions (O_2^-) by activated NADPH oxidases. This O_2^- is then processed to give hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), hypochlorous acid (HOCl), and oxidizing derivatives of nitric oxide ($NO^\bullet$) such

as peroxynitrite ($ONOO^-$) (Müller *et al.*, 2003). Consequently, the common defense strategies against oxidative and nitrosative stresses include detoxification enzymes and repair systems that enable the cells to resist ROS and RNS (Figures 1.2 and1.3).



Source: Modified after Clark *et al.*, 2007

Figure 1.2: Predicted superoxide anion radical detoxification system of *Entamoeba histolytica*. Superoxide radical anions are detoxified by an iron-containing superoxide dismutase (FeSOD). Molecular oxygen is reduced to hydrogen peroxide by a NADPH:flavin oxidoreductase (thioredoxin reductase, p34). Hydrogen peroxide is converted to water by rubrerythrin (Rbr). The nature of its redox partner is unknown. Hydrogen peroxide can also be converted to water via a classical thioredoxin redox system consisting of thioredoxin reductase (TrxR), thioredoxin (Trx) and peroxiredoxin (Prx).



Source: Modified after Clark *et al.*, 2007

Figure 1.3: Predicted nitric oxide detoxification system of *Entamoeba histolytica*. Nitric oxide is reduced by an A-type flavoprotein (FprA) to nitrous oxide and water. For this reaction, FprA receives electrons from NADH oxidase (Far).

Within the *E. histolytica* genome at least 32 genes coding for putative antioxidant enzymes have been identified. So far for only a few of them, the involvement in protection against oxidative stress has been shown and the respective reaction pathways were elucidated. Thus, for the majority of the various putative amoeba antioxidant enzymes, the biochemical pathways are yet not understood. In this work, constructs of some putative antioxidant genes were made. These constructs included thioredoxins 1 and 5, flavoproteins A1 and A2, flavoprotein B2, flavoproteins C1 and C3, flavoproetein D3, and NADH oxidase. Additionally, the regulation of some other enzymes was investigated namely iron-superoxide dismutase, rubrerythrin, thioredoxin reductase, proxiredoxin, and iron-hydrogenase.

1.3.4.1 Superoxide dismutase (SOD)

Prominent among antioxidant enzymes are the superoxide dismutases (SOD), a group of metalloenzymes that detoxify the highly reactive superoxide anion (O_2^-) to H_2O_2 and O_2 by a dismutative reaction (Choi *et al.*, 2000, McCord and Fridovich, 1969):

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H_2^+ \rightarrow O_2 + H_2O_2$$

The regulation of superoxide dismutase in a protozoan parasite was first reported by Bruchhaus and Tannich, 1994. Superoxide dismutases (SODs), which catalyze the conversion of superoxide radicals into H_2O_2 and molecular oxygen, are part of the cellular defense system that helps to protect the cells against toxicity and damage caused by oxygen

metabolites (Bruchhaus and Tannich, 1994). Using copper/zinc (Cu/ZnSOD), manganese (MnSOD), or iron (FeSOD) as metal cofactors, analysis of *E. histolytica* genome revealed only a single gene coding for FeSOD, and no sequences encoding MnSOD or Cu/ZnSOD (Clark *et al.,* 2007).

Detailed studies on the regulation of SODs have used *Eschericha coli*, which possesses a FeSOD and a MnSOD (Keele *et al.,*1970; Yost and Fridovich, 1973). The FeSOD is expressed constitutively under both aerobic and anaerobic conditions, whereas the MnSOD is induced either by aerobiosis or in the presence of superoxide radical ions (Schiavone and Hassan, 1988).

1.3.4.2 Rubrerythrin (Rbr)

Rubrerythrin (Rbr) is a nonhemeiron protein that was originally isolated from the cytoplasm of the anaerobic bacterium *Desulfovibrio vulgaris* (Weinberg *et al.,* 2004). Later, in 2005, Pütz, reported about Rbr in *Trichomonas vaginalis*, which was found to be homologous with that of *E. histolytica*, although both seem to be of independent prokaryotic origin. *E. histolytica* Rbr shares some similarities with several archaebacterial sequences.

The identification of Rbr in mitosomes of *E. histolytica* adds another dimension to the hydroperoxide detoxification capabilities. Amoebal mitosomes are minute but highly abundant cellular structures that occupy up to 2% of the total cell volume. In addition, protein colocalization studies allowed identification of the amoebal hydroperoxide detoxification enzyme rubrerythrin as mitosomal protein (Maralikova *et al.,* 2010). Its functional Fe-S centers could allow Rbr to participate in the transfer of electrons from organic donors to oxygen via reduction of hydrogen peroxide, thus preventing its accumulation and toxicity inside these organelles (Maralikova *et al.,* 2010). Furthermore, Dolezal *et al.,* 2010, reported that Rbr is localized in the mitosomal matrix.

1.3.4.3 Peroxiredoxin (Prx)

Peroxiredoxins are the major thiol-containing surface antigen of *E. histolytica*. They belong to a widely dispersed group of proteins found from Archaea to plants to humans (Choi *et al.,* 2005).

They are an ubiquitous family of antioxidant enzymes that exert their protective role in cells through their peroxidase activity, whereby hydrogen peroxide, peroxynitrite and a wide range of organic hydroperoxides (ROOH) are reduced and detoxified using redox-active cysteines.

The reducing equivalents are provided by the thioredoxin reductase/ thioredoxin/ NADPH system. Peroxiredoxins are divided into two categories, the 1-Cys and the 2-Cys peroxiredoxins, based on the number of cysteinyl residues directly involved in the catalysis. The 2-Cys peroxiredoxins are divided into two classes called the 'typical' and the 'atypical' 2-Cys peroxiredoxins (Wood *et al.*, 2003, Bruchhaus *et al.*, 1997). The cystein residues are conserved and undergo a cycle of peroxide-dependent oxidation and thiol-dependent reduction during the reaction (Clark *et al.*, 2007).

In *E. histolytica*, five different genes coding for peroxiredoxins were identified. They all belong to the 2-Cys peroxiredoxin class. Four of them (Prx 1-4) share 98% sequence identity and have an unusual N-terminal Cys-rich repeat, whereas the fifth peroxiredoxin (Prx 5) lacks the Cys-rich N-terminal extension and shares only 30% identity with Prx 1-4 (Clark *et al.*, 2007).

Peroxiredoxins are released during invasive amebiasis. The trophozoites invade the epithelium and resist immune and non-immune host defences, including H_2O_2 and reactive oxygen species generated by the host immune cells. Therefore, the peroxiredoxins of *E. histolytica* are likely to play a key role in protection from oxidative stress during invasion (Choi *et al.*, 2005). In addition, peroxiredoxins play an important role in the protection of trophozoites against their own metabolically produced H_2O_2 (Cheng *et al.*, 2004).

1.3.4.4 Thioredoxin reductase (TrxR)

The thioredoxin reductase (TrxR)/thioredoxin (Trx) system is one of the enzymatic systems developed to resist the oxidative damage generated by ROS and to maintain the intracellular redox balance. It utilizes reduction equivalents from NADPH and is involved in different biological processes such as protection against oxidative stress, regulation of DNA synthesis, transcription, cellular growth, and apoptosis (Arias *et al.*, 2007).

As shown in Figure 1.2, detoxification starts by using the reducing potential from NADPH to eliminate ROS. After the reduction of Trx in a reaction catalyzed by TrxR, peroxiredoxin is then reduced, which in turn splits the hydrogen peroxide producing water.

Thioredoxin reductase had been originally sequenced with different annotation. It was called disulphide oxidoreductase (Eh34) (Leitsch *et al.*, 2007). Bruchhaus and Tannich (1995), were the first to identify this gene and to predict its putative role in protection of the amoebae against toxic oxygen metabolites.

Two distinct forms of TrxR have been evolved: a 35 kDa protein in prokaryotes, archaea, lower eukaryotes and plant; and a 55 kDa protein in higher eukaryotes and apicomplexan parasites (Müller *et al.*, 2003). The structural properties of EhTrxR indicate that the enzyme belongs to the low-molecular-weight family of TrxRs, having a redox active site containing two key cysteine residues (Arias *et al.*, 2007).

It is worth mentioning that the thioredoxin system presents a remarkable chemotherapeutic target, and several drugs are being developed to target either Trx or TrxR.

1.3.4.5 Iron hydrogenase

Hydrogenases are enzymes responsible for producing hydrogen gas by transferring electrons to two protons. They are oxygen sensitive and found in a wide range of living organisms. On the basis of the metal content of the catalytic subunit, hydrogenases can be grouped into two non-homologous classes. Those containing only Fe at the active site, called Fe-hydrogenases and those with Ni, Fe and sometimes Se, called [Ni-Fe]-hydrogenase and [Ni-Fe-Se]-hydrogenases. Fe-hydrogenase is a distinct class of hydrogen-producing metalloenzyme, present in a wide variety of prokaryotes and eukaryotes (Ali and Nozaki, 2007; Das *et al.*, 2006).

Fournier (2004) reported for the first time that in the bacteria *D. vulgaris*, the periplasmic Fe-hydrogenase was up-regulated in response to oxidative stress, indicating a new function of Fe-hydrogenase in the protective mechanism of *D. vulgaris*.

E. histolytica, *T. vaginalis*, and *S. barkhanus* (a diplomonade similar to *G. lamblia*) were found to have genes that encode Fe-hydrogenase, even though these protists lack hydrogenosomes (Nixon *et al.*, 2003). Fe-hydrogenase is not directly involved in the detoxification system of *E. histolytica*, but since it is sensitive to the concentration of molecular oxygen, it would be likely to play an indirect role in response to oxidative stress.

1.4 Transcriptional analysis using microarrays

Microarray analysis has emerged in the last few years as a flexible method for analyzing large numbers of nucleic acid fragments in parallel. Its origins can be traced to several different disciplines and techniques. Microarrays can be seen as a continued development of molecular biology hybridization methods, as an extension of the use of fluorescence microscopy in cell biology, as well as a diagnostic assay using capture to solid surface as a way to reduce the

amount of analytes needed. In molecular biology, analysis of nucleic acids by hybridization is a universally adopted key method for analysis (Müller and Röder, 2006).

The Microarray technology offers the possibility to work with small amounts of samples to investigate large number of genes of interest. For transcriptome studies, mRNA probes are used to synthesize cDNA which is in turn is labeled with fluorescent dyes. The labeled cDNA are then brought into contact with immobilized sequences of known genes that are fixed on a matrix, and referred to as the target or spot. This results in base pairing between the probe and the target.

Fluorescent dyes, especially cyanine dyes Cy3 and Cy5, which have the green and red colors respectively, are used in transcriptome comparative studies. The advantage of using different labeling dyes in one experiment is the ability to detect two different signals in one experiment. Consequently, microarrays provide the possibility of accomplishing comparative studies of two or more samples at the same time.

A number of different microarray platforms have been developed for *E. histolytica*. Oligonucleotide microarrays of selected amoebic genes were developed when genome annotation improved, providing a more detailed inventory of the transcriptome of *E. histolytica*. This type of microarrays makes it possible to distinguish between many gene family members, and have substantially greater sensitivity for low abundance transcripts (Ehrenkaufer and Singh, 2008).

In addition, transcriptome comparison of related organisms, symbiotic organisms, or different clones of the same species could be investigated using microarrays. Upon treatment of organisms with different oxidative substances, microarrays provide a very effective tool for understanding the mechanisms used by organisms to overcome and to adapt to the environmental condition on the transcriptional level. The effect of overexpression of some genes, and comparing mutant stains with parental strains could be investigated as well.

1.5 Treatment of *Entamoeba histolytica*

Among the many antiparasitic drugs, metronidazole (α-hydroxyethyl-2-methyl-5-nitroimidazole) has established itself as the most effective treatment of amebiasis. Metronidazole was initially described in 1959 as an agent with specific trichomonicidal activity (Speck *et al.*, 1975). In 1966, Powell published data concerning the usefulness of

metronidazole (Flagyl) in the treatment of intestinal and extraintestinal amebiasis (Everett, 1974).

In the microaerophilic parasites *G. intestinalis*, *T. vaginalis*, and *E. histolytica*, which lack mitochondria, metronidazole enters the cell and its organelles via passive diffusion. It is relatively inert until its 5-nitro group is reduced (producing a nitro radical) by an appropriate electron donor such as ferredoxin, thus the metronidazole is activated (Leitsch *et al.*,2007, Ali and Nozaki, 2007). DNA is the major target of activated metronidazole since the nitro radicals bind to it to disrupt or break the nucleotide strands, leading to cell death.

Due to the fact that TrxR, an enzyme that is required for the antioxidative defence, is directly involved in the nitroimidazole mode of action, it is important to elucidate how nitroimidazole gained its effectiveness.

Covalent adduct formation with nitroimidazole metabolites diminishes thioredoxin reductase activity, which is likely to impair the cell's oxidative defence because peroxidases depend on thioredoxin for their function. In the presence of elevated concentrations of oxygen, this might lead to the accumulation of H_2O_2, which results in oxidation of thiols and proteins, and in the generation of highly toxic hydroxyl radical. Under low oxygen tensions, the depletion of thiols also leads to oxidative damage in the cell (Leitsch *et al.*, 2009).

Asymptomatic intestinal infections are treated with luminal amoebicides such as paromomycin and diloxanide furoate, which bind to the ribosomes preventing protein synthesis. They result in the eradication of luminal amoeba, and prevent tissue invasion. On the other hand, invasive amoebic infections are treated with tissue amoebicides such as metronidazole.

1.6 Aim of the study

Trophozoites of the intestinal protozoan parasite *Entamoeba histolytica* usually reside and multiply within the human gut, which constitutes an anaerobic or microaerophilic environment. However, during tissue invasion the parasite is exposed to elevated levels of reactive oxygen or reactive nitrogen species. Accordingly, amoeba trophozoites require antioxidant enzymes to survive the oxidative stress. The identification of genes coding for these enzymes and the understanding of their regulation are major targets of this investigation.

Within the *E. histolytica* genome at least 32 genes coding for putative antioxidant enzymes have been identified. So far, for only three of them has the involvement in protection against oxidative stress been shown and the respective reaction pathways were elucidated. Thus, for the majority of the various putative amoeba antioxidant enzymes, the biochemical pathways are yet not understood.

Using transgenic amoebae as well as respective recombinant proteins the function of these *E. histolytica* molecules will be characterized in detail. Furthermore, studies using two syngenic *E. histolytica* clones with substantial differences in their pathogenic properties have revealed no differences in susceptibility to superoxide radicals and hydrogen peroxide but remarkable differences in the susceptibility to nitric oxide or peroxynitrite. To facilitate the analysis of expression of a large number of *E. histolytica* genes, a microarray was designed containing oligonucleotides derived from a total of 152 amoeba genes, most of which encode peptidases, pore-forming peptides and antioxidant enzymes. The microarray analysis, in combination with real-time PCR, will be used to determine gene expression of antioxidant enzymes in *E. histolytica* isolates that have been exposed to various harsh conditions and that differ in their pathogenic properties.

Moreover, over-expression of some antioxidant genes would help increase our understanding of the function of these genes. Recombinant proteins were synthesized and used in the production of polyclonal antibodies. These antibodies were used to localize the corresponding antioxidant proteins in the trophozoites, using immune fluorescence analysis (IFA).

2. Materials and methods

2.1 Materials

2.1.1 Chemicals and reagents

Cy3 / Cy5 monoreactive dye	GE Healthcare, Munich, Germany
Diamond Vitamin Tween 80	Amimed, Allschwil, Switzerland
TRIzol® Reagent	Invitrogen, Karlsruhe, Germany
TiterMax® Classic Adjuvant	Sigma – Aldrich, Steinheim, Germany

2.1.2 Enzymes and enzyme inhibitors

E-64	Sigma – Aldrich, Steinheim, Germany
Fast Digest® Enzymes	Fermentas, St. Leon – Rot, Germany
Restriction enzymes	Fermentas, St. Leon – Rot, Germany
RNase – free DNase	Qiagen, Hilden, Germany
RNase Out	Invitrogen, Karlsruhe, Germany
Superscript III™ Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
Taq – Polymerase	Promega, Mannheim, Germany
T4 – DNA Ligase	Fermentas, St. Leon – Rot, Germany

2.1.3 Immune Reagents

Anti – his – peptide IgG primary antibody	Quiagen, Germany
Anti – tap (human) primary antibody	Sigma – Aldrich, Germany
Anti – human – secondary antibody	Thermo Scientific, USA
Anti – rabbit – HRP secondary antibody	Dako A/S, Glostrup, Denmark
Anti – mouse – ALEXA Fluor® 488	Invitrogen, Karlsruhe, Germany
Anti – mouse – ALEXA Fluor® 594	Invitrogen, Karlsruhe, Germany
Anti – mouse – HRP secondary antibody	Dako A/S, Glostrup, Denmark
Anti – Prx (rabbit) primary antibody	Bruchhaus
Anti – TrxR (rabbit) primary antibody	Bruchhaus
Anti – FeSOD (rabbit) primary antibody	Bruchhaus
Anti – Rbr (mouse) primary antibody	This work
Anti – Fe-hyd (mouse) primary antibody	Biller

2.1.4 Marker

GeneRuler™ 1 kb Ladder	Fermentas, St. Leon – Rot, Germany
GeneRuler™ 100 bp Ladder	Fermentas, St. Leon – Rot, Germany
PageRuler™ Prestained Protein Ladder	Fermentas, St. Leon – Rot, Germany
6 x Loading Dye (DNA loading buffer)	Fermentas, St. Leon – Rot, Germany

2.1.5 Kits

BCA Protein Assay	Pierce Thermo Scientific, Rockford, USA
Bradford Protein Assay	BIO – RAD, Hercules, USA
Fast Plasmid Mini Kit	Eppendorf, Hamburg, Germany
660 nm protein Assay	Pierce Thermo Scientific, Rockford, USA
NucleoBond® Xtra Midi/Maxi Kit	Macherey – Nagel, Düren, Germany
NucleoSpin Extract II Kit	Macherey – Nagel, Düren, Germany
RealMasterMix SYBER Green Kit	Eppendorf, Hamburg, Germany
RNase – free DNase Kit	Qiagen, Hilden, Germany
RNeasy®– Mini Kit	Qiagen, Hilden, Germany
SuperScriptIII First Strand Synthesis Kit	Invitrogen, Karlsruhe, Germany
SuperScript™ Indirect labeling Kit	Invitrogen, Karlsruhe, Germany
TOPO TA Cloning® Kit	Invitrogen, Karlsruhe, Germany

2.1.6 Oligonucleotides

The oligonucleotides used in this work were synthesized by the company Eurofins MWG Operon (Ebersberg, Germany). The stocks solution had a concentration of 100 pmol, whereas a concentration of 5 pmol was used in the Real-time PCR analysis. The recognition sequences of the restriction enzymes are shown in **bold**. All sequences are oriented in the direction of 5´→ 3´. (Abbreviation: S = sense; AS = antisense)

2.1.6.1 PCR oligonucleotides used for cloning into the pNC-tap vector

EhRub-S (*KpnI*)	GG**GGTACC**ATGGCAACTCTCATTAATCTT
EhRub-AS (*BamHI*)	GG**CTCGAGGGATCC**AACCATTTTAAATAGAAACTT
EhPrx-S (*KpnI*)	GG**GGTACC**ATGTCTTGCAATCAACAAAAAGA

EhPrx-AS (*BamHI*)	GG**CTCGAGGGATCC**ATGTTAAATATTTCTTAATTCCA
EhTrx1-S (*KpnI*)	GG**GGTACC**ATGGCTGTACTTCATATTAACG
EhTrx1-AS (*BamHI*)	GG**CTCGAGGGATCC**ATGCTGTTTCAACCATTTGTTT
EhTrx5-S (*KpnI*)	GG**GGTACC**ATGACTATACCACTTAATACTACAAA
EhTrx5-AS (*BamHI*)	GG**CTCGAGGGATCC**TAGTTTTGATGCTTCGTCC ATAAATG
EhTrxR1-S (*KpnI*)	GG**GGTACC**ATGAGTAATATTCATGATGTTGTGT GATTATCG
EhTrxR1-AS (*BamHI*)	GG**CTCGAGGGATCC**ATGAGTTTGAAGCCATTTTTC ACAGCTTAAGG
EhTrxR2-S (*KpnI*)	GG**GGTACC**ATGAGTAATATTCATGATGTTGTG
EhTrxR2-AS (*BamHI*)	GG**CTCGAGGGATCC**GTAAACAATTTAAATGGTTGTGTTG
EhFprA1-S (*KpnI*)	GG**GGTACC**ATGACAAGTAAAGTGTTAGAAGTACT
EhFprA1-As (*BamHI*)	GG**CTCGAGGGATCC**ATTTTCTTCATCTTCTTCTTTTATTG
EhFprA2-S (*KpnI*)	GG**GGTACC**ATGAAAGCATTGGAAGTAGTAAAAG
EhFprA2-AS (*BamHI*)	GG**CTCGAGGGATCC**AGCTTTAAGGGCCTCAGCAAATTTCT
EhFprB2-S (*KpnI*)	GG**GGTACC**ATGACAAAACAAATTAAGGTATTATTG
EhFprB2-AS (*BamHI*)	GG**CTCGAGGGATCC**CTCAACTATTCTCTTAAGAACAAATG
EhFprC1-S (*KpnI*)	GG**GGTACC**ATGGTTGTTAAAGTACTTGTTCTTCTTG
EhFprC1- AS (*BamHI*)	GG**CTCGAGGGATCC**AAGTTTATTAAAAGCCTTAATTCCAG
EhFprC3-S (*KpnI*)	GG**GGTACC**ATGGTTGCTAAAGTTCTTGTTCTTCTTG
EhFprC3-AS (*BamHI*)	GG**CTCGAGGGATCC**TAATTTCTCACCACATTCAATTCCTG
EhFprD3-S (*KpnI*)	GG**GGTACC**ATGTCTTTAAAAGTTCTTACACTTCTTG
Eh-FprD3-AS (*BamHI*)	GG**CTCGAGGGATCC**AATTTTTTGTGCAATTTTTTCTCCA
EhFar-S (*KpnI*)	GG**GGTACC**ATGAATATAAATGTACAATATCGTTG
EhFar-AS (*BamHI*)	GG**CTCGAGGGATCC**AAAATATGTTTTCCATTTTTCTATTAG

2.1.6.2 PCR oligonucleotides used for cloning into the pNC and pJC45 vectors

In pNC:

EhRbr-S (*kpnI)*	GG**GGTACC**ATGGCAACTCTCATTAATCTTTG
EhRbr-AS: (*BamHI*)	GG**GGATCC**TTACAGAAAAATTATTGGATAAAAG

In pJC45:

EhRbr – S (*NdeI*)	GG**CATATG**GCAACTCTCATTAATCTTTGTAAG
EhRbr – AS (*BamHI*)	GG**GGATCC**TTACAGAAAAATTATTGGAT AAAAG

2.1.6.3 Oligonucleotides used for quantitative RT-PCR

(See Appendix I)

2.1.6.4 Oligonucleotides used in TOPO sequencing analysis and for cDNA synthesis

M13F	TOPO	GTAAAACGACGGCCAG
M13R	TOPO	CAGGAAACAGCTATGAC

Oligo $(dT)_{12-18}$ Primer

2.2 Devices and consumables

Unless otherwise mentioned, all the consumed materials used were manufactured by Biozym (Oldendorf, Germany), B. Braun (Melsungen, Germany), Carl Roth (Karlsruhe, Germany), Eppendorf (Hamburg, Germany), Greiner Bio-One (Frickenhausen, Germany), Millipore (Schwalbach, Germany) and Sarstedt (Nümbrecht, Germany).

Advalytic Epoxy AD100 Microarray	Ocimunbio, Ijsselstein, Netherland
Hybridization Chambers	Amicon / Omnilab, Bremen, Germany
Micronon YM-30	Millipore, Schwalbach, Germany
Certomat® S	B. Braun Biotech Int., Pennsylvania, USA

2.2.1 Devices

Confocal laser scanning microscope PV10	Olympus, Japan
Fluorescence microscope DMBR	Leica, Wetzler, Germany
GeneAmp PCR system 9700	Applied Biosystems, Carlsbad, USA
Rotor Gene 3000	Corbett, Sydney, Australia
ScanArray Express HT Scanner	PerkinElmer, Boston, USA

2.2.2 Buffers and Solutions

Unless otherwise mentioned, all buffers and solutions have the concentration of [1x].

MOPS (10 x)	200 mM MOPS, 50 mM Na-Acetate, 20 mM EDTA; pH was adjusted to 7.0 with NaOH; autoclaved
NaPBS (1 x)	6.7 mM Na_2HPO_4, 3.3 mM KH_2PO_4, 140 m M NaCl; pH was adjusted to 6.8 with HCl; sterile filtered
SSC (20 x)	3 M NaCl, 300 mM Sodium citrate; pH was adjusted to 7.0 with HCl; autoclaved
TBE (10 x)	890 mM Tris, 890 mM Boric acid, 25 mM EDTA; pH was adjusted to 8.0 with HCl; autoclaved
TBS (10 x)	100 mM Tris, 1.5 M NaCl; pH was adjusted to 7.2 with HCl; autoclaved

Nickel affinity chromatography buffers

Buffer A	6 M Guanidine hydrochloride, 100 mM NaH_2PO_4, 10 mM Tris; pH was adjusted to 8.0 with HCl
Buffer B	8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris; pH was adjusted to 8.0 with HCl
Buffer C	8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris; pH was adjusted to 6.3 with HCl
Elution Buffer	Buffer C, 100 – 500 mM Imidazol

Microarray buffers

Hybridization buffer	Ocimumbio, Ijsselstein, Netherlands
Blocking buffer	4 x SSC, 0.5% SDS (w/v), 1% BSA (w/v); volume was adjusted to 200 mL with H_2O_{dd}
Washing buffer 1	10 x SSC, 0.1% SDS (w/v); volume was adjusted to 250 mL with H_2O_{dd}
Washing buffer 2	5 x SSC; volume was adjusted to 250 mL with H_2O_{dd}
Washing buffer 3	2.5 x SSC; volume was adjusted to 250 mL with H_2O_{dd}

SDS – PAGE buffers

Electrophoresis buffer (10 x)	0.25 M Tris, 0.5 M glycine, 1% SDS, pH was adjusted to 8.3 with HCl; autoclaved

Stacking – gel buffer	0.5 M Tris, 0.4% SDS, pH was adjusted to 6.8 with HCl, volume was made to 500 mL; sterile filtered
SDS – sample buffer	125 mm Tris, 20% glycerin (v/v), 2% SDS (w/v), 20 mM DTT, 0.001 % bromophenolblue (w/v), pH was adjusted to 6.8 with HCl; sterile filtered
Resolving – gel buffer	1.5 M Tris, 0.4% SDS, pH was adjusted to 8.8 with HCl, volume was made to 500 mL; sterile filtered

Western blot buffers and ECL detection

Blocking buffer	5% non – fat dried milk in TBS (w/v)
Transfer buffer	25 mM Tris, 192 mM glycine, 1.3 mM SDS, 20% Methanol (v/v); pH was adjusted to 8.3 with HCl
Washing buffer	0.05% Tween in TBS (v/v)
Solution A	0.1 M Tris, 1.25 mM Luminol, pH was adjusted to 8.6 with HCl; volume was made to 200 mL with H_2O_{dd}; stored at 4 ˚C
Solution B	6.7 mM Parahydroxycoumaric acid in 10 mL DMSO; stored in the dark at RT

Solutions for the transfection of *E.histolytica*

Cytomix incomplete solution	120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4 25 mM HEPES, 2 mM EGTA, 5 mM $MgCl_2$; pH was adjusted to 7.6 with KOH, sterile filtered
Cytomix complete solution	To the incomplete cytomix solution, 4 mM ATP and 10 mM reduced Glutathione were added

Protein staining

Coomassie staining solution	50% methanol, 40% H_2O_{dd}, 10% acetic acid (v/v) 0.05% Coomassie brilliant blue R – 250 (w/v)
Coomassie de-staining solution	50% methanol, 40% H_2O_{dd}, 10% acetic acid (v/v)

2.2.3 Antibiotics

Ampicillin	Roche Applied Science, Mannheim, Germany
G – 418 Sulfate	PAA Laboratories, Pasching, Austria
Penicillin	Grünenthal, Aachen, Germany
Streptomycin	Riemer Arzneimittel, Greifswald, Germany
Hygromycin	PAA Laboratories, Pasching, Austria
Kanamycin	Sigma-Aldrich, Steinheim, Germany

Table 2.1 Antibiotics

Antibiotic	Stock solution in H_2O_{dd}	Working solution
Ampicillin	100 mg/mL	100 µg/mL in LB – Medium
G – 418 Sulfate	50 mg/mL	10–50 µg/mL in TY–I–S33 medium
Penicillin	2×10^5 U/mL	200 U/mL in TY–I–S33 medium
Streptomycin	200 mg/mL	200 µg/mL in TY–I–S33 medium
Hygromycin	50 mg/mL	10 µg/mL in TY–I–S33 medium
Kanamycin	50 mg/mL	50 µg/mL in LB-Medium

2.2.4 Culture media

LB Agar	32 g of Lennox L Broth Agar were suspended in 1L H_2O_{dd}, then autoclaved
LB medium	20 g of Lennox L Broth Base were suspended in 1L H_2O_{dd}, then autoclaved
Freezing medium for *E.histolytica*	10 mL DMSO, 6 mL 50% sucrose, 84 mL complete TYI-SS-33 medium
TYI-S-33 medium	100 g Trypticase, 50 g yeast extract, 50g glucose, 10 g NaCl, 3.8 g K_2HPO_4, 3 g KH_2PO_4, 5 g L – Cysteine, 1 g ascorbic acid, 114 mg Iron-ammonium-citrate, volume

	was made to 4.350 liters, pH was adjusted to 6.8 with NaOH; sterile filtered
Completed YI-S-33 medium	To 800 mL of incomplete medium, 100 mL inactivated (twice, each time for 30 min at 56˚C) bovine serum, 30 mL Vitamin – mix *Diamond Vitamin Tween 80*, 120 mg Streptomycin, and 100 mg penicillin were added

2.3 Organisms and Plasmids

2.3.1 Organisms

2.3.1.1 *Entamoeba histolytica*

Domain: Eukaryota

Phylum: Amoebozoa

Class: Archamobae

Order: Amoebida

Genus: *Entamoeba*

Species: ***Entamoeba histolytica***

Table 2.2: *Entamoeba histolytica* clones

E. histolytica cell line	Properties
HM-1:IMSS clone A	It was originally isolated from a patient with colitis in 1967 (ATCC Nr. 30459). Since 2001 in axenic culture in Bernhard Nocht Institute for Tropical Medicine – Hamburg
HM-1:IMSS clone B	It was originally isolated from a patient with colitis in 1967 (ATCC Nr. 30459). Since 1991 in axenic culture in Bernhard Nocht Institute for Tropical Medicine – Hamburg

In this work, two clones of *E. histolytica* were used. Both originate from the culture isolate HM-1:IMSS, which is registered by the American Type Culture Collection (ATCC) under the catalog number 30459. These cells were isolated in 1967, from colon biopsies of male adult patients with amoebic colitis in Mexico. A sample culture of the HM-1:IMSS clone A was obtained from the university of Virginia (USA) in 2001. Since that time it has been cultivated axenically at the Bernhard Nocht Institute for Tropical Medicine – Hamburg. HM1:IMSS clone B was obtained directly from the ATCC in 1991. Since that time it has been cultivated axenically at the Bernhard Nocht Institute for Tropical Medicine – Hamburg.

2.3.1.2 Bacteria

Table 2.3: *Escherichia coli*

Bacteria	Relevant genotype	Reference
One Shot Top 10^{TM}	F^-mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 lac X74 deoR rec A1 araD139 Δ(araleu)7697 gal U gal K rpsL (Str^R) endA1 nupG	Invitrogen
BL21(DE3) pAplacI^Q	HsdS, gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene1)[pAPlacI^Q]	O.Fayet, Toulouse

2.3.1.3 Plasmids

Table 2.4: Cloning and expression Vectors

Plasmid	Size	Characteristics	Reference
pCR®II- TOPO	4.0 kb		Invitrogen
pJC45	2.4 kb	Amp^r, colE1 ori, T7-Polymerase under λpL- lac Operator, 10His, Factor Xa, Terminator	Clos and Brandau, 1994
pNC	6.0 kb	Neo^r, 5´/3´-Actin range and Lectin promotor from *E. histolytica*	Wassmann *et al*., 1999

The plasmids were maintained in the suitable *E. coli* cells, and stored at – 70 ˚C as bacterial stocks.

2.3.1.4 *Mus musculus*

Genus: *Mus*

Species: ***Mus musculus*** (house mouse) (LINNAEUS, 1758)

In this work, female mice of the strain BALB/c were used for the generation of polyclonal antibodies. This strain is an albino, laboratory – breed of the house mouse which is suitable for generating polyclonal antibodies.

2.4 Methods

2.4.1 Cultivation and storage of *E. histolytica*

Trophozoites were cultivated under microaerophilic (maximum 5% molecular O_2), and axenic conditions in 75 mL polystyrene culture flasks at 36 ˚C. Cultivation was done in complete TY–I–S33 medium. Every 48 hours, the medium was poured out of the culture flasks, and then 5 mL of fresh complete medium were added to the flasks. Cells were then detached by placing the flasks on ice for 5 – 10 minutes. Depending on the density of growing cells, 5 – 10% of the cell suspension was transferred into new culture flasks filled with fresh completed medium.

In order to make stocks of the culture, cells were harvested in the logarithmic growth phase, by addition of 5 mL freezing medium to the cells in the flask, and placed on ice to allow detachment. The cells were collected in Kryo reaction tubes and stored for 24 h at – 70 ˚C, before storing them in liquid nitrogen.

2.4.2 Harvest of *E. histolytica*

1 x 10^6 trophozoites were cultured in 75 mL flasks for 24 hours before harvesting them. In this time, the trophozoites form a monolayer in the culture flasks. The number of trophozoites was determined by using the hemocytometer. Cells were detached from the flask walls by placing the flask on ice for 5 – 10 minutes. Finally, cells were sedimented by centrifuging at 400 x *g* and 4 ˚C for 4 minutes. The cells were then washed twice with cold NaPBS.

2.4.3 Cultivation and storage of bacteria

The cultivation of *E. coli* was performed in LB – medium (Sambrook and Gething, 1989) under aerobic conditions in Erlenmeyer flasks. Cultures were incubated overnight in a shaker (Cirtomat® S) at 150 – 200 rpm and 37˚C.

In order to make stocks, bacteria that had reached the exponential growth phase, were mixed with 15% (v/v) sterile glycerin in LB – medium. Stocks were then stored at – 70 ˚C.

2.4.4 Immunofluorescence analysis in *E. histolytica*

Using this technique, the intra – and extracellular proteins of *E. histolytica* could be localized using fluorescent secondary antibodies. 2×10^6 trophozoites were harvested (section 2.4.2), then fixed - using a shaker - in 3% paraformaldehyde in NaPBS solution (v/v) for 30 minutes at RT. To permealise the cell membrane, trophozoites were incubated in 0.05% saponin in NaPBS(w/v). Free aldehyde groups were blocked with 50 mM NH_4Cl for 15 minutes. The cells were washed with NaPBS. Unspecific antibody binding sites were blocked with 2% FCS in NaPBS (v/v) for 10 minutes. The cells were washed with NaPBS and then incubated for 1 hour (in the dark) with the primary antibody (in different dilutions in NaPBS). The cells were washed three times with NaPBS and incubated for 1 hour with the DNA – marker DAPI (100 mg/mL, diluted 1:100 in NaPBS), and the fluorescence marked secondary antibody (1:300 dilution in NaPBS). The secondary antibody is directed against the IgG of the first antibody. The cells were then washed three times, and then suspended in 100 µL NaPBS. The confocal laser scanning microscope (Olympus) was used for capturing the pictures at a slice thickness of 0.3 µm, starting at top of the cell and ending at the bottom. In addition, the exposure conditions were the same for all captures. The software Adobe Image Ready CS2 and Adobe Photoshop CS5 were used for the analysis.

2.5 Protein Biochemical Methods

2.5.1 Isolation of NaPBS-soluble proteins and urea-soluble proteins form *E. histolytica*

Harvesting of trophozoites was performed as described previously (section 2.4.2). In order to minimize the autoproteolytic process, 50 µM trans – epoxysuccinyl – L – leucylamino – (4 – Guanodino) butane (E-64), were added to the trophozoites which were suspended in 100 µL NaPBS. The cell suspension was freez-thawed five times using liquid nitrogen and a heating

block (Thermomixer compact) at 37˚C. To the cells fragments, another 50 µM E-64 were added, followed by centrifugation for 15 minutes at 4˚C and 16000 x *g*. The supernatant, which contains the NaPBS soluble proteins, was then transferred to a new tube. The sediment, which contains the Urea soluble proteins, was washed twice with NaPBS, and finally suspended in 8M Urea. Both soluble and insoluble proteins were then stored at – 70˚C.

2.5.2 Determination of protein concentration

2.5.2.1 Determination of protein concentration using bicinchoninic acid (BCA)

This procedure is based on the Biuret test, a chemical reaction used to detect the presence of protein. In a positive test, a copper (II) ion is reduced to copper (I), which forms a complex with the nitrogen and carbon of the peptide bonds in an alkaline solution. A violet color indicates the presence of proteins. The test (Pierce) was carried out according to the producer's protocol. The measurement was performed photometrically at a wavelength of 562 nm, and protein concentrations determined using a calibration curve with 0 – 2 mg/mL BSA.

2.5.2.2 Determination of protein concentration using Pierce 660 nm Protein Assay test

The Pierce 660 nm Protein Assay is based on the binding of a proprietary dye-metal complex to protein in acidic conditions, which causes a shift in the dye's absorption maximum, measured at 660 nm. The dye-metal complex is reddish-brown and changes to green upon protein binding. The color change is produced by deprotonation of the dye at low pH, facilitated by interactions with positively charged amino acid groups in proteins. The dye, therefore, mainly interacts with basic amino acids such as histidine, arginine, lysine, and to a lesser extent tyrosine, tryptophan and phenylalanine. The test (Pierce) was performed according to the producer's protocol, and the measurement carried out using a calibration curve with 0 – 2 mg/mL BSA.

2.5.3 SDS polyacrylamide gel electrophoresis (SDS – PAGE)

SDS-PAGE uses an anionic detergent (SDS) to denature proteins. The protein molecules become linearized. These protein molecules move in the 12% gel (towards the anode), with their speed of migration based on their molecular weight, and are thus separated.

Table2.5: Composition of the solutions for SDS – PAGE

Reagents	Resolving gel (12% acrylamide)	Stacking gel (4% acrylamide)
Acrylamide (37.5:1)	3 mL	0.65 mL
Gel buffer	1.88 mL	1.25 mL
H_2O_{dd}	2.6 mL	3.00 mL
25% APS	25µL	25 µL
TEMED	5µL	5µL

The samples were mixed with 2x Laemmli sample buffer at a ratio of 1:2 and heated on a heating block (Thermomixer compact) at 95 ˚C for 3 minutes. The samples were then run on the gel in 1x electrophoresis buffer at 120 V.

2.5.4 Coomassie blue staining of protein

The principle of this staining method is based on the binding of the dye Coomassie Brilliant Blue nonspecifically to all proteins. The gel is soaked in a solution of the dye for at least 30 minutes, followed by three destaining steps each 20 minutes. The gel was then rehydrated in H_2O_{dd} at 4 ˚C.

2.5.5 Western blot detection and analysis

This analytical technique allows the transfer of proteins from SDS-polyacrylamide gel to a nitrocellulose membrane, where they are detected using antibodies specific to the target proteins. In this work, the wet blot technique was used. The transfer carried out in transfer buffer at 400 mA for 1 hour.

In order to prevent non-specific binding, the membrane was soaked in non-fat milk solution (blocking buffer) for 30 minutes. The membrane was then placed on a roller in the blocking buffer containing the primary antibody (at different dilutions) overnight at 4 ˚C. The membrane was washed three times with washing buffer, and then placed in the blocking buffer for 30 minutes. After that, the membrane was placed in blocking buffer to which the HRP – conjugated secondary antibody was added (at a dilution of 1:3000) and incubated on a roller for 2 hours at RT. The membrane was then washed three times before beginning the

ECL procedure. The membrane was incubated in freshly prepared ECL solution (5 mL solution A, 500 µL solution B, and 1.5 µL 30% H_2O_2) for 2 minutes at RT, and then directly exposed to a film for 1 sec – 5 min. This film was then developed.

2.5.6 Producing polyclonal antibodies

In order to produce polyclonal antibody against the recombinant protein EhRbr, three female mice of the type Balb/c were immunized. To do the immunization, 50 µg of the purified protein were mixed with complete TiterMax® Classic adjuvant (Sigma) in a proportion of 1:1 (v/v) respectively. The mix was injected into the mice intra peritoneal. With three time intervals, each of two weeks, a booster injection was given to strengthen the immunological response. The booster injection, containing 50 µL of the purified protein mixed with 50 µL of incomplete TiterMax® Classic adjuvant, was given using an identical procedure to above. After further two weeks, mice were sacrificed and the blood was collected.

The blood was stored at 4 ˚C until completely coagulated, then sedimented at 400 x *g* and 4˚C for 5 min. The supernatant which contains the serum was transferred to a new tube and centrifuged at 2300 x *g* and 4˚C for 5 min. The supernatant contains the polyclonal antibodies against the recombinant protein. The antiserum was used for specific protein detection in western blot analysis and for the localization of proteins using immunofluorescence analysis.

2.6 Molecular methods

2.6.1 Isolation of total RNA from *E.histolytica*

With the help of *TRIzol*® reagent (Invitrogen), which is a single phase solution of phenol and guanidine-isothiocyanate, total RNA could be isolated from cells or tissues. After harvesting trophozoites (section 2.4.2), the cell sediment was suspended in 1 mL TRIzol®- reagent, and incubated at RT for 5 min. 200 µL chloroform were added to the tube, shaken well, and incubated at RT for 3 min. The solution was then centrifuged at 4˚C (15 min, 11500 x *g)*. Three distinguishable phases were formed. The upper watery phase, which contains the RNA, was transferred into a new tube. RNA was precipitated with 500 mL isopropanol (incubation for 10 min at RT and centrifugation for 10 min at 4˚C). After washing with 70% (v/v) ethanol, and drying the pellet at 56˚C for about 10 minutes, the RNA was dissolved in 100 µL H_2O_{dd}.

2.6.2 RNA purification and DNA digestion

Cleaning of the isolated RNA was performed with the *RNeasy*®*-Mini*-Kit (Qiagen). The 100 µL containing the isolated RNA were mixed with 350 µL RLT buffer and 250 µL 100% ethanol. The whole mixture was run through the silica – matrix of the provided column, and then washed with 350 µL RW – 1 buffer. DNA was digested according to the standard protocol provided with the Kit (RNase – free DNase set). The RNA was eluted in 25 µL H_2O_{dd}, and was either used directly or was precipitated with sodium acetate and stored at -70 ˚C.

2.6.3 Precipitation of nucleic acids

The RNA or DNA solution was precipitated with 10% of its volume of 3M sodium acetate and 2.5% of its volume of isopropanol. The cations of the salt attach to the nucleic acids in the presence of alcohol and thus the nucleic acids precipitate. In order to dissolve and purify the precipitated nucleic acids, the solution was centrifuged for 10 min at 4˚C and 15000 x *g*. The pellet was washed twice with 70% ethanol, dried at 56˚C, and then suspended in H_2O_{dd}.

2.6.4 Measuring concentration and purity of nucleic acids

The concentration measurement of dissolved DNA and RNA samples was determined using a BioPhotometer (Eppendorf) at a wavelength of 260 nm. For an optical density (OD) of 1, the concentration of double-stranded DNA is assumed to be 50 µg/mL, and that of RNA to be 40 µg/mL. The purity was determined in regard to the ratio of $OD_{260nm/280\ nm}$. A pure DNA has the $OD_{260nm/280\ nm}$ value of 1.8, and pure RNA has the ration of 2.0 (Sambrook *et al*., 1989). The quality of the purified nucleic acids was additionally assessed by agarose gel electrophoresis.

2.6.5. Isolation of plasmid DNA from bacteria

2.6.5.1 Plasmid – Mini – preparation

The isolation of small amounts of plasmid – DNA was performed using the Fast Plasmid Mini Kit (Eppendorf) according to the producer's standard protocol. A single solution is used for cell resuspension, lysis, and DNA trapping. This solution contains a mixture of enzymes and detergents that lyse bacterial cells, denature and solubilize cellular components, degrade RNA, and trap DNA on a matrix. For the preparation, 1.5 mL of an overnight culture of

transformed bacteria were used. The plasmid – DNA was eluted in 50 µL H_2O_{dd}, and stored at – 20 ˚C if not directly used.

2.6.5.2 Plasmid – Maxi – preparation from *E. coli*

For the transfection of *E. histolytica*, larger amounts of plasmid – DNA were needed. 500 mL of overnight culture were prepared for the isolation, which was performed with the *NucleoBond*®Xtra Maxi kit (Macherey – Nagel). The standard protocol provided with the kit was used for the isolation. The isolated DNA was suspended in 0.5 mL $H_2O_{dd,}$ and stored at -20˚C.

2.6.6 cDNA synthesis

The synthesis of cDNA was accomplished using the SuperscriptIII-First-Strand Synthesis System Kit (Invitrogen). In order to produce cDNA only from mRNA, the oligo(dt12-18)-primer (Invitrogen) was used. It first anneals by T:A base-pairing to the poly(A) sequences universally present at the 3′ end of nearly every mRNA. The reverse transcriptase then extends from the annealed oligo(dT) primer along the mRNA template, resulting in the copying of mRNA sequence into cDNA sequence. The single-strand cDNA is then converted into double-strand cDNA. The reaction mixture (20 µL) was prepared as follows:

Synthesis buffer (5x)	4 µL
DTT (0.1 M)	2 µL
dNTPs [10 mM]	2 µL
Oligo (dT 12-18) primer	2 µL
RNaseOut	0.5 µL
SuperScript III	1 µL
$MgCl_2$ [25 mM]	0.4 µL
RNA	1 µg
H_2O_{dd}	up to 20 µL

The mixture was incubated for 1 hour at 42 ˚C. The produced cDNA (if not directly used for quantitative Real – Time PCR) was stored at -20 ˚C.

2.6.7 Quantitative Real – Time PCR

The Real-Time – PCR method is used for quantitative analysis of gene expression. It allows the detection of PCR product development. SYBR® Green I (Absorption at 498 nm), is a fluorescent dye that binds to the DNA double helix. Upon binding to amplified dsDNA, the fluorescence is enhanced and thus increases proportionally to the amount of amplified product. The increased fluorescence intensity is analyzed against the background fluorescence level. The cycle in which the fluorescence signal exceeds the background fluorescence is defined as threshold cycle (C_T). The C_T-value of the sample is correlated with that of the control.

In this work, the concept of relative quantification was implemented. The expression of the gene of interest (GOI) was measured and normalized in relation to a reference gene (*β – actin*) which does not undergo regulation (housekeeping gene). This normalizing gene serves as an internal standard, through which the cDNA output could be equalized.

The relative quantity was determined using the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

$$2^{-\Delta\Delta C_T} = 2^{-((C_T\,P_{GOI} - C_T\,P_{Ref}) - (C_T\,K_{GOI} - C_T\,K_{Ref}))}$$

Where:

C_T = Cycle of threshold
P = Probe-clone (HM-1:IMSS-clone B)
K = Calibrator-clone (HM-1:IMSS-clone A)
GOI = Gene of interest
Ref = Reference gene (β-*actin*)

The difference (Δ) in the C_T value between the GOI and the normalizing gene is calculated according to the following equation:

$$\Delta C_T = C_{T\,GOI} - C_{T\,\text{normalizing gene}}$$

The reaction mixture was prepared using the RealMasterMix SYBR Green kit (Eppendorf) according to the following:

Real Master Mix (2.5x)/SYBR Green + ROX (20x)	9 µL
Sense primer (5 pmol/ µL)	2 µL
Antisense primer (5 pmol/ µL)	2 µL
HPLC H_2O	6 µL

cDNA	1 µL

For the negative control 1 µL HPLC H_2O was used instead of 1 µL cDNA.

The amplification was performed using the following cycler program:

Initial denaturation	95˚C	for 150 sec
35 cycles:		
Denaturation	95˚C	for 15 sec
Annealing	58˚C	for 20 sec
		(with 1˚C touch down through the first 6 cycles)
Elongation and detection	68˚C	for 20 sec

The specificity of the amplified product was determined using a melting curve. For this purpose, the temperature was raised from 55 ˚C to 95 ˚C with increments of 1˚C. The change in fluorescence intensity was measured at increments of 8 seconds.

The reaction was performed using Rotor gene 3000 (Corbett Life Sciences), and the evaluation was performed with the corresponding software. The limiting value for a differential gene expression was set to be 2.5.

2.6.8. Amplification of DNA using Polymerase Chain Reaction (PCR)

This technique was used to amplify a certain gene of interest (GOI) using specific sense and antisense primers designed for the amplification and gDNA as a template. The reaction consists of a repeating cycle of three major steps:

1. Denaturation of the DNA molecule
2. Annealing of the primers
3. DNA synthesis

AccuPrime™ *Pfx* DNA Polymerase kit (Invitrogen), was used for the amplification. The DNA polymerase possesses a proofreading 3′ to 5′ exonuclease activity that provides high fidelity. In addition, it possesses a fast chain extension capability. 10x AccuPrime™ *Pfx* Reaction Mix contains Thermostable -AccuPrime™ proteins, $MgSO_4$, and dNTPs. In a final volume of 50 µL, 5 µL 10X AccuPrime™ *Pfx* Reaction Mix (final concentration of 1X), 1.5 µL primer mix (10 µM each), 0.7 µg gDNA template, and 1µL AccuPrime™ *Pfx* DNA Polymerase were mixed, and the volume was made 50 µL with H_2O_{dd}. The denaturation was at 95 ˚C for 40

seconds. The annealing temperature was determined according to the length of the primers and their melting temperature (T_m). It was applied for 40 seconds. The DNA synthesis depends on the size of the fragment to be amplified. Using this enzyme mix, 1 kb is amplified each minute at 72 ˚C. The reaction was placed in the thermocycler, and the cycles were repeated 35 times.

Direct cloning of DNA amplified by proofreading polymerases into the TOPO® vector (Invitogen) is often difficult since the proofreading polymerases remove the 3´ A-overhangs necessary for the cloning. For this reason, 1 unit of *Taq* polymerase was added to the amplified product, and incubated at 72 ˚C for 15 min.

2.6.9. Agarose gel electrophoresis

Agarose gel electrophoresis was used for the analytical separation of negatively charged DNA or RNA molecules. Depending on the size of the target fragment, the concentration of agarose ranged from 1 – 2 % (w/v) in 1x TBE buffer. In order to be able to detect the separated bands under UV light, 5 µL of ethidium bromide (1 mg/mL) were added to each 100 mL agarose during the preparation. Before loading the gel, samples were mixed with loading buffer (Fermentas). Different GeneRuler™ markers were used depending on the size of the fragments. The gel was run at a constant 120 V, using 1x TBE as a running buffer.

2.6.10 Extraction of DNA and PCR products from agarose gels

The desired bands were cut from the gel using a scalpel under weak UV light. The elution of DNA from the agarose was done using the NucleoSpin® Extract II Kit (Macherey-Nagel), according to the provided protocol.

2.6.11 Restriction analysis of DNA

With the help of restriction endonucleases, specific sequences of the DNA molecule could be cleaved. This technique is helpful for identifing a cloned DNA fragment and for linearizing the circular vector DNA. FastDigest® enzymes (Fermentas) were used as indicated by the producer.

2.6.12 DNA sequence analysis

Sequence of plasmid DNA was performed by the company MWG, using the value read sequencing service. The evaluation of the sequences was done using the software MacVector 10.0 (Accelrys).

2.6.13 Cloning and transformation with the TOPO TA Cloning® kit (Invitrogen)

The PCR-amplified DNA was cloned into the pCR®2.1-TOPO vector. The plasmid vector contains topoisomerase I from *Vaccinia* virus that works as a restriction enzyme. It allows the ligation of PCR products with poly-A-overhangs. Direct cloning of DNA amplified by proofreading polymerases into TOPO TA Cloning® vectors is difficult because proofreading polymerases remove the 3´A-overhangs necessary for TA Cloning. To be able to clone the blunt – ended fragments, *Taq* polymerase, which adds 3´A-overhangs, was added to the vial containing the PCR product, and then was incubated at 72 ˚C for 15 minutes. Transformation into TOP 10 cells was performed as indicated by the manufacturer's procedure.

2.6.14 Ligation of DNA fragments

DNA fragments were inserted into linearized vectors such as pNC, pNC*tap*, or pJC45 with the help of T4-DNA-ligase. The relative concentration of both the insert and the vector were estimated by means of 1% agarose gel electrophoresis. The ligation reaction was performed as indicated in the producer's manual.

2.6.15 Preparation of competent bacterial cells

In order to allow the bacteria able to take up foreign DNA more efficiently, the cells were incubated with a high concentration of $CaCl_2$. To produce the competent cells, *E. coli* bacteria were cultured in 40 mL LB medium at 37 ˚C, until they reached an OD_{600} of 0.4. The cells were sedimented at 200 x *g* and 4˚C. The cell sediment was suspended in 20 mL solution containing 50 mM $CaCl_2$, and incubated for 20 minutes on ice. The cells were sedimented once more under the same conditions. The sediment was suspended in 4 mL $CaCl_2$ (50 mM). For a long storage time, 50% Glycerin (v/v) was added to the cells, and the suspension was stored at – 70˚C.

2.6.16 Transformation of *E. coli*

For the transformation of plasmid DNA into bacteria, 50 µL competent bacterial cells were incubated with 4 µL of the ligation solution. The mixture was incubated for 10 minutes on ice; the cells were exposed to heat stress for 30 seconds at 42 ˚C, and then once more incubated on ice for 10 minutes. 250 µL SOC medium were added to the mixture and shaken at 800 rpm (Thermomix compact – Eppendorf) for 1 hour at 37˚C. Finally, the transformed bacterial cells were incubated on LB-amp agar plates overnight at 37 ˚C. In order to identify and to select the transformed cells, 40 µL of 20% X-Gal (5-brom-4-chlor-indolyl-beta-D-galactopyranosid) in DMSO (w/v) were added to the cells before plating them. X-gal is cleaved by β-galactosidase yielding galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter is then oxidized into 5, 5'-dibromo-4,4'-dichloro-indigo, an insoluble blue product. In successful cloning, the cleavage is prohibited, yielding white bacterial colonies.

2.6.17 Recombinant expression of proteins in *E. coli*

Recombinant expression of the *E.histolytica* molecule Rubrerythrin was carried out in pAPlacI^{Q} bacterial cells. The coding sequence was amplified with PCR from gDNA, and ligated in the bacterial expression vector pJC45. The vector contains an N-terminal His – *tag*, which allows the purification of the recombinant protein. The constructed plasmid pJC45–Rbr was transformed into pAPlacI^{Q}, and incubated in LB – amp medium overnight at 37˚C with 160 rpm. The overnight culture was transferred into 500 mL LB medium containing ampicillin (100 µg/mL) and kanamycin (50 µg/mL). The culture was incubated at 37 ˚C and 160 rpm. Protein synthesis was induced by adding 1 mM IPTG. The culture was incubated under the same conditions for 5 hours. Bacterial cells were sedimented at 4 ˚C and 5000 rpm for 20 minutes, and then stored at – 20 ˚C.

2.6.18 Purification of recombinant protein through Nickel chelate affinity chromatography

Using this method (described in details below), His-tagged recombinant proteins can be purified. The proteins bind through their N-terminal Histidine residues to the Ni^{2+} ions in the column matrix. The nickel ions, in turn, are attached to agarose (held within a column) through nitrilotriacetic acid (giving the name Ni – NTA Superflow column). The attached proteins could be eluted from the matrix using different concentration of the competitor imidazol.

The sedimented bacteria were suspended in 15 mL Buffer A, lysed on ice by ultrasound, and then centrifuged for 30 min at 10000 x g and 4 ˚C. The supernatant, which contains the soluble proteins, was collected. The pellet was suspended in Buffer A with guanidinium hydrochloride, treated in an ultrasound water bath for 10 min, and then centrifuged for 10 min at 10000 x g and 4 ˚C. The supernatant, which contains the insoluble proteins, was collected. By means of SDS-PAGE, it was observed that the protein of interest was found in the insoluble extract.

Buffer A was used to equilibrate 4 mL of the Ni – NTA Superflow (Qiagen) on the Ni – NTA Superflow column. The supernatant that contains the insoluble protein was loaded on the column. This was followed by washing steps with buffer A, buffer B and buffer C. The column was then washed with Buffer C that contains 20 mM imidazol. Finally the protein was eluted using buffer C that contains 500 mM imidazol in 1 – 1.5 mL fractions. The protein fractions were checked by means of SDS-PAGE and western blot analysis using the anti-histidin antibody. Eluted fractions with the highest content of the desired protein were used for immunizing of mice.

2.6.19 Transfection of *E. histolytica*

The transfection of *E. histolytica* through electroporation is based on a method described by Hamann *et al.* (1995). The cell membrane of the trophozoites is made permeable temporarily, which allows the entrance of the desired plasmid DNA.

The trophozoites (about 1 x 10^7) were harvested (section 2.4.2). The cell pellet was washed twice with 40 mL NaPBS and once with 40 mL incomplete cytomix. The cell pellet was suspended in 2.4 mL complete cytomix. In each electroporation cuvette (0.4 cm diameter), 800 µL of the cell suspension were mixed with 100 µg plasmid DNA by pipetting up and down. The mixture was then electroporated twice at 1200V and 25 µF. The time constant was between 0.4 and 0.6 ms. The transfected cells were then directly transferred to 75 mL culture flasks containing prewarmed TYI-S-33 medium.

The cells were then incubated at 36 ˚C for 48 hours to recover. Due to the resistance conveyed by the plasmid, cells were placed under selection pressure of a neomycin analogue, G418-sulfate. The selection was started with the concentration of 10 µg/mL, and raised gradually to reach 50 µg/mL G418.

2.6.20 Microarray studies

The microarray technique was used in this work to analyze the gene expression profile in experimental *E. histolytica* cells. With the help of this technique, the gene expression in the control cells was compared with the experiment cells. Two biological replicates were analyzed, and dye-swap experiments (exchange of fluorescent dye) were performed. Figure 2.1 illustrates the schematic run of the experimental procedure.

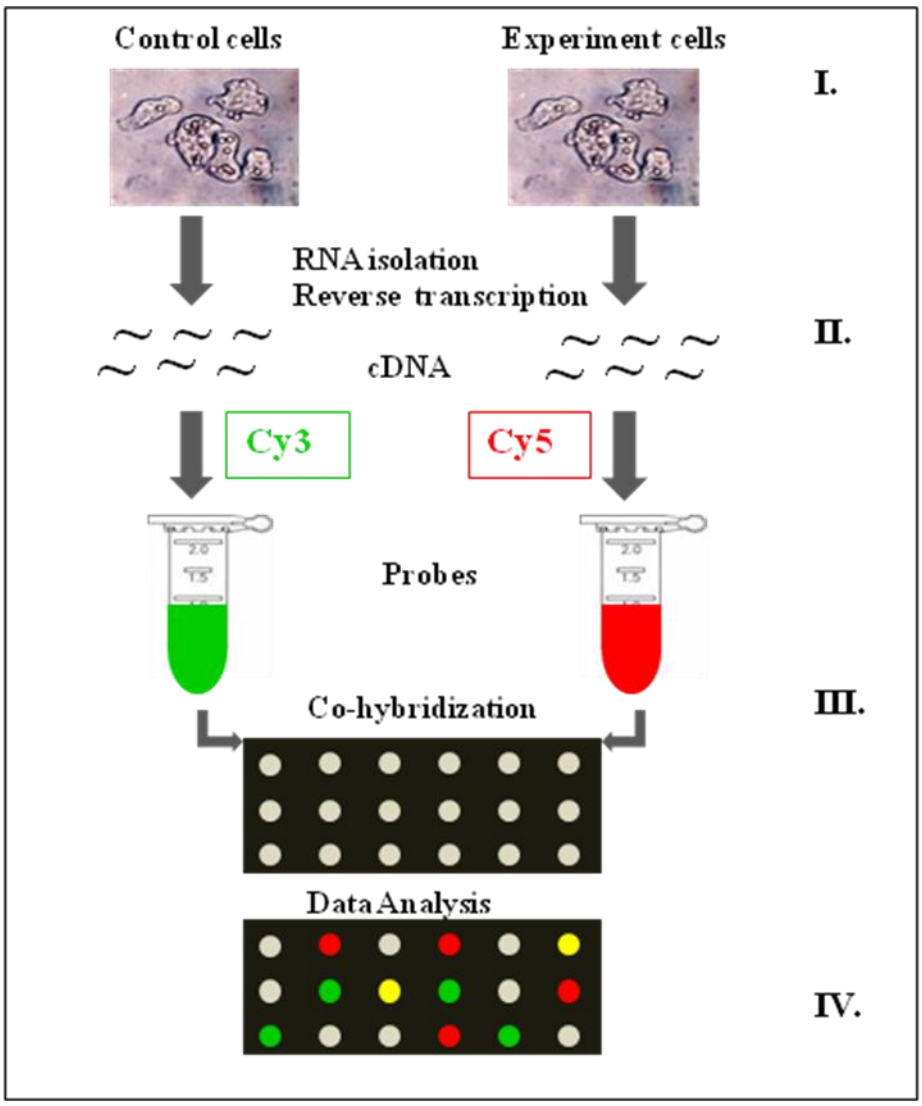


Figure 2.1: Schematic representation of a microarray experiment. I. Isolation of RNA from sample cells II. cDNA synthesis and indirect fluorescence labeling of the probes III. Co-hybridization of both probes on an oligonucleotide microarray IV. Detection of the Cy3 and Cy5 hybridization signals. The signals are overlaid through calculations, which lead to an overlay representation. Differentially expressed genes are reflected by green or red, while yellow reflects an equal expression of genes in both samples.

2.6.20.1 The principle of the microarray technique

Microarrays are classified into oligonucleotide (oligo – array), on which short and single stranded DNA (20 – 70 Bp) are bound, and DNA microarrays, on which the DNA – arrays are made up of much longer DNA fragments (about 1000 bp). In this work, the oligo – array technique was used. An oligo – array carries specific oligonucleotide sequences, that have sequences complementary to the investigated genes. The oligonucleotides are immobilized on the glass microscope slide (Matrix) through covalent bonding in defined positions (spots) of a raster (array) (Maskos and Southern, 1992). Next, the oligonucleotides can be synthesized directly on the glass slide nucleotide by nucleotide by a photolithgraphic procedure (Fodor *et al*., 1993). This method was developed by the Affymetrix company (Affymetrix, Santa Clara, USA).

To measure the relative expression differences between a probe and a reference, the RNA is isolated and transcribed into cDNA. The cDNA is fluorescently labeled (Kallioniemi *et al.*, 1992), and hybridized with the oligonucleotides on the array (competitive hybridization). Hybridization signal is then measured by means of scanner. The intensity of the hybridization signals is directly proportional to the relative level of transcription. In order to get statistically representative results, the experiments were repeated and the data was normalized. For each probe, two biological replicates were used.

2.6.20.2 Microarray design

The oligonucleotides that were chosen as probes for the microarrays had a GC content of 35% and an average of melting temperature (T_m) of 71.6˚C with a standard deviation of 1.17 (66˚C - 74˚C). The oligonucleotides were synthesized by the company Eurogentec in quadruplicate replications with a concentration of 50 µM. They were fixed on advalytic epoxy AD100 glass slides. The microarray was produced by the company Ocimumbio (Ijsselstein, Netherland).

2.6.20.3 cDNA synthesis and indirect fluorescence labeling

Total RNA was isolated from the trophozoites using Trizol reagent (section 2.6.1), purified, and treated with DNase (section 2.6.2). The concentration and purity of the isolated RNA was measured photometrically. The synthesis of cDNA was accomplished with the help of the SuperScript™ Indirect cDNA labeling System Kit (Invitrogen) according to the directions of the producer. For each probe, 7 µg RNA were used for the cDNA synthesis. As the cDNA

was produced, RNA was denatured using RNase H, which was provided in the kit. The cDNA was purified using the S.N.A.P.™ column purification of the Purification module kit (Invitrogen), according to the directions of the producer. The cDNA was precipitated after mixing with 300 µL ethanol, 10 µL 3 M Na – acetate, 2 µL 20 mg/mL glycogen, and storing at – 20 ˚C for 45 min. The probe was sedimented at 4 ˚C and 11500 x *g* for 15 min, and washed with 75% ethanol (in H_2O_{dd}). The probe was resuspended in 5 µL 2x coupling buffer (Invitrogen).

The Cy3 and Cy5 monoreactive dye aliquots (GE Healthcare) were dissolved in 40 µL DMSO in preparation for the labeling of the amino-modified cDNA. The cDNA was mixed with 10 µL of the matching dye, and incubated for 1 hour in the dark at RT. In order to remove the unreacted dye, the probes were once again purified using the S.N.A.P.™ column purification, and eluted in 50 µL HPLC-H_2O. The efficiency of fluorescence labeling was determined spectrophotometrically. The absorption of the Cy3 labeled probes was measured at a wavelength of 550 nm ($A_{550\ nm}$), and that of the Cy5 labeled probes was measured at a wavelength of 650 nm ($A_{650\ nm}$). The amount of the coupled fluorescent dye was calculated according to the following equation:

$$\text{pmol Cy3} = A_{550\ nm} \times \text{volume of probe (µL)} / 0.15$$

$$\text{pmol Cy5} = A_{650\ nm} \times \text{volume of probe (µL)} / 0.25$$

All probes must have 250 pmol of coupled dye. The corresponding volumes were mixed together, and concentrated to a volume of 20 µL using microcon centrifugation filter YM-30 (Millipore) by centrifuging at 11500 x *g* for 10 min.

2.6.20.4 Hybridization

Before hybridization, the microarrays were soaked in blocking buffer for 30 min at 42˚C. This step was necessary in order to block the unspecific binding sites. The microarrays were washed five times with H_2O_{dd}, dried, and embedded in the hybridization chambers (Amicon). The area on which the arrays are found was surrounded with sticky plastic frames. The probes were suspended in 120 µL hybridization buffer which was warmed to 42 ˚C (Ocimumbio). The mix was incubated at 95˚C for 3 min, and then was distributed on the microarray without allowing the formation of air bubbles. In order to reduce the drying of

sample during hybridization, 10 µL H_2O_{dd} were added to the hybridization chamber, which in turn was closed tightly and incubated overnight at 42˚C with slight swirling.

The microarrays were washed sequentially with three washing buffers, all warmed to 30˚C. This was an important step to minimize the background signals during the evaluation. All washing steps were 5 minutes, in a glass slide container, and with a continual up and down swirling mixing. Finally the microarray slides were washed with H_2O_{dd} and dried up by centrifugation.

2.6.20.5 Evaluation and normalization of the microarray data

Each microarray was analyzed, using ScanArray Express HT Scanners (PerkinElmer), at 550 nm for Cy3 and 650 nm for Cy5 with a resolution of 5 µm. The collection and evaluation of data were performed using the ScanArray software (PerkinElmer). For the data analysis, the mean signal intensity (pixel) minus the mean local background (pixel) of each spot was calculated. With this step, the error in the marked spots was excluded. For the evaluation of data, the global mean normalization method was used. This method provides that most of the genes represented on the array are experimented under the same conditions. Calculation of the normalizing factor was done according to the following formula:

$$\text{Normalizing factor} = \Sigma_{\text{spot intensity of experiment}} / \Sigma_{\text{spot intensity of control}}$$

The values of the spots intensity of the control were multiplied by the individual calculated value of the normalizing factor. After normalization, the genes that had the $2 \leq \text{ratio} \leq 0.5$ were considered as differentially expressed.

2.7 Incubation of trophozoites with radical releasing chemicals (Viability tests)

2.7.1 Preparation of trophozoites suspension for the reaction

The following experiment was carried out using cultured trophozoites (section 2.4.1). The trophozoites were harvested (section 2.4.2), and sedimented at 200 x g for 4 min. The cell pellet was washed twice with ice cold 1x NaPBS, and was then suspended in 2 mL of 1x NaPBS. The number of living cells in the suspension was determined with the help of a hemocytometer. A total number of $5x10^5$ cells/mL were suspended in 1 mL NaPBS in a 1.5-mL tube. After adding the radical producing chemicals, the reaction tubes were incubated at

37˚C in a heating block (Thermomixer compact) for 90 min. As control, trophozoites were incubated in NaPBS under the same conditions. The numbers of viable cells were determined using Trypan blue. Experiments were performed twice in duplicates.

2.7.2 Preparation of transgenic trophozoites suspension for the reaction

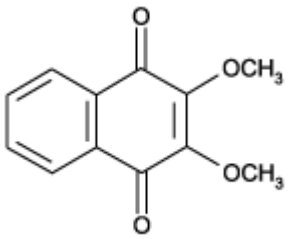
The following experiment was carried out using transgenic amoeba in which various antioxidant genes were overexpressed. Trophozoite cultures (section 2.4.1) were supplemented with 50 µg/mL of the selection marker G418. Transfected cells were able to multiply under selection pressure.

The trophozoites were harvested (section 2.4.2), and sedimented at 200 x g for 4 min. The cell pellet was washed two times with ice cold 1x NaPBS, and was then suspended in 2 mL of 1x NaPBS. The number of living cells in the suspension was determined with the help of a hemocytometer. A total number of $5x10^5$ cells/mL were suspended in 1 mL NaPBS in a 1.5-mL tube. After adding the radical producing chemicals, the reaction tubes were incubated at 37˚C in the heating block (Thermomixer compact) for 90 min. As control, trophozoites were incubated in NaPBS under the same conditions. The numbers of viable cells were determined using Trypan blue. Experiments were performed in duplicates two times. The concentration of the radical producing chemicals used in each experiment was adjusted to kill about 50% of the cells that contain only the pNC vector.

2.7.3 Radical producing chemicals

2.7.3.1 2,3-Dimethoxy-1,4-naphthquinon (DMNQ)

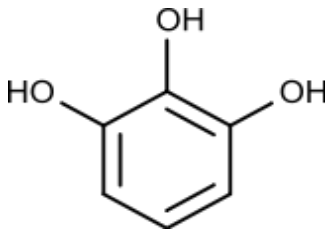
DMNQ is a redox-cycling agent that induces intracellular superoxide anion formation. It does not react with free thiol groups, is non-alkylating and adduct-forming in contrast to other quinones. Thus, DMNQ is a valuable tool for the generation of reactive oxygen species (ROS) in order to study the role of ROS in cell toxicity and apoptosis. Below is the structural formula of DMNQ.



The preparation for the DMNQ viability test was carried out as described above (section 2.7.1). The DMNQ reagent was prepared in methanol fresh before the experiment. In this experiment, a final concentration of 0.025 mM DMNQ was used for the non-tranfected cells and 0.04 mM DMNQ for the transfected cells.

2.7.3.2 Pyrogallol

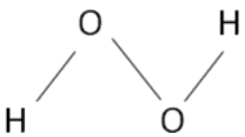
Pyrogallol or benzene-1,2,3-triol, is a powerful reducing agent. It oxidizes in the presence of molecular oxygen and produces superoxide radicals. This reagent is used as a donor of extracellular superoxide radicals. The release of the radicals takes place rapidly (in about 10 min). Below is the structural formula of pyrogallol.



The preparation for the pyrogallol viability test was carried out as described above (section 2.7.1). The pyrogallol reagent was prepared in H_2O_{dd} directly before the experiment. In this experiment, a final concentration of 80 mM pyrogallol was used for the non-tranfected cells and 100 mM pyrogallol for the transfected cells.

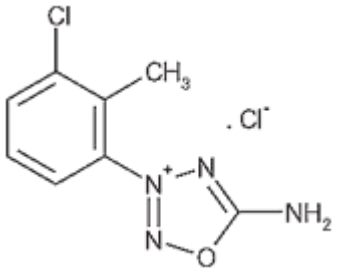
2.7.3.3 Hydrogen peroxide (H_2O_2)

Hydrogen Peroxide is a strong oxidizing agent and a weak acid in water solution. The structure is similar to that of water, with an extra atom of oxygen attached, H_2O_2. It is completely soluble in water. Below is the structural formula of H_2O_2.



The preparation for the H_2O_2 viability test was carried out as described above (section 2.7.1). The H_2O_2 reagent was prepared in H_2O_{dd} fresh before the experiment. In this experiment, a final concentration of 0.05 mM H_2O_2 was used for the non-tranfected cells and 0.08 mM H_2O_2 for the transfected cells.

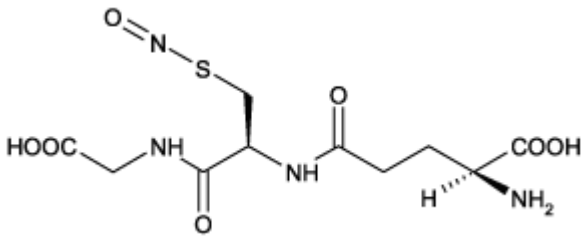
2.7.3.4 [1,2,3,4-oxatriazolium,5-amino-3-(3-chloro-2-methylphenyl)-,chloride] (GEA 5024)



The preparation for the GEA 5024 viability test was carried out as described above (section 2.7.1). The GEA reagent was prepared in H_2O_{dd} directly before the experiment. In this experiment, a final concentration of 0.2 mM GEA5024 was used for the non-tranfected cells and 0.4 mM GEA5024 for the transfected cells.

2.7.3.5 S-Nitroso-L-glutathion (GSNO)

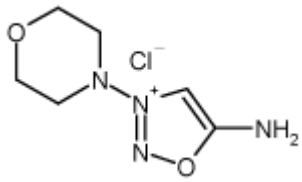
GSNO is a nitric oxide donor that spontaneously releases NO under physiological conditions through hemolytic fission. It has a half-life of 159 hours under physiological conditions. Below is the structural formula of GSNO.



The preparation for the GSNO viability test was carried out as described above (section 2.7.1). The GSNO reagent was prepared in H_2O_{dd} directly before the experiment. In this experiment, a final concentration of 1 mM GSNO was used for both transfected and non-transfected cells.

2.7.3.6 3-Morpholinosydnonimine (SIN-1 chloride)

SIN-1-Cl is considered to be a peroxynitrite releasing compound. Using molecular oxygen it generates superoxide and nitric oxide (NO) that together spontaneously form peroxynitrite. Below is the structural formula of SIN-1-Cl.



The preparation for the SIN-1-Cl viability test was done as described above (section 2.7.1). The SIN-1-Cl reagent was prepared in H_2O_{dd} directly before the experiment. In this experiment, a final concentration of 20 mM was used for the non-transfected cells, and 40 mM for the transfected cells.

3. Results

3.1 Characteristics of clones A and B of the *E. histolytica* isolate HM-1: IMSS

In this study two syngenic amoeba clones (clone A and clone B), which are derived from the *E. histolytica* isolate HM-1: IMSS were investigated. Clone A has been cultivated at the Bernhard Nocht Institute since the year 2001, while clone B was received by the Bernhard Nocht Institute directly from the ATCC in 1991. Both clones are cultivated under axenic conditions in the same laboratory. Early on, Biller *et al.*, (2009) noted that the cells from the two cultures differed in size and growth rate. Compared with cell line A, cells from cell line B are about one-third larger in size and the doubling time is about 30% faster.

An additional phenotypic difference between clones A and B is that the two clones differ in their virulence. Clone A is incapable of inducing liver abscess in rodents, while clone B is capable of producing large liver abscesses that reach up to 30% of the liver size (Biller *et al.*, 2009). During the invasive phase of amoebiasis, the trophozoites of *E. histolytica* are faced with oxidative stress in the form of reactive oxygen species and reactive nitrogen species. Due to the fact that *E. histolytica* lacks mitochondria (Lohia A, 2003), a conventional respiratory system is absent. It cannot transfer the molecular oxygen into water molecules. Despite that, trophozoites of *E. histolytica* succeed in traveling with the blood stream to the liver and (in the case of clone B) causing liver abscess. Consequently, trophozoites require different antioxidant enzymes to stand the stress. The antioxidant genes in clones A and B were investigated in *in vitro* experiments in an attempt to understand the regulation of these genes.

3.2 Viability of clones A and B of *E. histolytica* trophozoites upon treatment with oxidative reagents

In order to characterize the differences between the clones A and B, an experiment was designed in which trophozoites of both clones were exposed to substances that release oxidant radicals into their surroundings. In order to compare clone A with clone B, both were exposed to the same concentration of oxidative reagents. Series of experiments were done to estimate the concentrations of the oxidative reagents that were enough to kill about 50% of the cells of the control. As a control, trophozoites were incubated in NaPBS under the same conditions. For some oxidative reagents such as DMNQ, the best solvent is not water, but

rather an organic substance (such as methanol). In this case, a volume of solvent that is equal to the volume of oxidative reagent solution was added to the control.

The oxidative reagents were chosen to provide a variety of oxidant radicals that the trophozoites of amoeba face in the invasive phase of amoebiasis. Radicals are released in the form of intra- and extracellular superoxide radicals, hydrogen peroxide radicals, and nitric oxide radicals (table 3.1).

Table 3.1: Percent of the viable cells upon the treatment with oxidative regents. DMNQ: 2,3-Dimethoxy-1,4-naphthquinone, H_2O_2: Hydrogen Peroxide, pyrogallol: benzene-1,2,3-triol, SIN-1-Cl: 3-Morpholinosydnonimine, GEA 5024:[1,2,3,4-Oxatriazolium, 5-amino-3-(3-chloro-2-methylphenyl)-chloride], and GSNO: S-Nitroso-L-glutathione.

Oxidative reagent	Mode of action	Percent of viable cells	
		Clone A	Clone B
DMNQ (0.025 mM)	Intracellular superoxide anion donor	41 ± 1.5^{SD}	48 ± 9^{ns}
H_2O_2 (0.05 mM)	Hydrogen peroxide donor	58±3	68 ± 5^{ns}
Pyrogallol (80 mM)	Superoxide radicals donor	55±2	61 ± 4^{ns}
SIN-1-Cl (20 mM)	Superoxide and nitric oxide donor	45.5±7	72.5±5.5*
GEA5024 (0.2 mM)	Nitric oxide donor	27±4	46±4*
GSNO (1 mM)	Nitric oxide donor	42±1	71.5±8*

*$p < 0.05$; ns, no significance; SD, standard deviation.

The results in table 3.1 reveal that there is no significant difference between the trophozoites of clone A and clone B upon the treatment with oxidative reagents that produce intracellular superoxide anions, hydrogen peroxide, and extracellular superoxide radicals. However, clone A was found to be significantly more sensitive to oxidative reagents that produce nitric oxide radicals (GEA 5024 and GSNO). In addition clone A was also more sensitive than clone B when incubated in the presence of SIN-1-Cl. SIN-1-Cl produces both superoxide (O_2^-) and nitric oxide radicals ($NO^{\cdot}$) resulting in the generation of peroxynitrite ($ONOO^-$).

3.3 Expression profile of antioxidant genes

3.3.1 Expression profile of antioxidant genes of *E. histolytica* using microarrays

The use of microarrays gives the possibility of analyzing the expression of a large number of gene expressions within the same experiment. The microarray used in this study contained about 170 gene sequences of putative pathogenic factors of *E. histolytica*, from which there were representatives of different antioxidants. The objective was to investigate the expression profile of the antioxidant genes in the pathogenic clone B of the isolate HM-:IMSS.

First, it was determined whether antioxidant genes are expressed in the pathogenic clone B of the *E. histolytica* isolate HM-1:IMSS under axenic culture conditions, as well as their basal level of expression. The expression level of genes was classified in three categories. The expression was considered to be high when the value of the normalized spot intensity was above 10000 pixels, moderate when the value of the normalized spot intensity was above between 1001 and 10000 pixels, and low when the value of the normalized spot intensity was below 1000 pixels.

As it is shown in figure 3.1, 20 antioxidant genes of the 28 genes analyzed were shown to be expressed. The remaining 8 genes were excluded by the normalizing calculation method. Six antioxidant genes were highly expressed namely *ehfpra2*, *ehprx*, *ehfesod*, *ehtrxr*, *ehtrx1*, *ehtrx*6, and *ehtrx7*. Analysis reflects that 11 antioxidant genes were moderately expressed namely *ehfpra1*, *ehfprb2*, *ehfprc1*, *ehfprc3*, *ehfprd3*, *ehrbr*, *ehtrxr*, *ehtrx5*, *ehtrx8*, *ehtrx9*, and *ehtrx10*. And finally three antioxidant genes were found to be low expressed namely, *ehtrx2*, *ehtrx3*, *and ehtrx4*.

Furthermore, the same microarray technique was used in trophozoites to investigate the antioxidant genes that upregulate when faced with oxidative stress. Trophozoites were incubated with different oxidative reagents (section 3.2) for 90 min at 37℃. As a control, the trophozoites were incubated under the same conditions in NaPBS. Microarray analysis did not reveal any difference between the experiment and the control in terms of upregulation of antioxidant genes.

On the other hand, the expression of antioxidant genes in both clones was compared between cells that were collected directly from the standard culture medium TY and cells that where incubated in PBS. Microarray analysis revealed that the genes *ehprx*, *ehfesod*, *ehtrx1*, *ehfprB2*, and *ehfprC3* were upregulated in the cells incubated in PBS.

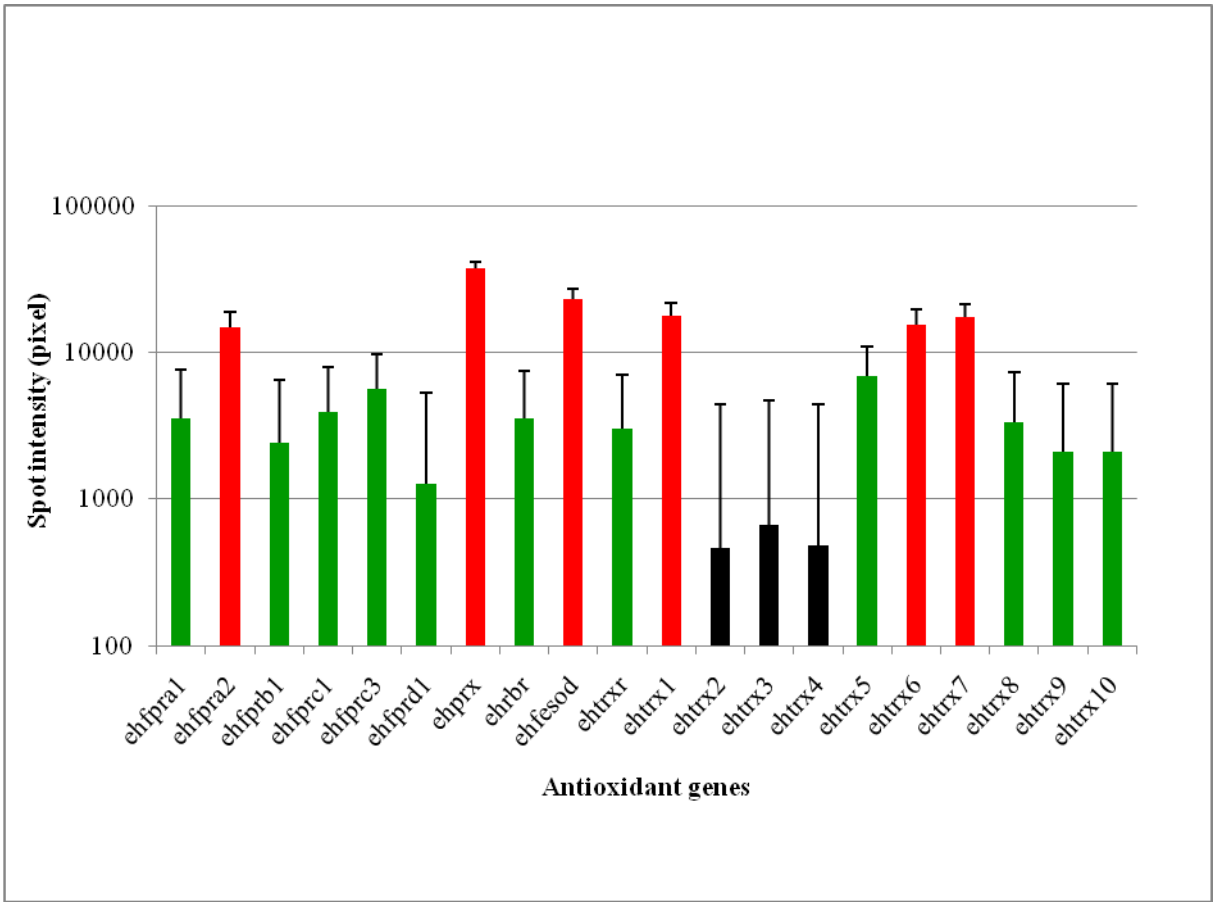


Figure 3.1: Expression profile of antioxidant genes in the pathogenic clone B of the *E. histolytica* isolate HM-1:IMSS. The data represent four independent experiments. Red bars represent a high (> 10000 pixel) expression ratio, green bars represent a moderate (1001 – 10000 pixel) expression ratio, and black bars represent a low (< 1000 pixel) expression ratio. The unit of spot intensity is pixel

3.3.2 Expression profile of antioxidant genes in clones A and B using RT-PCR

The difference in behavior between clone A and B in regard to cell viability upon radical exposure, and the microarray results showing different levels of antioxidant gene expression in clone B, suggests the importance of characterizing and comparing the expression profile of antioxidant genes in these clones. The transcriptional analysis of antioxidant genes in clones A and B was carried out by means of real time PCR (RT-PCR). In order to determine the relative amounts of each transcript, β-actin was used as normalizer. ΔC_T value of both clones was then calculated. ΔC_T value is the difference in the C_T value between the gene of interest

(GOI) and the normalizing gene (section 2.6.7). C_T is the value used to estimate the renaturing (complementary single strands of DNA) of DNA after it has been denatured (DNA molecule which has been broken into two individual single strands). Taking the definition of the C_T value into consideration, the smaller the ΔC_T value of a gene is, the higher it is expressed.

In order to verify the microarray results, RT-PCR was used to investigated the expression of 15 antioxidant genes. The thioredoxin-related genes were excluded from the analysis due to not being well defined within the predicted antioxidant system. The gene Fe-hydrogenase (*ehfehyd*) was included in the investigation because it is differentially upregulated in the pathogenic clone B (Biller, *et al.*, 2009). The same RT-PCR experiments were also used to compare the expression profile of antioxidant genes in both the apathogenic clone A, and the pathogenic clone B of *E. histolytica.*

Genes included peroxiredoxin (*ehprx*), thioredoxin reductase (*ehtrxR*), iron superoxide dismutase (*ehfesod*), rubrerythrin (*ehrbr*), and iron hydrogenase (*ehfehyd*), five thioredoxin genes (*ehtrx*1 – 5), two flavoprotein genes of the family A (*ehfprA*1 and *ehfprA2*), one flavoprotein gene of the family B (*ehfprB*1), one flavoprotein gene of the family C (*ehfprC*1), and one flavoprotein gene of the family D (*ehfprD*1). The oligonucleotides used for the transcriptional analysis are listed in appendix I.

Results in Figure 3.2 show that from the 15 chosen antioxidant genes, four genes were differentially less expressed in the apathogen clone A than in the pathogen clone B, namely *ehprx* (ΔC_T values 0.94 and – 0.3 respectively), *ehfesod* (ΔC_T values 2.4 and 0.4 respectively), *ehfehyd* (ΔC_T values 9.4 and 5.8 respectively), and *ehtrx4* (ΔC_T values 7.4 and 4.2 respectively). The rest of the genes were not differentially expressed in either of the clones. It is worth mentioning here that the gene *ehfprD*1 was not expressed at all in both clones A and B.

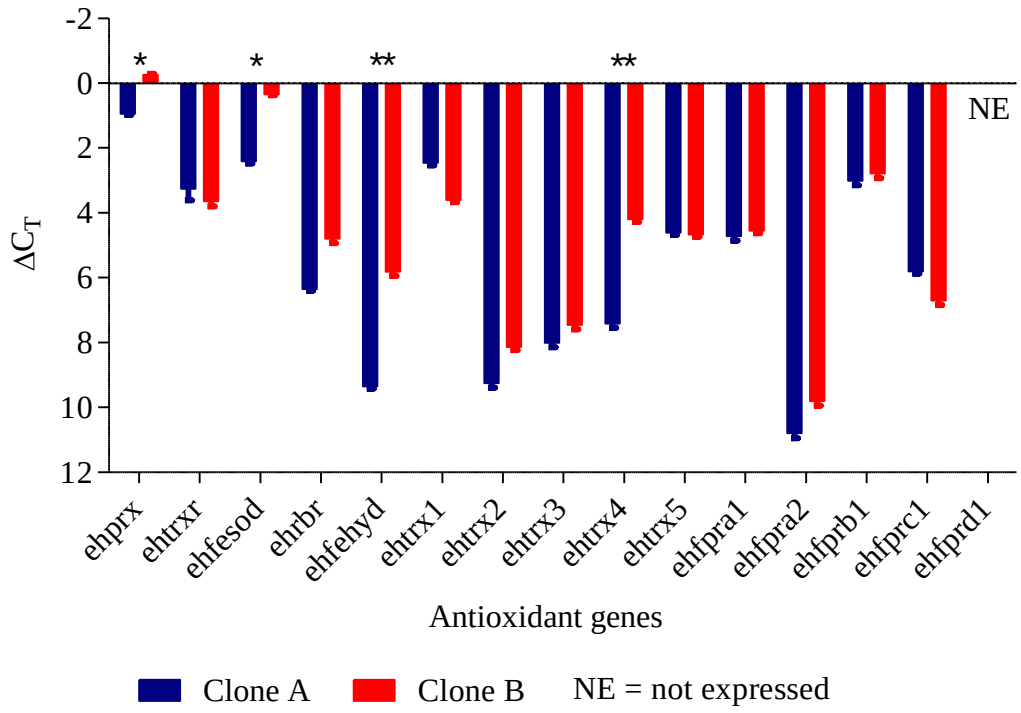


Figure 3.2: Rt PCR analysis for comparing the ΔC_T value of antioxidant genes in clones A(blue) and B(red). The Δ_{CT} value was calculated with the formula $\Delta C_T = C_{T\ GOI} - C_{T\ normalizing\ gene.}$ β-actin was used as normalizer. * $p < 0.05$, ** $p < 0.01$.

In regard to the verification of the microarray results, the antioxidant genes that were investigated by both techniques (microarray and RT-PCR) correlate with each other with one exception. The gene *ehtrx4* was found to be low expressed by microarray analysis, while the RT-PCR analysis revealed that the gene *ehtrx4* was moderately expressed.

In order to correlate the transcriptional level of the four differentially expressed antioxidant genes with the translational level, the respective proteins were localized in both clones A and B. In addition, two more proteins that correspond to the genes thioredoxin reductase (*ehtrxr*) and rubrerythrin (*ehrbr)* were localized in both clones. These two genes are predicted to play a role in detoxifying hydrogen peroxide radicals. Due to the fact that there was no specific antibody for the gene *ehtrx4* availabe, localization studies could not be performed.

3.4 Localization of antioxidant proteins in *E. histolytica* trophozoites using immunfluorescence analysis

In order to localize the antioxidants peroxiredoxin, thioredoxin reductase, iron-superoxide dismutase, rubrerythrin, and iron-hydrogenase in the *E. histolytica* clones A and B, immunfluorescence confocal microscopy was used. The cells were harvested, fixed with 3% paraformaldehyde, and then, a subset of fixed cells was treated with 0.05% saponin (a membrane pore-forming chemical), while the rest remained untreated. The treatment with saponin aimed at easing the entrance of antibodies into the cytoplasm of cells, and thus reacting with epitopes that are localized in the cytosol. On the other hand, cells which were not treated with saponin provide the possibility for antibodies to react only with the antigens located on the surface of these cells.

Trophozoites were then incubated with the corresponding antibody of each antioxidant protein. The detection and localization was done with the help of fluorescence marked secondary antibodies. The nuclei of trophozoites were stained with DAPI.

In order to make sure that the secondary antibodies do not cross react with unspecific molecules in the cell, a control was prepared in which the cells were not incubated with primary antibodies, but only with secondary antibodies. The reaction was negative in all treatments (data not shown).

Immunfluorescence analysis of rubrerythrin demonstrated no significant difference in the signal intensities of rubrerythrin antibodies between clones A and B. This suggests that the amount of rubrerythrin, which is located in the mitosomes (Maralikova *et al.*, 2010), is almost the same in both clones. It is also noticed that there is a strong association of rubrerythrin with the surface of the cells. Again there was no significant difference between clones A and B in regard to the amount of rubrerythrin on the surface of cells. This result matches up with the expression level investigated by means of RT-PCR, which reflected no significant difference between the two clones.

In order to make sure that rubrerythrin is located on the surface of cells and not underneath, antibodies against lectin, which is known to be located on the surface of trophozoites, were used as a means of co-localization with rubrerythrin (Figure 3.3).

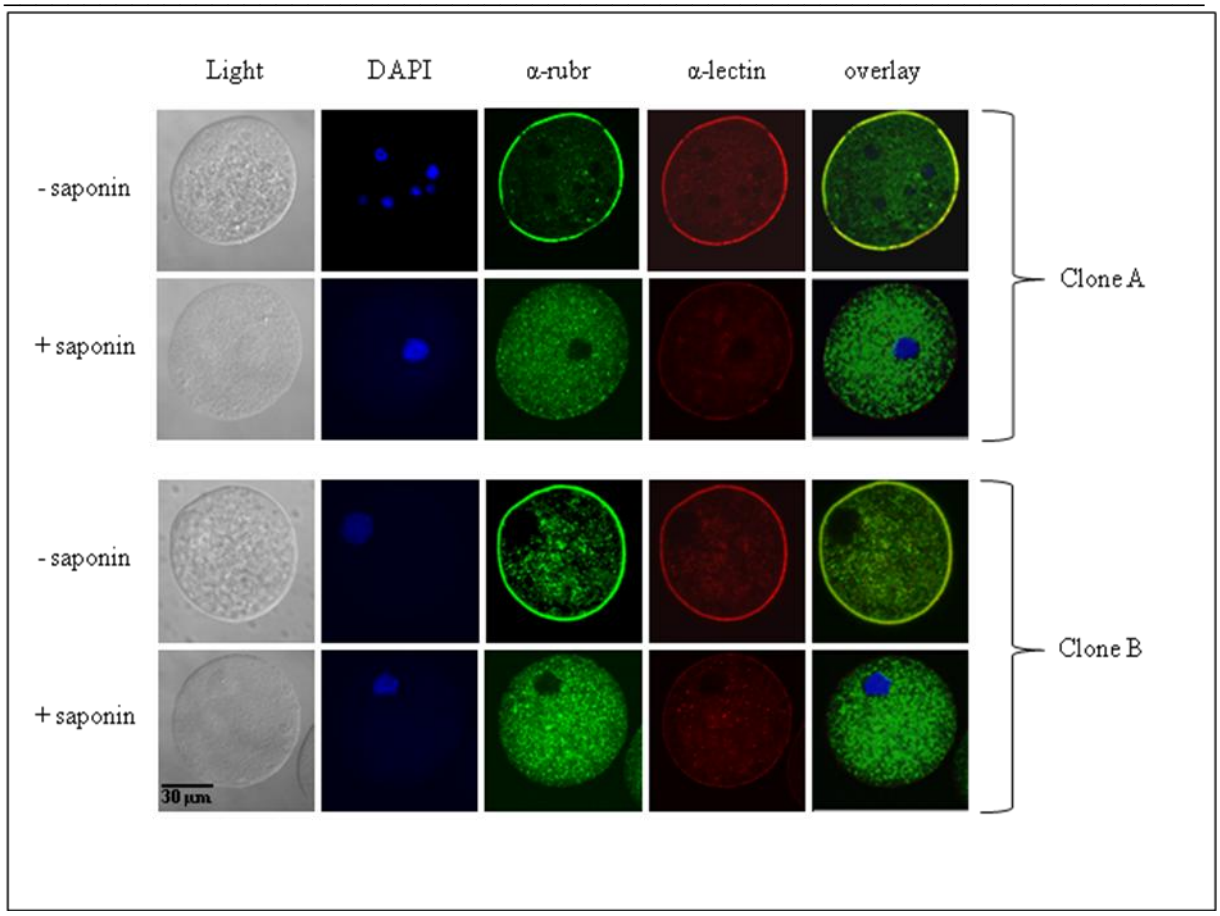


Figure 3.3: Immunfluorescence analysis of the localization of rubrerythrin in *E. histolytica* trophozoites. Cells of clones A and B are compared upon the treatment with and without saponin. The binding of anti-rubrerythrin antibody (1:100) was verified by the Alexa 488 marked secondary anti-mouse-IgG antibody (1:250). The binding of anti-lectin antibody (1:100) was verified by the Alexa 594 marked secondary anti-rabbit-IgG antibody (1:250). The nuclei were stained with DAPI (1:100). The overlay picture is a merge picture of DAPI, Alexa 488 and Alexa 594 treatments. The exposure conditions were constant for all pictures, showing the differences in the signal intensities, and thus the concentration of rubrerythrin and lectin in cells. All pictures were made of the same layer of the cell, which is about at the middle line of the cell. All slices were 0.3 µm thick.

Immunofluorescence analysis of thioredoxin reductase (Figure 3.4) demonstrated that there was no significant difference in the signal intensities of thioredoxin reductase antibodies between clones A and B. Analysis suggests that thioredoxin reductase is cytosolic and its amount is almost the same in both clones. It has no association with the surface of trophozoites. Both clones revealed no significant difference in regard to the amount of thioredoxin reductase. This result correlates with the RT-PCR analysis, in which there was no significant difference in the level of expression of the gene between the clones.

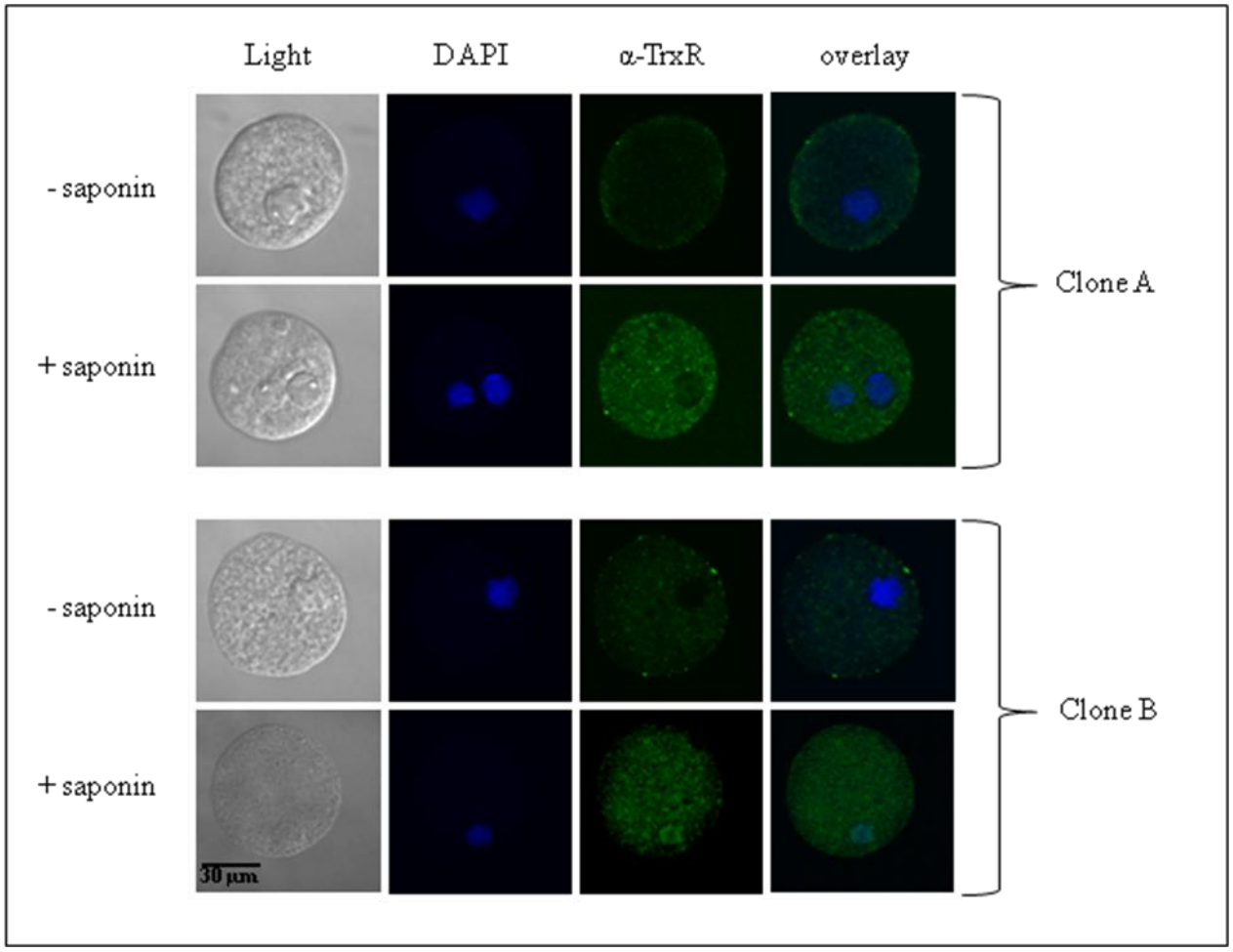


Figure 3.4: Immunfluorescence analysis of the localization of thioredoxin reductase in *E. histolytica* trophozoites. Cells of clones A and B are compared upon the treatment with and without saponin. The binding of anti-thioredoxin reductase antibody (1:100) was verified by the Alexa 488 marked secondary anti-rabbit-IgG antibody (1:250). The nuclei were stained with DAPI (1:100). The overlay picture is a merge picture of DAPI and Alexa 488 treatments. The exposure conditions were constant for all pictures, showing the differences in the signal intensities, and thus the concentration of thiredoxin reductase in cells. All pictures were made of the same layer of the cell, which is about at the middle line of the cell. All slices were 0.3 µm thick.

Signal intensity of peroxiredoxin antibodies was remarkably lower in clone A than in clone B according to immunfluorescence analysis. According to saponin-treated cells, peroxiredoxin is located mainly in the cytosol. In regard to its association with the cell surface (in cells which were not treated with saponin), peroxiredoxin in both clones A and B had the same degree of intensity (Figure 3.5).

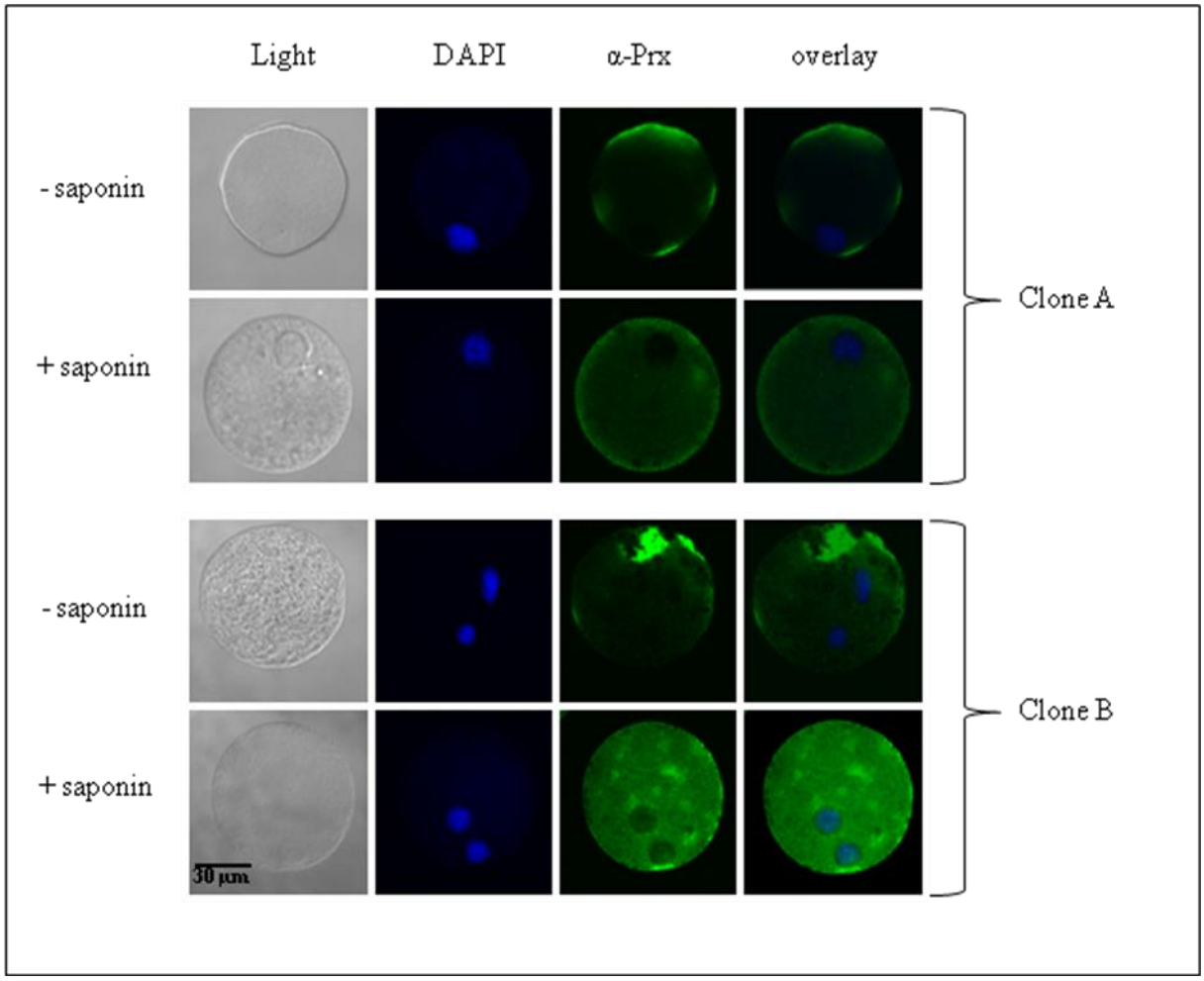


Figure 3.5: Immunfluorescence analysis of the localization of peroxiredoxin in *E. histolytica* trophozoites. Cells of clones A and B are compared upon the treatment with and without saponin. The binding of anti-peroxiredoxin antibody (1:100) was verified by the Alexa 488 marked secondary anti-rabbit-IgG antibody (1:250). The nuclei were stained with DAPI (1:100). The overlay picture is a merge picture of DAPI and Alexa 488 treatments. The exposure conditions were constant for all pictures, showing the differences in the signal intensities, and thus the concentration of peroxiredoxin in cells. All pictures were made of the same layer of the cell, which is about at the middle line of the cell. All slices were 0.3 µm thick.

The amount of iron superoxide dismutase was strikingly less in clone A than in clone B. The signal intensity of the iron superoxide dismutase antibodies in clone A was clearly weaker than that in clone B, reflecting that the amount of iron-superoxide dismutase in clone A is much less than in clone B. Analysis reveals that iron superoxide dismutase is located in the cytosol. It is also demonstrated that iron superoxide dismutase, in both clones, is associated with the cell surface but also with a weaker intensity in clone A than in clone B (Figure 3.6).

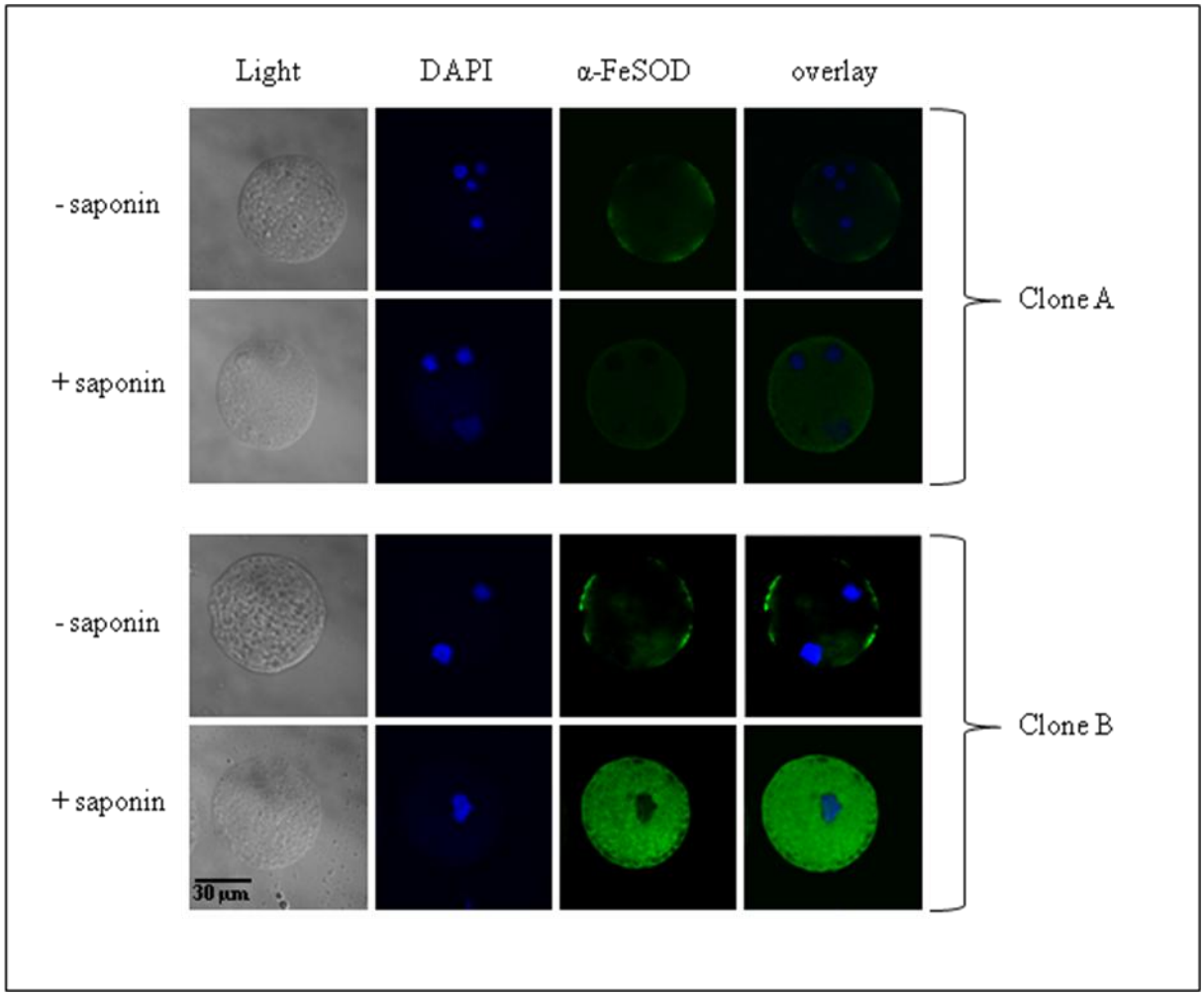


Figure 3.6: Immunfluorescence analysis of the localization of iron-superoxide dismutase in *E. histolytica* trophozoites. Cells of clones A and B are compared upon the treatment with and without saponin. The binding of anti-Fe-superoxide dismutase antibody (1:100) was verified by the Alexa 488 marked secondary anti-rabbit-IgG antibody (1:250). The nuclei were stained with DAPI (1:100). The overlay picture is a merge picture of DAPI and Alexa 488 treatments. The exposure conditions were constant for all pictures, showing the differences in the signal intensities, and thus the concentration of Fe-superoxide dismutase in cells. All pictures were made of the same layer of the cell, which is about at the middle line of the cell. All slices were 0.3 µm thick.

In the cells that were treated with saponin, immunfluorescence analysis revealed weaker intracellular (cytosolic) signals for the anti-Fe-hydrogenase antibodies in clone A than in clone B. In the cells that were not treated with saponin, immunfluorescence analysis did not show any reaction in both clones A and B. This indicates that the Fe-hydrogenase is a classical cytosolic protein. The pictures captured at almost the middle line of the cell show a cytosolic distribution of Fe-hydrogenase molecules (Figure 3.7).

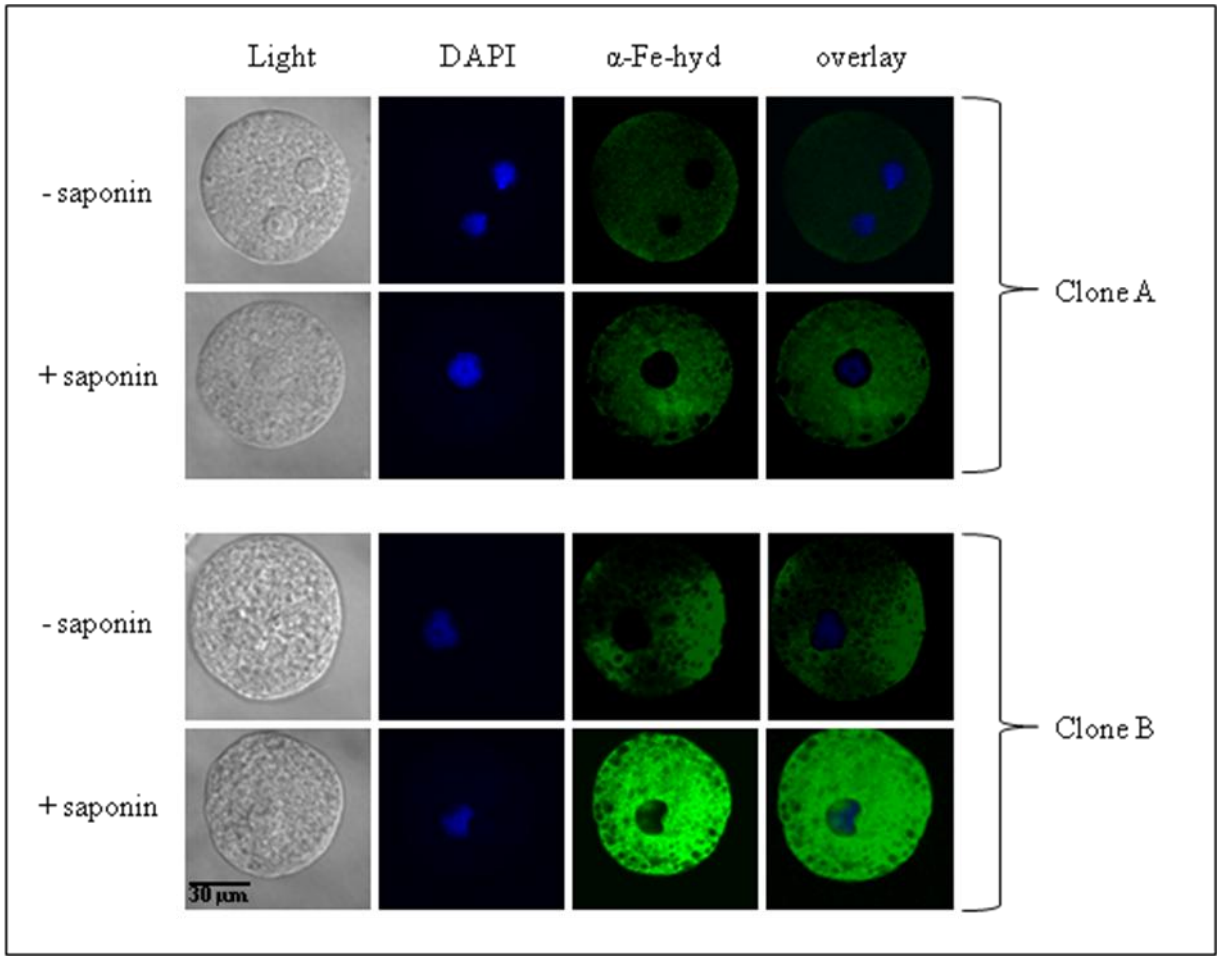


Figure 3.7: Immunfluorescence analysis of the localization of Fe-hydrogenase in *E. histolytica* trophozoites. Cells of clones A and B are compared upon the treatment with and without saponin. The binding of anti-Fe-hydrogenase antibody (1:100) was verified by the Alexa 488 marked secondary anti-mouse-IgG antibody (1:250). The nuclei were stained with DAPI (1:100). The overlay picture is a merge picture of DAPI and Alexa 488 treatments. The exposure conditions were constant for all pictures, showing the differences in the signal intensities, and thus the concentration of Fe-hydrogenase in cells. All pictures were made of the same layer of the cell, which is about at the middle line of the cell. All slices were 0.3 µm thick.

Comparing the results of the expression profile experiments and the immunfluorescence analysis, the three antioxidants peroxiredoxin, iron-superoxide dismutase, and iron-hydrogenase, which show an increased level of expression in clone B, also show an increased level in the corresponding amount of proteins. Also, the antioxidants thioredoxin reductase and rubrerythrin, which were not differentially expressed, reflected no difference in the amount of the corresponding proteins. Consequently, it could be assumed that the transcriptional level of antioxidant genes correlates closely with the translational level.

Interestingly, the genes *ehprx* and *ehfesod*, are involved in the detoxification of hydrogen peroxide and superoxide radicals (Figure 1.2), but not nitric oxide radicals. This result raises the possibility that these genes to contribute to the detoxification of nitric oxide radicals and

peroxynitrite. To test this hypothesis, antioxidant genes were overexpressed and the results are presented in the following section.

3.5 Expression profile of antioxidant genes in transgenic clones A and B

3.5.1 Constructs for the overexpression of antioxidant genes

For a better understanding of the role of antioxidant molecules in *E. histolytica*, antioxidants that are necessary for the detoxification process were overexpressed in the trophozoites of both clones A and B. Constructs of antioxidants were produced, namely of the genes peroxiredoxin (*ehprx*), thioredoxin reductase (*ehtrxr*), iron superoxide dismutase (*ehfesod*), rubrerythrin (*ehrbr*), and iron hydrogenase (*ehfehyd*). The goal was to test if the sensitivity of transgenic trophozoites toward nitric oxide radicals has changed, to examine whether these trophozoites have increased their ability to detoxify these oxidative radicals, and to study the effect of overexpressing these genes on the expression of other antioxidant genes.

In this work, the overexpression of rubrerythrin (*ehrbr*) is illustrated as an example. It was overexpressed in both the apathogenic clone A and the pathogenic clone B by using the expression vector neocassette (pNC).

The pNC vector encloses a neomycin phosphotransferase coded sequence that is flanked by 480 bp of the 5'untranslated and 600 bp of the 3'untranslated regions of actin genes of *E. histolytica*. The *ehrbr* gene, which has the size of 607 bp, was cloned at the cutting site of the restriction enzymes *Kpn* I and *Bam*H I. The cloning site is in turn flanked by the 485 bp of the 5' untranslated region of lectin genes and the 600 bp of the 3' untranslated region of actin genes. The expression vectors were then referred to as pNC-EhRbr, and the pNC vector was used a control in all experiments (Figure 3.8).

The overexpression vectors pNC-EhPrx, pNC-EhTrxR, pNC-EhFeSOD, pNC-EhRbr, and pNC-EhFehyd were used for the transfection of *E. histolytica* trophozoites of the clones A and B, followed by setting the cells under selection pressure of the neomycin analogue G418-sulfate (section 2.6.18).

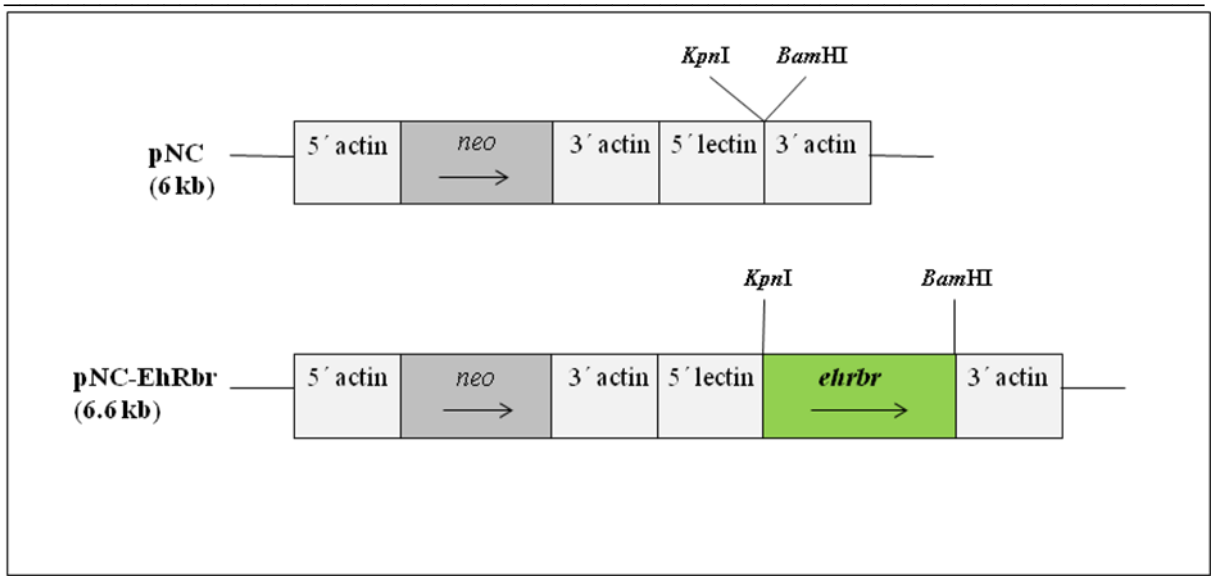


Figure 3.8: Scheme that represents the *E. histolytica* expression vectors pNC and pNC-EhRbr. The restriction enzymes *Kpn*I and *Bam*HI were used to perform the insertion of the *ehrbr* gene into the pNC vector. The arrows indicate the direction of transcription.

After 48 h of the transfection, the selection marker G418 was inducted into the culture at a concentration of 10 µg/mL. Those cells that could stand the selection pressure were able to reproduce under the elevated concentrations of G418, up to 50 µg/mL.

Real-time PCR was used to detect the expression profile of the above mentioned overexpressed antioxidant genes in both transgenic clones A and B. The pNC was used as a control, and β-actin as a normalizer. The oligonucleotides used for the expression analysis are listed in appendix I.

3.5.2 Producing polyclonal antibodies against the recombinant rubrerythrin

In order to deepen the investigation of rubrerythrin, it was recombinantly synthesized using the N-terminal Histidine-tag in pAPlacI^{Q} bacteria. Rubrerythrin was cloned in the vector pJC45 at the cutting site of the restriction enzymes *Nde*I and *Bam*HI, and then transformed into the pAPlacI^{Q} cells. The recombinant protein was purified using the nickel column (section 2.6.17) and collected in fractions. Using western blot, it was observed that the protein of interest was found in the insoluble extract and has the molecular weight of 22 kDa (Figure 3.21).

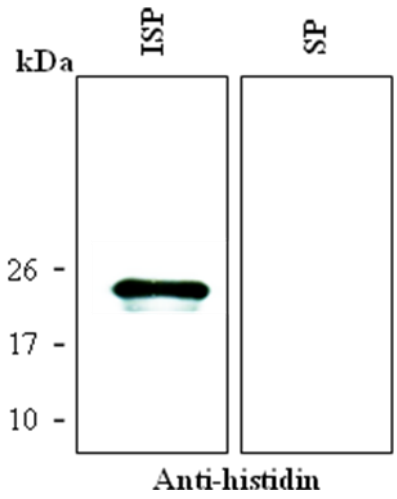


Figure 3.21: Western blot analysis of the recombinant rubrerythrin. The histedine taged protein was detected in the insoluble fraction of the extract using the antibody anti-histidin. SP = soluble protein fraction, ISP = insoluble protein fraction

Subsequently, the polyclonal antibodies were produced by immunizing female mice of the type Balb/c with the recombinant protein (section 2.5.6). Antiserum was collected from blood of mice and used for further western blot and immune staining investigations.

3.6 Effect of overexpression of antioxidant genes on the expression of other genes in transgenic clones A and B

The overexpression of antioxidant genes in *E. histolytica* clones A and B was intended to investigate whether the overexpression has an effect on the expression of other antioxidant genes. In addition to the five above mentioned antioxidants ((*ehprx*), (*ehtrxr*), (*ehfesod*), (*ehrbr*), and (*ehfehyd*)), the expression of 10 other antioxidant genes was investigated.

The expression of five thioredoxin genes (*ehtrx*1 – 5), two flavoprotein genes of the family A (*ehfpra*1 and *ehfpra2*), one flavoprotein gene of the family B (*ehfprb*1), one flavoprotein gene of the family C (*ehfprc*1), and one flavoprotein gene of the family D (*ehfprd*1), was investigated in trophozoites of clones A and B, in which one of the five main antioxidant genes was overexpressed. This was done by means of RT-PCR, in which the relative expression ($2^{-\Delta\Delta C}{}_{T}$) of antioxidant genes was calculated (Figures 3.10 – 3.14).

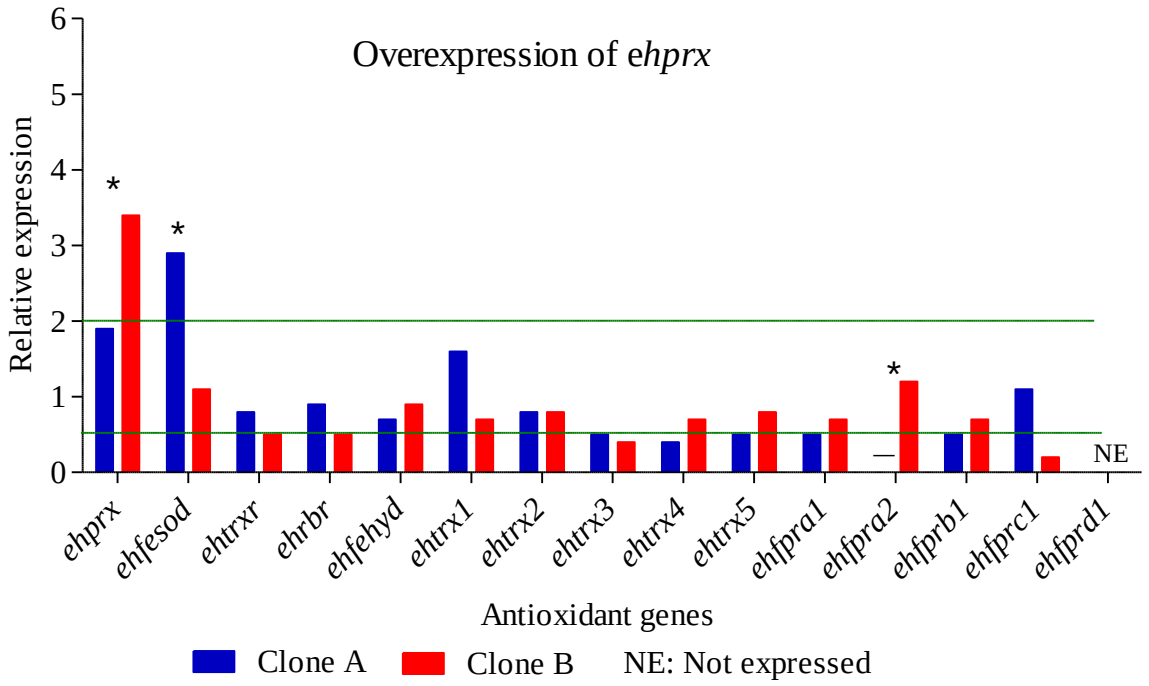


Figure 3.10: Effect of the overexpression of *ehprx* on the expression of other antioxidant genes in clones A (blue) and B (red). The relative expression of clones A and B was calculated using the $2^{-\Delta\Delta C}{}_{T}$ method. *β*-actin was used as normalizer, and the pNC was used as a calibrator. Genes which have a relative expression value of 2 or above are considered to be upregulated. On the other hand, genes which have a relative expression value of 0.5 or below are considered to be downregulated. * $p < 0.05$

In pNCEhPrx transfectants, the relative expression of the other antioxidant genes was investigated. Results of the relative expression of antioxidant genes in the transgenic clones A and B revealed that the gene *ehprx* is more highly expressed in clone B than in clone A (Figure 3.10). The overexpression of *ehprx* also has an influence on the expression of other antioxidant genes. E*hfesod* was differentially upregulated in clone A, e*hfprc1*was downregulated in clone B, and *Ehfpra2* was not expressed at all in clone A. E*hfprd1* was not expressed at all in the transgenic clones A and B, and the non-transfected clones as well.

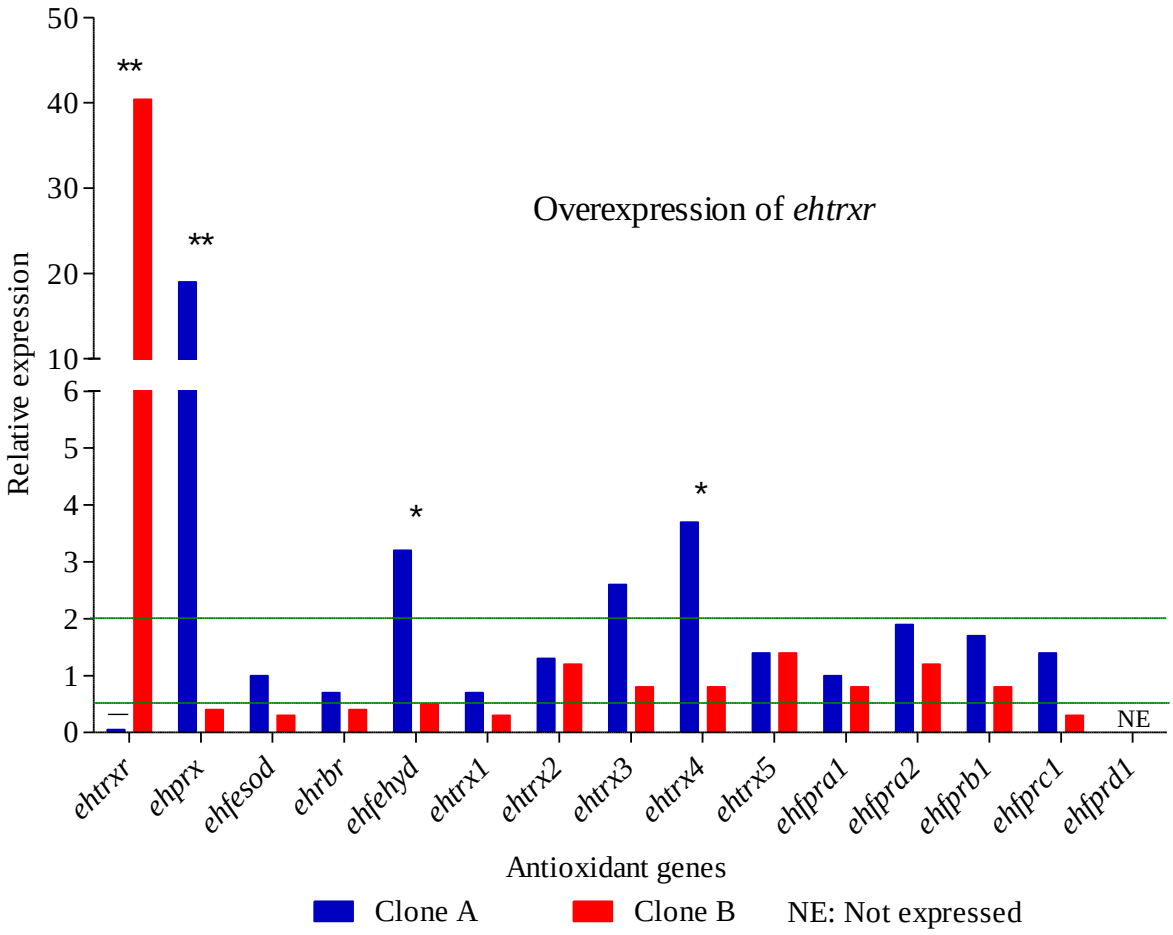


Figure 3.11: Effect of the overexpression of *ehtrxr* on the expression of other antioxidant genes in clones A (blue) and B (red). The relative expression of clones A and B was calculated using the $2^{-\Delta\Delta C}{}_T$ method. *β*-actin was used as normalizer, and the pNC was used as a calibrator. Genes which have a relative expression value of 2 or above are considered to be upregulated. On the other hand, genes which have a relative expression value of 0.5 or below are considered to be downregulated. * $p < 0.05$, ** $p < 0.01$.

In the transgenic clones A and B, which were transfected with the construct pNCEhTrxR, the intended overexpression of the gene *ehtrxr* (Figure 3.11) seems to lead to its silencing. E*htrxr* was almost inhibited in clone A (0.05), while its expression in clone B was significantly higher (40.4). The overexpression of the gene *ehtrxr* in both clones A and B has a remarkable influence on the expression of some other antioxidant genes. The gene e*hprx* was highly upregulated in clone A, while it was downregulated in clone B. The gene *ehfehyd* was upregulated in clone A, while it was downregulated in clone B. The gene *ehtrx4* was upregulated in clone A.

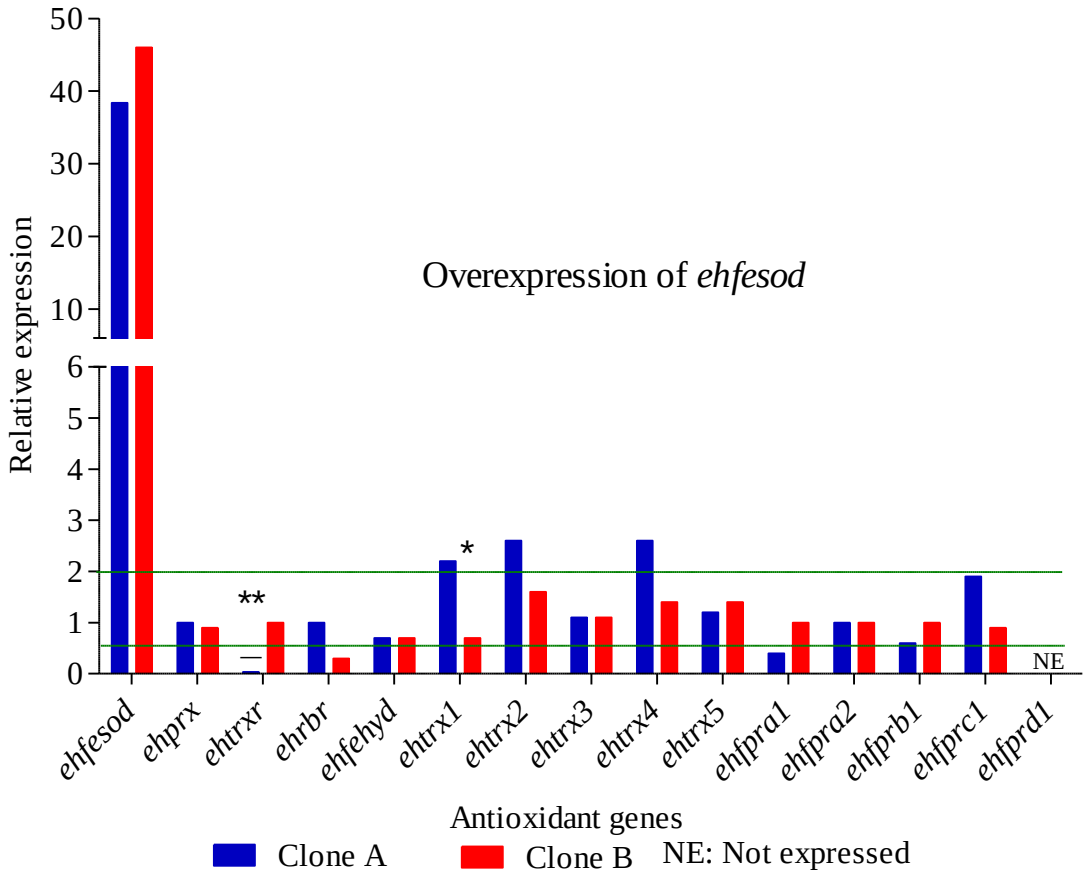


Figure 3.12: Effect of the overexpression of *ehfesod* on the expression of other antioxidant genes in clones A (blue) and B (red). The relative expression of clones A and B was calculated using the $2^{-\Delta\Delta C}{}_{T}$ method. *β*-actin was used as normalizer, and the pNC was used as a calibrator. Genes which have a relative expression value of 2 or above are considered to be upregulated. On the other hand, genes which have a relative expression value of 0.5 or below are considered to be downregulated. * $p < 0.05$, ** $p < 0.01$.

Iron superoxide dismutase in both transgenic clones A and B was highly expressed with no significant difference (38.4 and 46 respectively). The overexpression of the gene *ehfesod* in both clones A and B (Figure 3.12) has a lower effect on the expression of other antioxidant genes than the overexpression of the *ehprx* and *ehtrxr* genes. In almost all genes there was no significant difference in their expression between clones A and B. Differential regulation was observed in very few genes. The gene e*htrxr* was remarkably downregulated in clone A,while it was not regulated in clone B. The gene *ehtrx1* was upregulated in clone A.

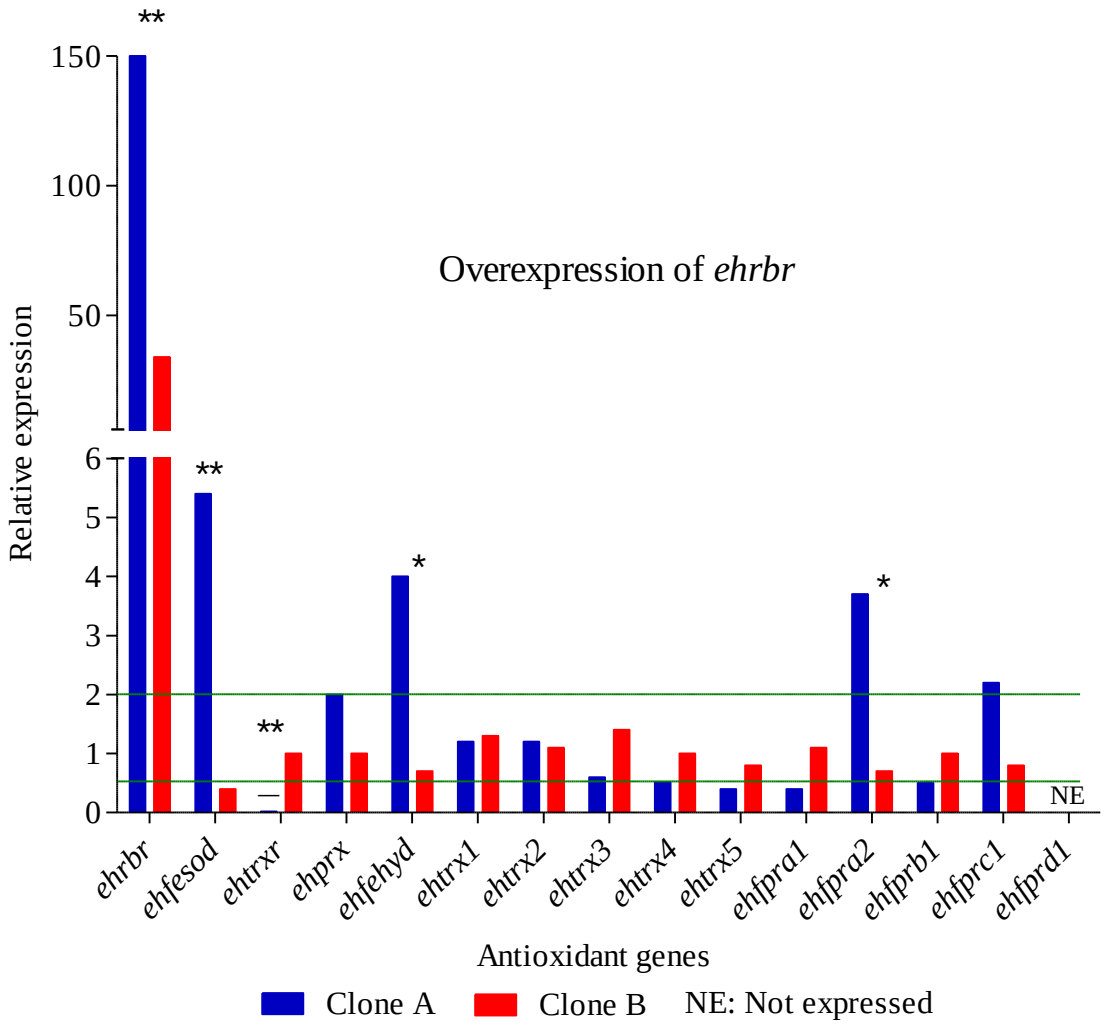


Figure 3.13: Effect of the overexpression of *ehrbr* on the expression of other antioxidant genes in clones A (blue) and B (red). The relative expression of clones A and B was calculated using the $2^{-\Delta\Delta C_T}$ method. *β*-actin was used as normalizer, and the pNC was used as a calibrator. Genes which have a relative expression value of 2 or above are considered to be upregulated. On the other hand, genes which have a relative expression value of 0.5 or below are considered to be downregulated. * $p < 0.05$, ** $p < 0.01$.

The overexpression of rubrerythrin leads to a significantly higher expression in clone A (150) than in clone B (34). The overexpression of the gene *ehrbr* in trophozoites of both clones A and B (Figure 3.13) also has a remarkable effect on the expression of other antioxidant genes. The effect was noticed mainly on the relative expression of genes *ehfesod*, *ehtrxr*, *ehfehyd*, and *ehfpra2*. The genes *ehfesod*, *ehfehyd*, and *ehfpra2* were differentially upregulated in clone A. On the other hand, the gene *ehtrxr* was significantly downregulated in clone A.

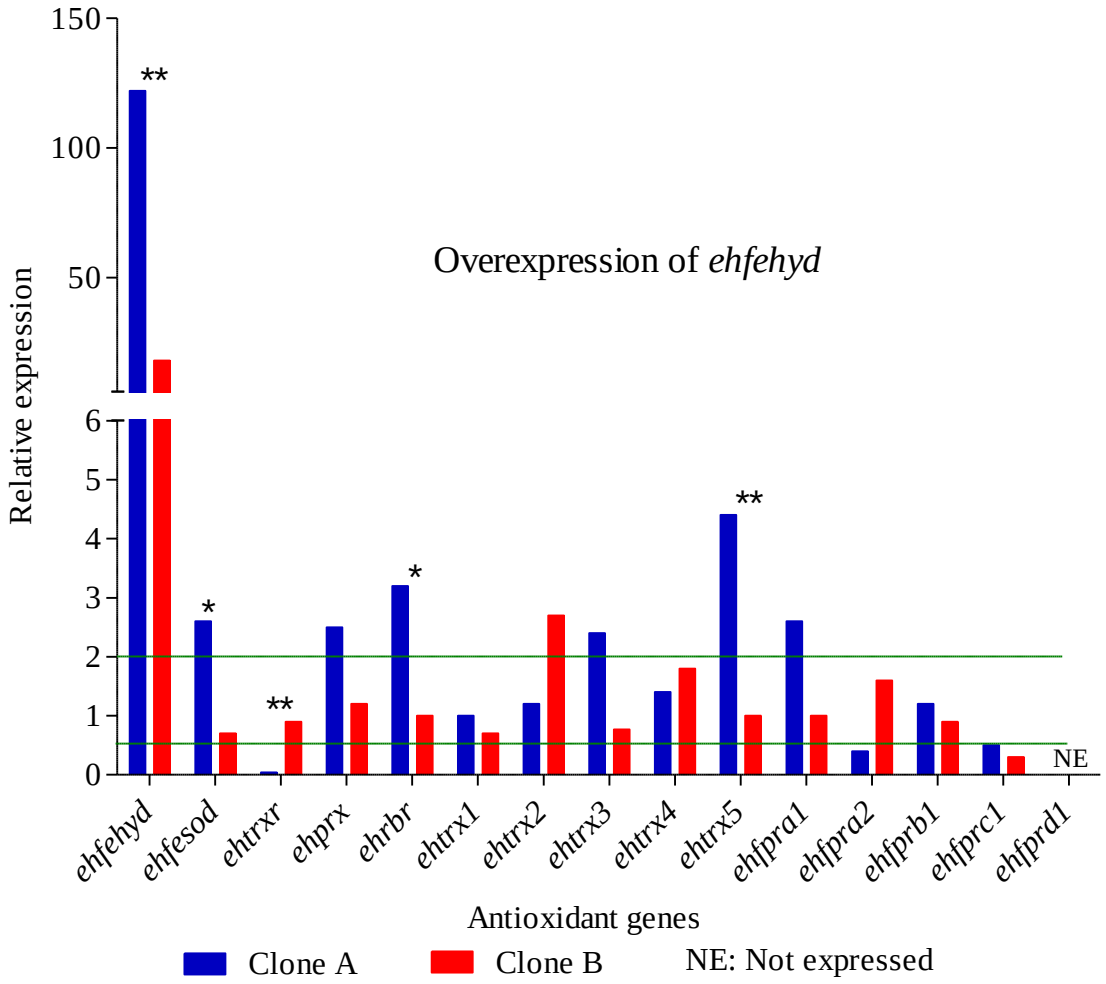


Figure 3.14: Effect of the overexpression of *ehfehyd* on the expression of other antioxidant genes in clones A (blue) and B (red). The relative expression of clones A and B was calculated using the $2^{-\Delta\Delta C}{}_{T}$ method. *β*-actin was used as normalizer, and the pNC was used as a calibrator. Genes which have a relative expression value of 2 or above are considered to be upregulated. On the other hand, genes which have a relative expression value of 0.5 or below are considered to be downregulated. * $p < 0.05$, ** $p < 0.01$.

Overexpression of iron-hydrogenase demonstrated a significant differential relative expression in clone A (122) and (18) in clone B. The overexpression of the gene *ehfehyd* in trophozoites of both clones A and B has a notable effect on the expression of other antioxidant genes (Figure 3.14). This effect was obvious on the relative expression of antioxidant genes in clone A, in which *ehfesod*, *ehrbr*, and *ehtrx5* were significantly upregulated. The gene *ehtrxr* was downregulated in clone A. The gene *ehfprd1* was not expressed at all in both clones A and B.

To summarize the effect of overexprssion of antioxidant genes in trophozoites of clones A and B, it was noticed that although the same constructs were used for the transfection of these clones, the expression level of the antioxidant genes ((*ehprx*), (*ehtrxR*), (*ehfesod*), (*ehrbr*), and

(*ehfehyd*)) in these clones was different. Moreover, the overexpression of each of these antioxidant genes has a different effect on the expression of other antioxidant genes. It would be interesting to investigate if these differences have an effect on the sensitivity of these transgenic clones toward oxidative reagents.

3.7 Viability of transgenic trophozoites of clones A and B upon the treatment with oxidative radicals

Results have shown that clone B is more resistant to nitric oxide radicals, and has the same level of resistance as clone A when exposed to superoxide and hydrogen peroxide radicals. In order to understand the role of antioxidant genes of *E. histolytica* in detoxifying oxidative radicals, the transgenic clones A and B, which were transfected with the constructs pNC-EhPrx, pNC-EhTrxR, pNC-EhFeSOD, pNC-EhRbr, and pNC-EhFehyd, were treated with the same oxidative reagents as clones A and B.

It was noticed that the concentration of oxidative reagents used to kill about 50% of the untransfected trophozoites was not enough to kill the same amount of the control pNC transfected trophozoites. The concentration was raised to be 0.04 mM of DMNQ, 0.08 mM of H_2O_2, 100 mM of pyrogallol, 40 mM of SIN-1-Cl, 0.4 mM of GEA5024, and 1 mM of GSNO. The results of treating the five antioxidant constructs are illustrated in figures 3.15-3.20.

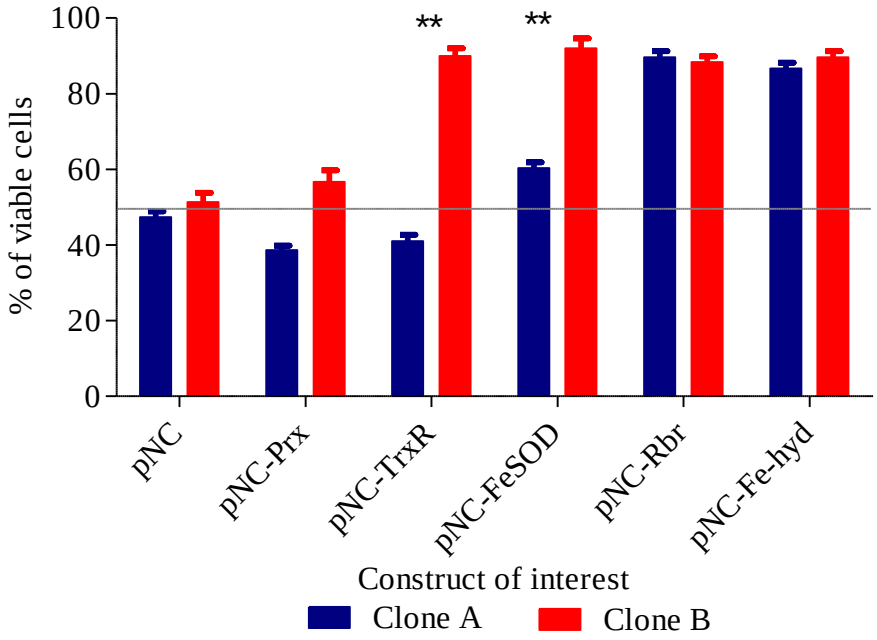


Figure 3.15: Comparison of the percent of viable cells of clones A and B constructs upon the treatment with 0.04 mM DMNQ. The concentration of 0.04 mM was found to be able to kill about 50% of the control (pNC) cells. ** $p < 0.01$

Results in figure 3.15 show that the overexpression of *ehrbr* and *ehfehyd* genes in clones A and B leads to the protection of cells of both clones A and B against intracellular superoxide radicals. The overexpression of *ehtrxr* and *ehfesod* genes revealed that the cells of clone B are more protected than those of clone A.

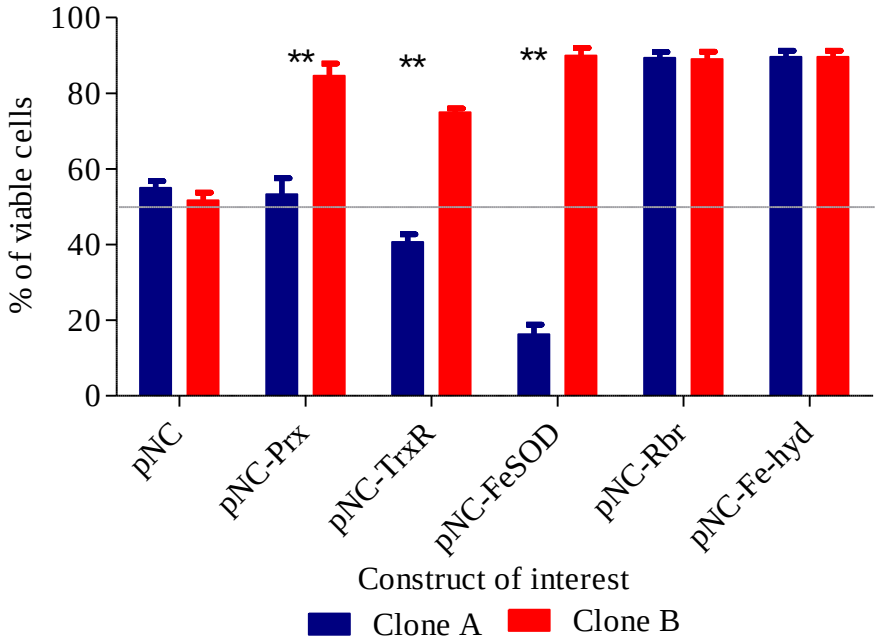


Figure 3.16: Comparison of the percent of viable cells of clones A and B constructs upon the treatment with 0.08 mM H_2O_2 The concentration of 0.08 mM was found to be able to kill about 50% of the control (pNC) cells. ** $p < 0.01$

Results in figure 3.16 show that the overexpression of *ehfesod* leads to the increase of protection level of cells of clone B against hydrogen peroxide radicals, while in cells of clone A, the sensitivity to these radicals increased. Moreover, the overexpression of *ehprx* and *ehtrxr* demonstrated that cells of clone B increased their protection level, while those of clone A provided no significant difference compared with the control. The overexpression of *ehrbr* and *ehfehyd* genes in clones A and B leads to the protection of cells of both clones A and B against hydrogen peroxide radicals.

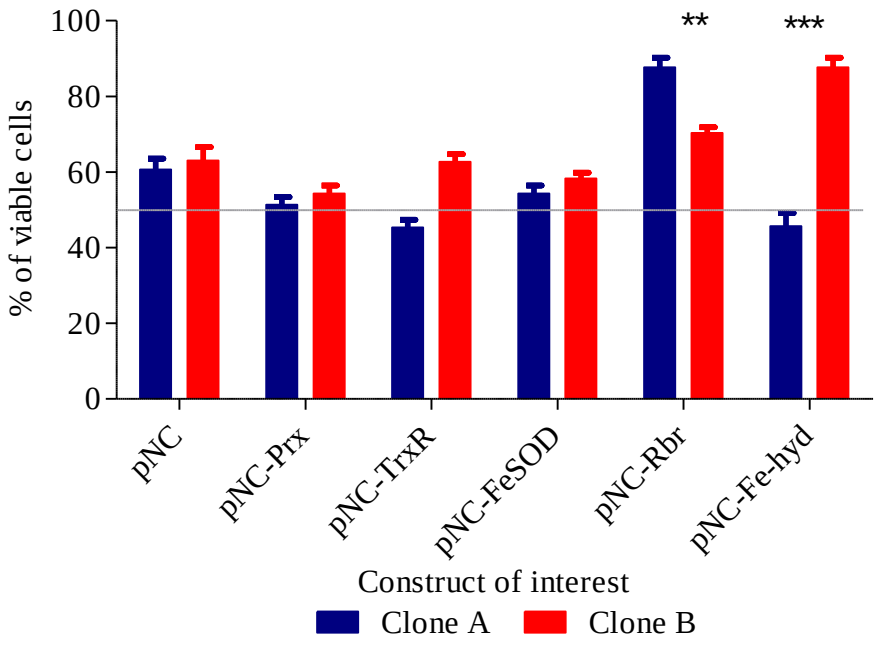


Figure 3.17: Comparison of the percent of viable cells of clones A and B constructs upon the treatment with 100 mM pyrogallol. The concentration of 100 mM was found to be able to kill about 60% of the control (pNC) cells. *** $p < 0.001$

The concentration of pyrogallol used in the treatment of the control cells of both clones A and B was lethal for about 40 % of cells, providing a viability percent of 60% of cells for both clones (Figure 3.17). The overexpression of *ehrbr* provided that cells of the clone A are protected against the extracellular superoxide radicals, and the overxpression of *ehfehyd* revealed that cells of clone B were protected against the same radical.

In figure 3.18, the overexpression all of the five antioxidant genes (*ehprx*, *ehtrxr*, *ehfesod*, *ehrbr*, and *ehfehyd*) reflected a high degree of protection against the peroxynitrite (which is formed by the reaction of superoxide and nitric oxide radicals) produced by the SIN-1-Cl reagent.

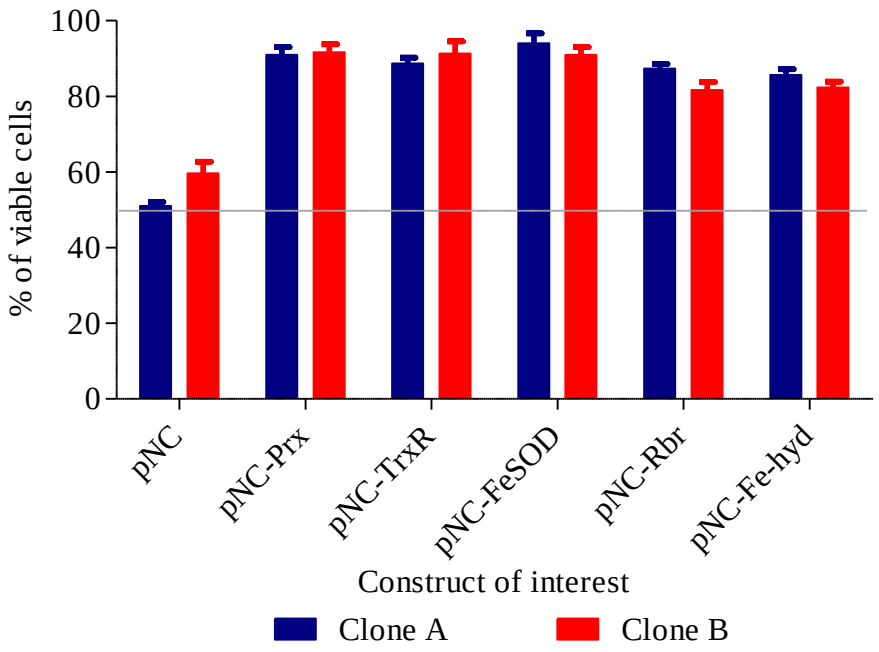


Figure3.18: Comparison of the percent of viable cells of clones A and B constructs upon the treatment with 40 mM SIN-1-Cl. The concentration of 40 mM was found to be able to kill about 50% of the control (pNC) cells.

Results in figure 3.19 show that the overexpression of *ehrbr* and *ehfehyd* genes in clones A and B leads to the protection of cells of both clones A and B against nitric oxide radicals. In comparison with the sensitivity of clone A (which is not transgenic) toward nitric oxide, the overexpression of these genes raised the protection level of clone A.

In regard to the overexpression of *ehfesod* gene, cells of clone A became very sensitive to nitric oxide radicals. On the other hand, cells of clone B raised their degree of protection by the overexpression of the gene *ehprx*.

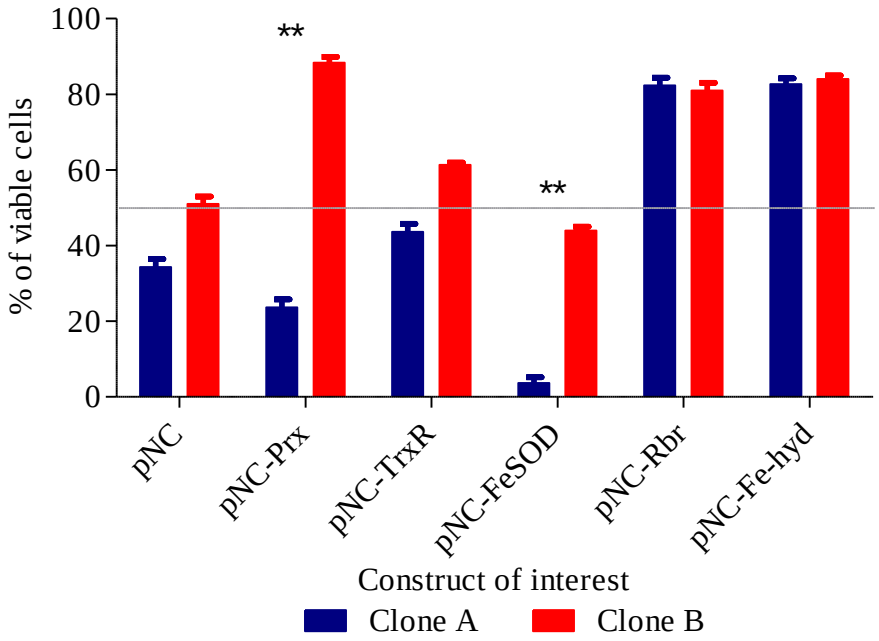


Figure 3.19: Comparison of the percent of viable cells of clones A and B constructs upon the treatment with 0.4 mM GEA5024. The concentration of 0.4 mM was found to be able to kill about 50% of the control (pNC) cells. ** $p < 0.01$

Results in figure 3.20 reflect that the overexpression of the gene *ehtrxr* has increased the protection of cells of clone B against nitric oxide radicals, while cells of clone A became more sensitive. Cells of clone A became more sensitive to the radicals after the overexpression of the gene *ehfesod*.

Cells of both clones, in which the genes *ehrbr* and *ehfehyd* were overexpressed, have the same degree of protection against radicals. Both clones were able to stand the stress of nitric oxide radicals induced by GSNO.

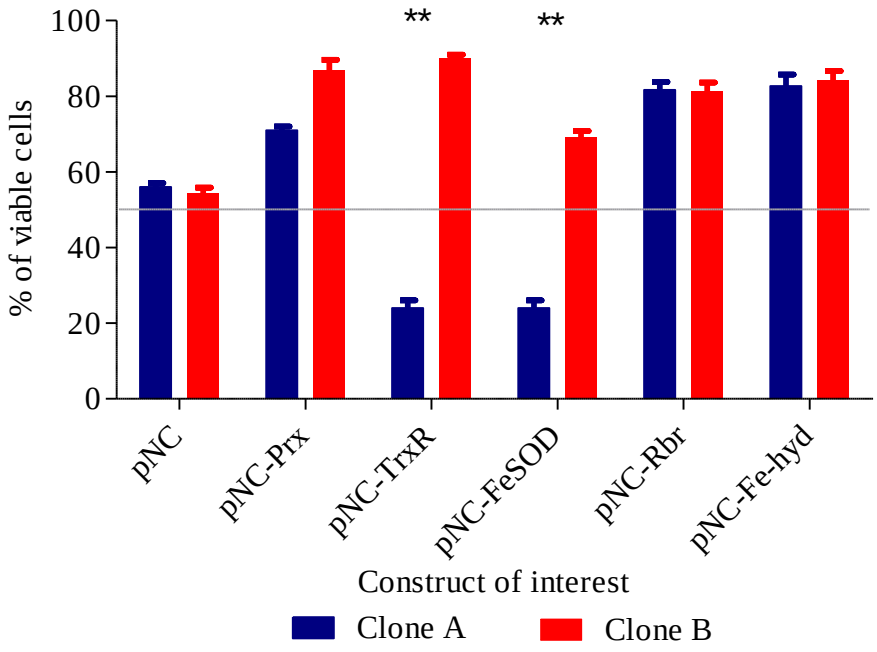


Figure 3.20: Comparison of the percent of viable cells of clones A and B constructs upon the treatment with 1 mM GSNO. The concentration of 1 mM was found to be able to kill about 50% of the control (pNC) cells. ** $p < 0.01$

4. Discussion

The protozoan parasite *Entamoeba histolytica* normally resides in the large intestine. Amoebae occasionally penetrate the intestinal mucosa and may disseminate into other organs (WHO, 1997). Antioxidant proteins in *E. histolytica* are considered to be defensive virulence factors that protects trophozoites against oxidative stress during the invasive phase of amoebiasis. On the other hand, *E. histolytica* has other virulence factors that are considered offensive one. These offensive virulence factors are resembled by the cysteine proteinases, amoebapores, and lectines. During the invasive phase of amoebiasis, the microaerophilic trophozoites of *E. histolytica* strains are able to stand the oxidative stress in the blood stream and tissues, and eventually reach different organs such as the liver. Some of them are able to develop abscesses mainly in the liver.

As a consequence of their oxygen metabolism, all eukaryotic cells generate reactive oxygen species (ROS) and reactive nitrogen species (RNS). Additionally, some immune effector cells exploit the biotoxic properties of ROS and RNS as a means to combat invading microbes, i.e. via the 'oxidative burst', which increases the oxidative burden on the microbe to lethal levels. An excess of ROS (such as superoxide anions, hydrogen peroxide (H_2O_2), hydroxyl radicals) and/or RNS (such as nitric oxide (NO) and peroxynitrite (ONOO-)) creates a potentially dangerous situation known as oxidative or nitrosative stress, respectively. Like other aerobic organisms, parasites protect themselves against these assaults by means of antioxidant networks consisting of enzymatic and non-enzymatic components (Vonlaufen *et al.*, 2008).

Moreover, it is clear that the mechanism of amebic killing by activated macrophages is contact dependent and involves both oxygen and nonoxygen-dependent pathways (Lin and Chadee, 1992). It is assumed that the ability of *E. histolytica* trophozoites to survive within the host is accomplished by the specific regulation of a number of amoeba proteins (Gomez *et al.*, 2010). Among these proteins are the antioxidant enzymes.

In this study, two clones of the *E. histolytica* isolate HM-1:IMSS were investigated with the aim of understanding the regulation of antioxidant enzymes. The choice of these clones was based on the fact that clone A is apathogenic, while clone B is able to introduce liver abscess in rodents.

4.1 Microarray analysis

As the microarray technique was developed in the 1990s, it provided a promising tool for molecular biology research. Since that time technical impovement in this technology have largely overcome the problems intially encountered with this complex technique. Further investigations using this approach by a number of groups unveil important insights into amoebic pathogenesis (MacFarlane *et al.,* 2005; Ehrenkaufer *et al.,* 2008).

In order to get stable data sets, all experiments were performed twice with two different biological replicates. In cDNA microarray experiments, a comparison between an experimental RNA sample and a reference RNA sample is made. The expression was seen under more or less anaerobic conditions. cDNAs are synthesized from the experimental and reference samples and stained with fluorescent dyes such as Cy3 and Cy5 (Beneš and Muckenthaler, 2003). In order to minimize the error in measurements of the fluorescence marked cDNA, which were photometrically done, the concept dye swap was used. Another accuracy measure was taken during the production of the microarray itself. The oligonucleotide probes were spotted in triplicates on the microarray to reduce the effect of local fluctuation in the hybridization process.

Microarray results show that during the standard cultivation of the pathogenic clone B of the *E. histolytica* isolate HM-1:IMSS, 6 antioxidant genes were highly expressed, 11 antioxidant genes were moderately expressed, and 3 antioxidant genes were low expressed (Figure 3.1). The highly expressed genes meet the expectations of playing a role in protecting the cells against oxidative stress. Counter examples of highly expressed antioxidant genes in pathogenic strains of *E. histolytica* were previously documented. McFarlane *et al.,* (2006) demonstrated that some antioxidant genes are highly expressed in the virulent strains, such as a type A flavoprotein and peroxiredoxin. In clone B, both genes (*ehfpra2* and *ehprx*) were shown to be highly expressed. Falvoproteins of the family A are enzymes that have significant nitric oxide reductase activity (Gomes *et al.*, 2002). As for peroxiredoxin, it is known that this protein is involved in the detoxification of the endogenous toxic H_2O_2 produced (Cheng *et al.*, 2004). In addition it plays a role in other organisms in signaling or differentiation processes (Hofmann *et al.*, 2002).

The high expression of iron-superoxide dismutase reveals that this antioxidant gene is important for the survival of amoeba even under standard conditions. It is an advantage for

the trophozoites in the invasive phase if the expression level of iron-superoxide dismutase increases in *in vivo*, hence better survival possibility. This may also be true for all other highly expressed genes.

The expression level of antioxidant genes is subjected to change if the trophozoites are faced with an environment with oxidative stress. *In vitro* studies have indicated that cultured *E. histolytica* trophozoites are indeed able to cope with lethal conditions, which is associated with up- or downregulation of various amoeba proteins (Bruchhaus *et al.*, 2002). In this regard, microarrays were also used to investigate the regulation of antioxidant genes upon the treatment with different oxidative reagents. No difference was observed in the level of gene regulation between the control which cells were incubated in NaPBS, and the experimental cells, to which the NaPBS-dissolved oxidative reagents were added (Section 3.2). Comparing the expression of antioxidant genes between cells that were collected directly from the standard culture medium TY and cells that where incubated in PBS, microarray analysis revealed that the genes *ehprx*, *ehfesod*, *ehtrx1*, *ehfprB2*, and *ehfprC3* were upregulated in the cells incubated in PBS.

This result strongly suggests that the antioxidant genes started to upregulate once they come into contact with an oxygen-rich environment, and continued to a maximum level over the time of incubation. Bruchhaus and Tannich (1994) reported that slight induction of SOD expression was found when amoebae were shifted from TYI, the standard culture medium, to PBS; this relates to the fact that incubation in PBS does not comprise strictly anaerobic conditions. This provides that even if the cells are exposed to oxidative reagents, no additional upregulation in the antioxidant genes will take place.

4.2 Viability of clones A and B upon treatment with oxidative reagents

As the microarray analysis demonstrated that there are highly expressed antioxidant genes in the pathogenic clone B, and that there was no notable differences in the expression of genes between the experiment and the control after exposing the cells to molecular oxygen, we performed the viability tests to compare both clones A and B in terms of their sensitivity to oxidative and nitrosative stresses created by different radical donors (Figure 3.1). Having set standard conditions for all experiments, both clones responded similarly to oxidative stress represented by the intra-and extracellular superoxide radicals and hydrogen peroxide.

Although imunfluorescence results demonstrated that the clone B contains larger amounts of peroxiredoxin and superoxide dismutase in the cytoplasm, almost similar amounts were associated with the surface. This suggests that cells of both clones have used peroxiredoxin and superoxide dismutase which are associated with the surface to deal with the oxidative environment. In *Schistosoma mansoni*, sporozoites were shown to be able to upregulate H_2O_2-scavenging enzymes, in response to environmental oxidative stress (Vermeire and Yoshino, 2007). This in turn reflects the similar level of protection in both clones.

On the other hand, the two clones responded differently to nitrosative stress represented mainly by nitric oxide radicals. Clone B was more resistant, while clone A was found to be more sensitive. Clone B seems to have a higher amount of antioxidant genes that are involved in the detoxification of nitric oxide and peroxynitrite. This is probably due the high expression of the gene *ehfpra2* in the pathogenic clone B.

4.3 Expression analysis by RT-PCR

Based on their differential behavior toward nitrosative stress, the differential gene expression of antioxidant genes in both clones A and B was investigated by means of real time PCR (Figure 3.2). Among the 15 investigated genes, 4 genes were differentially higher expressed in the pathogenic clone B. Otherwise there were no significant differences in the expression of other genes in both clones. Although all four differentially expressed genes (*ehprx, ehfesod*, *ehfehyd*, and *ehtrx4*) are predicted to be involved in the detoxification of superoxide and hydrogen peroxide radicals, it could be hypothesized that these genes are also involved in protecting cells against nitric oxide radicals and peroxynitrite. Indeed, Piacenza *et al.,* 2008 demontrated that peroxiredoxin efficiently detoxifies peroxynitrite in cells of *T. cruzi* and protects the parasite from the deleterious actions of this oxidant.

Another argument could suggest that the expression of these genes help the cells to overcome any oxidative stress that might arise during the treatment, and thus work parallel to the genes involved in the detoxification of nitric oxide and peroxynitrite that are already proven to be moderately or highly expressed in the pathogen clone B.

4.4. Localization analysis of antioxidant proteins

To gain a better understanding of the role of antioxidant enzymes in the survival of *E. histolytica*, cellular localization of the enzymes was studied. Furthermore clones A and B were compared with each other in terms of the amount of the corresponding proteins, which reflects the expression levels of the antioxidant genes.

In this work, it was the first time in which polyclonal antibodies against recombinant rubrerythrin (Rbr) in *E. histolytica* were used. Fluorescence microscopy shows that Rbr is produced in both clones A and B in almost equal amounts (Figure 3.3). This was expected, since RT-PCR results demonstrated that there was no significant difference in the expression level of Rbr between the two clones.

Rbr was found to be located in small compartments in the cytosol of *E. histolytica*, assuming that these compartments are the mitosomes (Dolezal *et al.*, 2010, Maralikova *et al.*, 2010). Providing this, it adds another dimension to the hydroperoxide detoxification capabilities of *E. histolytica.* The functional Fe-S centres of Rbr could allow it to participate in the transfer of electrons from organic donors to oxygen via reduction of hydrogen peroxide, thus preventing its accumulation and toxicity inside these organelles (Maralikova *et al.*, 2010). Whether oxygen metabolism occurs primarily in mitosomes or in the cytoplasm remains to be determined experimentally. However, the Rbr-dependent removal of toxic hydrogen peroxide in mitosomes could help the parasite survive the fluctuating low oxygen concentrations within the human intestine and, most importantly, the high concentrations of oxygen it encounters in the portal system during invasive amoebiasis (Stanley, 2003). Moreover, Mydel *et al.*, (2006) demonstrated that Rbr has a dual function in the protection of *Porphyromonas gingivalis* in *in vivo* not only against oxygen stress but also against reactive nitrogen species.

In addition, immune localization studies have shown also that Rbr is highly associated with the surface of the cell membrane indicating that this enzyme is transported to provide protection against environmental oxidative stress. This result was verified using colocalization studies with lectin, which is known as a classical cell surface glycoprotein in *Entamoeba histolytica* (Petri *et al.*, 1990).

Thioredoxin reductase (TrxR), through its immunfluorescence localization, was found to be localized in the cytosol (Figure 3.4). Comparing the two clones, there was no significant difference in the amount of the corresponding proteins between them. This finding suggests the importance of TrxR for both clones, as a redox partner within the TrxR/Trx system, in the metabolism pathway, which could be critical for the maintenance and virulence of the parasite when exposed to highly toxic reactive oxygen species (Arias *et al.,* 2008; Becker *et al.,* 2000). In addition, the thioredoxin redox system is involved in a variety of important cellular functions, which include the maintenance of the intracellular reducing environment, the reduction of ribonucleotides and hence DNA synthesis, and the regulation of transcription of certain genes by interaction with transcription factors (Krnajski *et al.,* 2002). The immune localization of thioredoxin reductase matches the results of RT-PCR, which demonstrated that there is no significant difference in its expression between the two clones A and B.

Immune localization of peroxiredoxin (Prx) (Figure 3.5) showed that it is located in the cytoplasm with an association to the cell surface. Son *et al.,* (2001) reported that peroxiredixin in *T. gondii* is located in the cytoplasm of Tachyzoites. Signal intensity of peroxiredoxin antibodies revealed that it is more produced in cells of clone B than in clone A. This finding matches with the RT-PCR results and provide the importance of Prx in the protection of cells against their own metabolically produced H_2O_2 (Cheng *et al.,* 2004). Cytoplasmic localization of peroxiredoxin was reported by other groups using indirect fluorescent antibodies and immunoblots of soluble versus membrane proteins. There is no structural basis to suggest that the peroxiredoxin is a transmembrane protein, but it is surface associated (Tachibana *et al.,* 1991; Bruchhaus *et al.,* 1997).

Indeed, our findings demonstrated that Prx was also found to be associated with the cell surface. The surface associated Prx was found to be not equally distributed, but rather assembled on certain locations of the cell (Choi *et al.,* 2005). *E. histolytica* contains a peroxiredoxin on the outer surface of the cell which reduces and detoxifies peroxides and peroxynitrites under oxidatively stressed conditions (Bruchhaus *et al.,* 1997). Our finding matches also with the finding of Choi *et al.,* (2005) who found that the enzyme was primarily surface localized in *E. histolytica*. Hughes *et al.,* (2003) provided an explanation for the surface association of peroxiredoxin of *E. histolytica*. It binds to the cytoplasmic domain of the *N*-acetylgalactosamine inhibitable (GalNAc) lectin, the major surface lectin of *E.*

histolytica mediating adherence to host cells. Thus, it could be said that Prx plays an important role in survival of the parasite in highly oxidative environments (Sen *et al.,* 2007).

Iron superoxide dismutase (FeSOD) was found to be produced in higher amounts in clone B than in clone A (Figure 3.6). This finding was verified with RT-PCR results, which reflected a higher expression of *ehfesod* in clone B than in clone A. Moreover, the signal intensity of FeSOD antibodies in clone B correlates with the microarray results that reflected a high level of expression in the pathogen clone B. Immune localization demonstrated that FeSOD is located in the cytosol of cells with an association to the cell surface. FeSOD was found to be cytosolic in other organisms such as *E. coli*. Its function is to protect the cytoplasmic enzymes against oxyradical damage (Hopkin *et al.,* 1992). Its occurrence in the peripheral of isolated cells suggests that it could function as a barrier against oxygen radicals both in the cytoplasm and in the extracytoplasmic area (Gaiola *et al.,* 1996).

Figure 3.7 shows that iron-hydrogenase is located in the cytoplasm of trophozoites (Horner *et al.,* 2000), with remarkably higher abundance in cells of clone B than in clone A (Biller *et al.,* 2010). Fournier et al. (2004) have reported the involvement of Fe-hydrogenase in the response of *D. vulgaris* Hildenborough to oxidative stress. When the cells were exposed to oxygen for 1 h, the hydrogenase activity in the periplasm was higher than the one in cells kept under anaerobic conditions. However, only iron-hydrogenase activity was reported to be affected by the presence of oxygen (Dolla *et al.,* 2006).

4.5 Effect of the overexpression of some antioxidant genes on the expression of other antioxidant genes.

Constructs of the five antioxidant genes *ehprx*, *ehrtxr*, *ehfesod*, *ehrbr*, and *ehfehyd* were produced and used to transfect both the apathogenic clone A and the pathogenic clone B. Both clones were successfully transfected and were able to reproduce under the selection pressure of G418. It was observed that the overexpression of antioxidant genes had an effect on the expression of other antioxidant genes. The measurement of the relative expression of genes was accomplished by means of RT-PCR.

4.5.1 Effect of the overexpression of *ehprx* on the expression of other antioxidant genes

Having in mind that *ehprx* was found to be higher expressed in the pathogenic clone B than in the apathogenic clone A (Figure 3.2), upon the overexpressing *ehprx*, the differential upregulation of the genes *ehprx* and *ehflpra2* in clone B suggests that transfectants of clone B would be protected against oxidative and nitrosative stress. Wassmann *et al.*,(1999) reported that the amoebae in which peroxiredoxins' expression was increased were more resistant to metronidazole. This indicates that peroxiredoxin may play a role in the protection of trophozoites against nitric oxide radicals. On the other hand, *ehfesod* was significantly upregulated in clone A. Since iron superoxide dismutase, trhough the detoxification of superoxide radicals generates H_2O_2 (Clark *et al.*, 2007), it is expected the transgenic cells of clone A would be more sensitive to superoxide radicals and hydrogen peroxide.

4.5.2 Effect of the overexpression of *ehtrxr* on the expression of other antioxidant genes

Surprisingly, the overexpression of *ehtrxr* led somehow to the silencing of the gene *ehtrxr* in the transgenic clone A (Figure 3.11). Earlier, Irmer *et al.*, (2010) demonstrated that transcriptional silencing by *trans* inactivation can contribute to the regulation of gene expression in eukaryotic cells. The mechanism of *AP-A trans* inactivation is largely unknown. Taking into consideration the predicted pathway of antioxidants in *E. histolytica* (Figure 1.2), the presence of thioredoxin reductase is essential for the activity of peroxiredoxin as redox partner, and thus the detoxification of hydrogen peroxide. Consequently, peroxiredoxin is not expected to be effective in protecting the transgenic trophozoites of clone A against oxidative stress although it differentially upregulated. In *P. falciparum*, Bozdech and Ginsburg (2004), reported that although the enzyme peroxiredoxin protects the parasite from oxidative and nitrosoative stresses, the availability of reduced Trx for these activities before 31 HPI when the TrxR transcript peaks remains to be established.

This has been also proven in the microaerophilic parasite *T. vaginalis*, in which diminished thioredoxin reductase activity consequently leads to reduced activities of antioxidant enzymes that depend on thioredoxin for their function. It is shown that the breakdown of H_2O_2 by peroxidases is slowed down congruently with the decrease in thioredoxin reductase activity

(Leitsch *et al.*, 2009). Moreover, the gene *ehtrx4* was differentially upregulated in clone A, but for being directly associated with TrxR as a redox patner, it is not expected to add any protection measures to the cells. The importance of the thioredoxin system was documented for other organisms such as *T. vaginalis*. The importance of the thioredoxin system as one of the major antioxidant defense mechanisms in *Trichomonas* was confirmed by showing that the parasite responds to environmental changes resulting in increased oxidative stress by upregulating thioredoxin and thioredoxin peroxidases levels (Coombs *et al.*, 2004). This finding puts forward that the transgenic clone A would be less resistant to oxidative stress than the transgenic clone B.

4.5.3 Effect of the overexpression of *ehfesod* on the expression of other antioxidant genes

As for the overexpression of *ehfesod*, the gene was upregulated in both transgenic clones with no significant difference. But for the reason that the gene *ehtrxr* was downregulated almost to the silencing level in clone A, it is more likely that the significant upregulation of *ehtrx1* alone in clone A would not help the cells to overcome the oxidative stress for the same reason mentioned above (section 4.5.3).

4.5.4 Effect of the overexpression of *ehrbr* on the expression of other antioxidant genes

The upregulation of *ehrbr* gene in both transgenic clones A and B is very important. It is true that the gene *ehtrxr* was downregulated, and that peroxiredoxin is not expected to fullfil its task, but rubrerythrin is expected to be able to take over the task done by peroxiredoxin in detoxifying H_2O_2. This finding suggests that transgenic clones of *E. histolytica* would be able to stand the oxidative stress. Rubrerythrin is involved with cellular redox potential in other organisms as well. For example, it was up-regulated in *Methanothermobacter thermautotrophicus* by H_2O_2 along with other redox enzymes (Kato *et al.*, 2008). In vitro analysis of rubrerythrin from *D. vulgaris* and *C. perfringens* found NADH peroxidase activity as part of a novel oxidative stress protection system found in these organisms (Lumppio *et al.*, 2001; Coulter *et al.*, 1999). Moreover, by the upregulation of *ehfpra2* in clone A, it is expected that this clone would also be able to stand the nitrosative stress resembled by nitric oxide radicals and peroxynitrite.

4.5.5 Effect of the overexpression of *ehfehyd* on the expression of other antioxidant genes

The upregulation of the gene *ehfehyd* in both clones, which was accompanied by the upregulation of other genes such as *ehfesod*, *ehrbr*, and *ehtrx5* in clone A, implies the same argument elucidated by the overexpression of *ehrbr*, which provides the expectation that clone A would get a better protection against oxidative stress, especially that the gene *ehtrxr* was again downregulated in clone A. Although *ehfehyd* is considered to be a putative response gene (MacFarlane and Singh, 2006), the exact mechanism of iron-hydrogenase within the antioxidant system of *E. histolytica* is not fully unserstood. Futher investigation about its role is a necessity.

4.6 Viability of transgenic clones upon the treatment with oxidative regents

In this part of the work the target was to examine if the overexpression of the antioxidant genes in clones A and B leads to a better resistance to oxidative and nitrosative stress. Transgenic trophozoites of both clones were treated with the same oxidative reagents used to treat the non-transfected cells of clones A and B (Section 3.7).

4.6.1 Treatment of transgenic clones with DMNQ

By the treatment with 0.04 mM DMNQ (Figure 3.15), which releases intracellular superoxide radicals, the overexpression of *ehprx* was not able to increase their protection against the radicals. This could be due to the fact that the gene *ehprx* was not highly enough overexpressed in either of the two clones to cope with the amout of hydrogen peroxide produce in the cells. On the other hand, the significantly higher degree of protection in pNC-EhTrxR and pNC-EhFeSOD transfectants of the clone B is most likely to be due to the absence of *ehtrxr* in clone A.

As for the pNC-EhRbr and pNC-EhFehyd transfectants, cells of both clones demonstrated a high degree of protection against superoxide radicals. It is most likely that the protection in clone A, in which *ehtrxr* was silenced, was due to the compensating pathway of detoxification of hydreogen peroxide represented by the presence of rubrerythrin.

4.6.2 Treatment of transgenic clones with H_2O_2

Upon the treatment with 0.08 mM H_2O_2, Overexpression of *ehprx* in both clones provided the protection of clone B cells. This is mainly due to the significantly higher overexpression of *ehprx* in this clone B. Moreover, the higher degree of protection in clone B than clone A by the overexpression of *ehtrxr* and *ehfesod*, is expected to be due to the blocking of the peroxiredoxin pathway resulting from the silencing of *ehtrxr* in clone A. As for the overexpression of *ehrbr* and *ehfehyd*, cells of both clones demonstrated a high level of protection against hydrogen peroxide radicals. The above above mentioned assumption concerning the silencing of *ehtrx* could be adopted here also.

4.6.3 Treatment of transgenic clones with pyrogallol

Pyrogallol is an oxidative reagent that produces extracellular superoxide radicals. The reason why the overexpression of the genes *ehprx* did not increase the defense capacity in both clones A and B is not fully understood. The amount of peroxiredoxin that is associated with the cell surface might be not enough to provide the protection. The overexpression of *ehfesod* could not provide protection to cells of both clones. Temperton *et al.,* (1998) described such a case in *T. cruzi* and provided an explanation for it. The generation of O_2^- in cells in which overexpress SOD causes an imbalance in the *T. cruzi* oxidative defense system resulting in an increased rate of H_2O_2 production. In a situation where the ability to metabolize H_2O_2 is limited, this could enhance the generation of $HO^{\cdot}$ which is catalyzed by SOD. This explains the enhanced sensitivity to superoxide radicals of *E. coli* which overexpress FeSOD (Yim *et al.,* (1990).

Rubrerythrin, which was found to be notably associated with the cell surface, has offered protection. This protection was significantly higher in clone A because the overexpression of *ehrbr* was at a higher level in this clone than in clone B. Although the role of iron hydrogenase is not fully understood in the antioxidant defense mechanisms of *E. histolytica*, transgenic cells of clone B were significantly more protected by the overexpression of *ehfehyd*.

4.6.4 Treatment of transgenic clones with GEA5024 and GSNO

The oxidative reagents GEA 5024 and GSNO are considered as nitric oxide donors. In clone B, the overexpression of *ehprx* led to the upregulation of *ehfpra2*, which is expected to be the reason behind its protection aginst nitric oxide radicals. The gene *ehfpra2* was silenced in clone A. According to the predicted pathway of antioxidants in *E. histolytica* (Figure 1.2), flavoproteins of the family A are involved in the detoxification of nitric oxide radicals.

The overexpression *ehfesod* did not add to the protection ability of clone B because it is not involved in the detoxification of nitric oxide radicals. Cells of clone A, in which *ehtrxr* was silenced, were very sensitive toward these radicals, which supports the argument that thioredoxin reductase has an indirect effect on the detoxification of nitric oxide radicals. As for the overexpression of *ehrbr* and *ehfehyd*, the reason that allowed cells of clone A to be more resistant to nitric oxide radical, and to reach the level of protection of Clone B, is that the genes *ehfpra2* and *ehfpra1* were upregulated in clone A.

4.6.5 Treatment of transgenic clones with Sin-1-Cl

The oxidative reagent SIN-1-Cl is considered to release both nitric and superoxide radicals. The protection gained by the overexpression of all five antioxidant genes (*ehprx*, *ehtrxr*, *ehfesod*, *ehrbr*, and *ehfehyd*) it is to be explained as folows. Peroxynitrite is a cytotoxic oxidant formed from the diffusional-controlled reaction between nitric oxide and superoxide radicals. A variety of enzymes participate in the detoxification of peroxynitrite such as glutathione peroxidase, flavonoids (Trujillo *et al.*, 2008). In addition, genes involved in the assembly of iron-sulfur clusters also show enhanced transcription as a response to peroxynitrite (McLean *et al.*, 2010). This suggests that the overexpression of each antioxidant gene together with the other expressed antioxidant genes provide protection to both clones.

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Appendix I

List of oligonucleotides used in quantitative RT-PCR

Protein name	Accessory no.	Sense	Antisense
Fe-Superoxide dismutase	XM_643735	TGCTGCTTTTGGAGGATTAG	ACCATCATGTTCAACCAACC
Rubrerythrin	XM_647039	GCTGCTATTGCTGGTGAAAC	GCAATTTGAGCATGTCCTTC
Peroxiredoxin	XM_642815	CAGGAATTGCAAGAAGAGGA	TTTCTTCCGTTGATCTTCCA
Thioredoxin 1	XM_651634	TTTGAAGAACTTGCTGGTCA	AGAACAAATGTTGGCATTGA
Thioredoxin 2	XM_649815	TTCGTTGTATGCCGACATTT	GCAGCCCACATTACCATATC
Thioredoxin 3	XM_651803	TTGCACAAGACTATCCCCTTA	AAAAGTTGGCATTGAACGAA
Thioredoxin 4	XM_644472	GATTTTCCTGAAATTGCTCAAG	TGCTCCTGTAAATCTTTTGGTT
Thioredoxin 5	XM_646791	GTTGCTGTTGTTCAATTCCA	AGTTGTTGTTGGTGCTTGTG
Thioredoxin 6	XM_655940	GCAGCTACTGAATTCCCAAA	TTCTTGGACCTTCAAACTCAA
Thioredoxin 7	XM_645650	GAAGAAGTCGCACAAGCATT	TCCATGTTCTTGGCAAAGTT
Thioredoxin 8	XM_649760	TGCAAATAAACAAGGATTGG	TGTGGACAATATGGTGAAAAGA
Thioredoxin 9	XM_648410	CGCCAATTAACTCAAAACAA	TGTATTGCTTCGTCGAGTTG
Thioredoxin 10	XM_647543	CGCCAATTAACTCAAAACAA	TGTATTGCTTCGTCGAGTTG
Thioredoxin reductase 1	XM_643726	TGCAACAGGTGCTACAGCTA	AAAATAGGAACAGCCCCATC
Thioredoxin reductase 2	CAA56112	GTTGTGATTATCGGCTCAGG	CCTGCCATAAACCCTTCATA
Flavoprotein A1	XM_646535	GTTGGTGTTGAGGTGTCCAT	TTGGCGTCCCAAGTAATAAA
Flavoprotein A2	XM_641854	TCACCAACCTGTTGAACCTT	CTTTAAGGGCCTCAGCAAAT
Flavoprotein A3	XM_648931	CAACACTTGTTGGTGAAGCA	TCCACCCATAAGTTCCAAAA
Flavoprotein A4	XM_646723	GACGCAACACCTCCTTTTTA	TCCAAATGACCCAAAACATT
Flavoprotein B1	XM_644658	CTGAACCAGATCACAGTGGA	TAATGCTGCCATAGTTCCAA
Flavoprotein B2	XM_650038	TGCTGATGTACCTGGTGTTG	CCATAACAACTGCTGCTCCT
Flavoprotein B3	XM_645918	CCAGTCATGGATGAGTTGCT	TGACAACTCCTGGAACGTCT
Flavoprotein C1	XM_647032	GCAAAAGCATTTCTTGAAGG	TTCCTTTGGTGAACAAGCAT
Flavoprotein C2	XM_643101	TTGCTTGTGTTCCTCACAAA	AGCCAAAATACTGGATGTGC
Flavoprotein C3	XM_647345	TTGCTTGTGTTCCTCACAAA	AGCCAAAATACTGGATGTGC

Protein name	Accessory no.	Sense	Antisense
Flavoprotein D2	XM_649865	GTTGGATGTGATGGAGAAGG	CCCGTAAATGTTCTACACCAA
Flavoprotein D3	XM_644279	TGATGATGGATTGGAACACA	CGATCATAACCAGCAGAACC
NADH oxidase	XM_650619	TTTGCATGTGGAGATTGTTG	TCCTGCTATTGCCTTACCAC

Appendex II. List of the oligonuceotide sequences used in the microarray analysis

Protein name	Spot position	Accession-Nr.	Oligonucleotide sequence (5'—3'-Oriented)
	A1		
	A2		
Actin	A3	XM_651518	CTACATTCCAAAACATGTGGATTACCAAGGAAGAATATGATGAATCTGGACCAGCTATTG
AsP22-1	A4	XM_648987	GAAGTTATGTTGACAGTAGCAACTCATGTTGACGGACCAATCAAATTTATCTTCCCTAAA
AsP22-4	A5	XM_648604	ATGTTGATTCAAACCCATTCACTCTATCATCTACACATGGAAAAGGAGTAAAGTTAGGAC
AsP22-2	A6	XM_647728	ATATTATTGATATGGAATTATCAAATAGGCATACCTGTGTTACTGTGCATAGTTCCAGCA
AsP22-3	A7	XM_652471	ACAAATTCTCTTCCTGAACTTAGTCTACCTTCTGACTTCTCACATGAATCTCCTGTCTTT
EhCP-A1	A8	XM_645064	TCACTGTCATTTTGATGTTTTATATTGGATATGGGATTGATTTCAATACATGGGTTGCCA
EhCP-A2	A9	XM_645550	ATGTTTGCTTTTATTTGTTTACTTGCTATTGCAAGTGCTATTGATTTCAATACATGGGCT
EhCP-A3	A10	XM_648162	CAACAACATGTGTGGTATTGGAAGAGATTCTAACTATCCAACCGGAGTCAAGTTAATTTA
EhCP-A4	B1	XM_651510	TTCACTCCAAAAGTTCAAACTACTGGTTTAACTCATGTTACTCCAACTGAAGAAGCTTTA
EhCP-A5	B2	XM_645845	TGCTGTTAAAATTACTGGACAAAAATTAGTTAGACCAGGAAGTGAAAAAGCACTTATGCG
EhCP-A6	B3	XM_652272	ATGTTTGGTTTACTCTTTGTTACTCTCATTTCATTGAGCAATGCTATTAGCTTTGATAAA
EhCP-B1	B4	XM_646489	ACTGTTGATGGTTATGGAGAATGTGATGGACATAAATTCCTTTGGGTAAGAAATTCATGG
EhCP-A8	B5	XM_652354	AATGTCCAGAAATAAGAATAATCAATGTGGTATTTGCACAGGAATTTCATTCCCAGTTGG
EhCP-A10	B6	XM_646598	GTAATAGTTGGGGTGATTGGAAATGGGGAGAAGATGGATATATGAGACTTTATAGAGGAG
EhCP-B2	B7	AY156069	GTGGATGTGGAGGAGGATTTGCTGAAGATGTTCTTGATTCTGTAGATGGAATTTATTATG
EhCP-B3	B8	XM_651655	CTTGTGGGTCATGTTATTGTGTAAGTAATGCTCTTGCTCTTCAATTAAAATGGGCTAATC
EhCP-B4	B9	XM_643409	AGAATAGTTATTATTGTGAGGGAGGTACTTCAGATGAACCGTTAATGTCTTCTCATTACG
EhCP-B5	B10	XM_647579	CTGCTAAGTTATCTGCTGACAGTTTATGTGGAATAGGAAATTGTGATGGTGAGAATGTTC
EhCP-B6	C1	XM_647373	AAACATCAAGCATTCAGCACACAACAAATCATTGATTGTTCAAACAATAATGGGTGTAGT
EhCP-B7	C2	XM_645308	AGAGAATCACAACGATGTTATTCATGTAAAAGGTTCTATCGACTAAATCAATATTCTTGT
EhCP-A10	C3	XM_646598	GTAATAGTTGGGGTGATTGGAAATGGGGAGAAGATGGATATATGAGACTTTATAGAGGAG
EhCP-B8	C4	XM_645957	ACCATGGATAGTCGTGGTATGTGTTTAGATAATTCTTATCCTTCAATTCCAGAAGATGCT
EhCP-A11	C5	XM_646598	CTTACTCGTGAAGAAAGTGTGGCTATTGCACAAGGAATTCATATAGACAAATCTGATCTT
EhCP-C1	C6	XM_649361	ACTTTTAAACGTGGGGTATGCAAAACTAGAGAGATGGGAGATGGACTTATTGTTTATCTA
EhCP-B10	C7	XM_643214	ACATTAAAATGTTCTGCTTGTAAAGCAAATACTACTCTTGATGCAAGAGGAATGTGTGTT
EhCP-C2	C8	XM_651540	TCAGCATATGTTATTCCTGAAAATAGAATCACTCCAGTTAAAGGACAATTAGCTAGAGGT
EhCP-A12	C9	XM_648731	CCAATTAAATCAAGTGGAACAAGATATTTTGAAGCAACAATTGATGGTATTGAAGCAGCA
EhCP-C3	C10	XM_650036	CGTTCAACTTGTTGGTCTTTTGTTACTTCTGGTTTCTTAGAGTCTGCCTATAATTCTGAG
EhCP-C4	D1	XM_650708	TATCCTCAATAAACATTTACCATTCTGGGGAGGGTATGTTGGAAGTCATTTTGAAATTGA*
EhCP-C5	D2	XM_649708	TGCACCATTTATCAAAGAGAATGAAATAGTATCGACGTTTGAAAAGTCTGTAGGAAATGT
EhCP-C6	D3	XM_646461	ACTTGTGGAGCATTTATTATTAGAGATTCTACTGATGATACTCTTGTTTATGGCAGTCAT
EhCP-C7	D4	XM_652181	AATGGTATTAGAAAAGGATTTCTTAAAGAAAATGAATATCTTAGACTATCACCTCAAGCA
EhCP-C8	D5	XM_652181	AGAAAGTTCATATAGAGCTTATGGTCTTCGACATAATCTTCTTAACTCAACAGAGTATGT
EhCP-C9	D6	XM_649919	GTACCATGTCCATCATCTTTAGGAGGGGATTGTGTTGTACTTACTTTTAATCCATATTCC
EhCP-A13	D7	AY156071	CATAAATCGTACACCAACAAAGCAGAGTATCTTTATCGTCTCGCCGTCTTCTTAGACAAC "
EhCP-C10	D8	XM_649737	TGCACCATTTATCAAAGAGAATGAAATAGTATCGACGTTTGAAAAGTCTGTAGGAAATGT
EhCP-C11	D9	XM_642991	TGGGTGTCGAGATTTAGAAACGATAAATAAGTCATTTGATCCAGATTTTGCTCCTTCATT
EhCP-C12	D19	XM_645737	TTAGTTCGTAACGAATTTCAAGGTGATTGTATTAATGACCAACTTAAAAGTGAGTGTCAA
EhCP-C13	E1	XM_651464	GCTACCATTGGTCTTTTGGAACAATCATATAGAGATAATGACTATCACCTCAAGCATATG
EHCP-B11	E2	XM_642921	AAAGAGTATTTGACTATGATGAGAGAGCACAATGCAAAAGGAAGTTCTTATAGAATGGGA
EhAUTO1	E3	XM_646294	ATCACATTCACTGAACATCAACTAATTTTAGACTCATTAGCTCTTTCTCAAAGTCGAGGA
EhAUTO2	E4	XM_648706	TTCATGGAAATTATGTCCTATTAGATGTGTTATGTGTTCAAATGTTTCAATACCAACTCA
EhUBHY	E5	XM_652264	CAGTCTTTAAAAAAGTGGTTTGAAATTAATAACGAAGAGGTGAAAGAAGCATTTTTCCCA
EhAUTO3	E6	XM_646951	ACACCTTTCAACTTTGTTTCGAATCACTTATAGAAATGGCTTTACTTACCATTTACCTCA
EhAUTO4	E7	XM_651632	TTTCTTTGGGATACATCACAATCAATTACTTTTTCTTGACCCTCATTTTGTACGTCCATG
EhCALP1	E8	XM_644830	ATGTCTAAAACACCGAGAGAAAGAGCAGGAAGAGAACCTAAAATGGGAAAAGTAGGAATG
EhCALP2	E9	XM_652220	AGCTTTAAGTGATTTAACAGGAATGCCAGTAAAACGTATATCTACCAGAGAAACAGACGT
EhOTU	E10	XM_648921	TAAAATGGGTTCCGATTAGAAGAAAAATGGCTGCTGATAATTCCTGTTTATTTCATTGCT
EhCP-B9	F1	XM_647901	CTCAAAGCATTGCGTTTAATCTAGAACTCCCCGTAGACAAATTGTTTATTCAGTTCAAAA
EhCP-I-1	F2	XM_648163	TGTTTTAGTATACAGAAGACCTTGGGCACCAAATGCTAATGACCGTACTTTTACTTTAAA
EhCP-I-2	F3	XM_644271	TCAACTTAGAACAAATCCATCAACAGGATATGCTTGGAATATTGAATACCCAACTGACAC
Hexokinase	F4	XM_650873	GGGTATTGATGTTGGTGGAACTAATCTTAGAGTCTTATTATTAGAAATCCCTGAACCTGG
Phosphofruktokinase	F5	XM_648631	AAAGGTCTTGATCTTGGTAGTGATAAATCAGGAAATATTGTTCATTGGGATGCTGTTACA
Enolase	F6	XM_644069	TGAAACTGAAGATAC-TTTCATTGCTGATCTTGTTGTTGGACTTAACTGCAAACAAATCAA
Histin H2A	F7	XM_647378	AGATGTTACTATTTCTTATGGTGGAGTATTCCCTAATGTTCC-TACTGCCGTTAATTCAAA
EhMP8-1	F8	XM_650302	TTTTGAAGACCTAGGAACTTATTCAGTTAATTATAGTGCTGCTGAACCATTAACATGGGG
EhMP8-2	F9	XM_647540	CTGGTGTAAGGAGGTGTACTAATGACCGTTCTGCTATTGGTATATGTGATGGAAATTCAT
EhMP16-1	F10	XM_649757	ATAAGGTTAGAGTTGAAGGTGGTGCTTATGGAAGTTGGATGTCTTATTCATATAGTGGAA
EhMP1-1	G1	XM_647466	TTTGGTGATTTAGTTACAATGAAATGGTGGAATGATCTTTGGCTTAATGAAGGATTTGCT
EhMP24-5	G2	XM_644888	TGGTTCCCAATATAAAGAAGGATGTACTACTGATGTAACAAGAACTGTTCATTATGGAGA
EhMP24-4	G3	XM_645554	AGAACCATTATTATTCCACGTATAAAAGATAGTTATGTAATTCAGTGGGTTGAATCTAAT
EhMP18-1	G4	XM_651526	AACTAGGAGAAGAGGTTGGAGTAAAGTTTCAAAGAACTGTTAAACGACAAGAAAAAGGAG
EhMP24-3	G5	XM_649119	TATTAACTGATCTTGCTGATGTTAATTGGGCTTTCAATATTAGAGCACATGATATTCCTT

Proteinname	Spots-Position	Accession-Nr.	Oligonucleotidsequenz (5'—3'-Orientierung)
EhMP20-3	G6	XM_651453	GGAGATGATGGAACTGGAGTTGCATGTGGACTTGCATATATGGAACTTAGAGATAAATTC
EhMP20-4	G7	XM_650524	TTTATCAGCACAAGGAACTACTCTTGGAGGAGATGATGGAACTGGAAGATAAATTCCAAC
EhMP18-2	G8	XM_645374	GTACAGAAAAGACTTGAGTCTGCTGGTTATGTCCGTCTTAAAGAAAATGAGGTTTGGAAC
EhMP49-1	G9	XM_649181	CTTTAAGTCACTTGGAAGTGCAATGGAAGAGTGTAGAGCTGAAGCTGTTGGATTATTCTT
EhMP24-1	G10	XM_646447	CAATTCATAAAAGTGTTAGACAATGGGCTCAACAATGGATTAAACCAGGAATGTCAGATC
EhSP26-2	H1	XM_646699	GGTCTGGTGCTACTACTGTTGAACATGGAAATTCTACTAAATTCGTACTTCCTACTGAAT
EhMP48-1	H2	XM_643678	ATGTTGTAGATCCATTTGTCTCTACAATTGAAAACTCTCACCCAAATCTTGTAGAACGAA
EhU48-1	H3	XM_651374	AGAGGAGTGTTTATACCATACCTGTTGACAAACGGATGCACATCAATATTTACATTTATC
EhMP22-1	H4	XM_647200	ATCTAATGTCTAAGAGTGGTCAGTTTACGCCTATAGAAGATGCAACAGTACATCAAAGAT
EhMP3-1	H5	XM_644785	TCAACTAAATTCAAAACTTCTATCCTTGAAATGGGTGGTTCTAAACCAGCAAGTGAATTA
EhMP3-2	H6	XM_644508	AGATCAGACATTGAAGAATATCGAAAAGCATATGATGAAATTCTCCCTAAAGTAACAGAA
EhMP24-6	H7	XM_648239	TTGGAGAAGAAGCAACAGGAGATAAAGCAACTATTATTGAAGCAGGATATACTGCACTTC
EhMP20-1	H8	XM_651336	AAATAATAACTTCTAGTGGTGATACATTACTTGGTGCAGACGATAAATGTGCTGTTGCTA
EhMP20-2	H9	XM_645060	TCACCATAAATAGTCAAAGTAATGAATCAACTCATGTCACTCCTTCAACCCAATGTCAAT
EhMP24-2	H10	XM_651993	AGACTCAATTATTTTCTTAGAAGGTGGTCTTGAACTTCCATTTTATGATACTGATGGTGA
EhSP9-1	I1	XM_650173	AGGATATGATAATCAACCATTATTTAATAATGATGGAAATAAAAGTGATCAATCAGTATT
EhSP9-2	I2	XM_650130	TGGAAGTCTTTTATATTATCTTTCAATGTCAGCACCTAAAGATGAAAGTGATAAATCAGT
EhSP9-3	I3	XM_651288	GGTCTTACAGATGGAATTTCTTATTAATGGCATCAGAAGGGTACATTATTATTGCACCGA
EhSP9-5	I4	XM_650584	GTTATTATATTTTCCCGACGAAAACCACTGGGTTGTTAAAGCACAAAATGGGATGTTATG
EhSP28-3	I5	XM_646997	GCATGGCAAATCTGTAGTGAATACAGTTATTTCCAACCAGTTAATGAAAGTCTTCCATTT
	I6		
	I7		
	I8		
	I9		
	I10		
	J1		
	J2		
EhSP26-1	J3	XM_648050	TATTCAAAATGTTACTCAGTTTGGATTAATTGTTGCATCTGCTGTAATATTGTGGAAAGC
EhSP28-3	J4	XM_646997	GCATGGCAAATCTGTAGTGAATACAGTTATTTCCAACCAGTTAATGAAAGTCTTCCATTT
EhSP28-1	J5	XM_651670	CAGTTACGCAAAACTCTCACTCAACCAACAAGTAATGCAACAAATATCTCAATCATTCAT
EhSP28-2	J6	XM_643899	TATCAATGTTAGATATGGAGGAAAGAAACCATGTGTAACCAATGTTGCATTTACAAATGG
EhSP-I	J7	XM_645170	ATTTGATGAACGTGCAGATTTTAGTAAAATGGCAAAAGGACATTTTTGTGTTTCAGAAGC
SOD	J8	XM_643735	TTTGGGAACATGCTTATTACATTGACACTAGAAACAACAGAGCTGCTTACTTAGAACATT
Rubrerythrin	J9	XM_647039	AAAGTATGTCCACTTTGTGGTGAACCAGGTGACTTCTTCAGAGTTCAAGTTTCTATTTAA
Peroxiredoxin	J10	XM_642815	CAAATTGGAAAAGAAGCACCAGAATTTAAAGCACCAGCATATTGTCCATGTGGTTCAATC
Thioredoxin1	K1	XM_651634	TGTAGACCAAGCTGAAGAAATTTGTGTTAATTATAAAGTTAGATCAATGCCAACATTTGT
Thioredoxin2	K2	XM_649815	TTTTCTGGAGCTGATAAGAACAAATTAAATGATATGGTAATGTGGGCTGCTTTTCAATAA
Thioredoxin3	K3	XM_651803	AGCAAGACAATGTAATATTCGTTCAATGCCAACTTTTAGATTTTATAGACAAGGAGGGTT
Thioredoxin4	K4	XM_644472	AATGGAGTTTCAACCAAAAGATTTACAGGAGCATATAGAGATGAAGTAGAGAGAATGATT
Thioredoxin5	K5	XM_646791	CAAGCACCAACAACAACTTATCTTGACATTGACATTGATAAAGCTCCAGAATTGAAGAAA
Thioredoxin-related 6	K6	XM_645940	TCCCAAATTTAATTGTTGCTGAAGTTGATTGTACTCAAAACCAAGATATTTGTGAACATG
Thioredoxin-related 7	K7	XM_645650	CATTATTGCTGAAGTAAACTGTGACGATTACAGAGAACTTTGCCAAGAACATGGAATTAG
Thioredoxin-related 8	K8	XM_649760	TGTTGCTGAATTAAATTGTGTTGACTTTAGAGATTTATGTGGGTTCTATAAAATTAGAGG
Thioredoxin-related 9	K9	XM_648410	TTTATCATCTGCTTATATCTGGGAATTTGATCCAAATAAACTACAACGCCAATTAACTCA
Thioredoxin-related 10	K10	XM_647543	GTAAAAGTCGATGTTGATCAAGGCACTGACATTGCCCAAAGGTATGGTGTTCGTTCTATG
Thioredoxin-Reduktase	L1	XM_643726	TTTCAGGAGAATACAAAGTTGTTCCAGTAGCTGGATTGTTTTATGCTATTGGACATAGTC
P34 (Disulfidoxido-reduktase)	L2	X79603	GCAGGAGGACAACTAACTACCACTACTATCATTGAGAATTTCAGGATTTCCAGGATTTCA
FprA1	L3	XM_646535	GGAATCTTTCAGCAAGACTTCAACAACTAAAAGCAAAACAACCAGTTGAGCCATTGTCTT
FprA2	L4	XM_651854	ATTGTAATAGATTTGTTCAATGCTTTGGTTCATTCGGTTGGAGTGGTGAAGGTGTTAAAA
FprA3	L5	XM_648931	AAATTCTAAAGGATTATTATTAGGAACTCCAACACTTGTTGGTGAAGCACTTCCACCAAT
FprA4	L6	XM_646723	GATTGTTGGAGACGCAACACCTCCTTTTTATGATTTACTTGGACATCTGAATCCATTTAT
NADH-Oxidase	L7	XM_650619	TCTGTAGGAATTATTGAAGGAGAGTCTATACTAAAACAAAATGGAAATGAAGTGATGGAA
FprB1	L8	XM_644658	CCCAATTAGAAAGAAAGGTAGGAGTTGCAGTTGTTGTACCATGTAAAGGAGGTTCAGTTT
FprB2	L9	XM_650038	AGTACCATTGTCTGTTAAAAGTAGAGCACTGAATAGAAAAGTAGGAGCAGCAGTTGTTAT
FprB3	L10	XM_645918	TCAACCAATTTATTACAACAAAGAAACACTTAGAAGAAAAGTTGCAGCGGCAGTTGTTAT
FprC1	M1	XM_647032	TGTTGAGTCATATGGAGCTAAGTATCAAGGAGTTTATTTATCTGGTACACAAAGAGAAGA
FprC3	M2	XM_647345	AAATTGTAGAAGAACAACAAAAGAAACTGCTGTGAATGCTGAAAAGCCTATTGAATGTTT
FprD1	M3	XM_649789	AAAGACGACTTTTATTGTGTCCAAAAGGACGAGACATTCAAACTAGCGAAGGATATTTTG
FprD2	M4	XM_649865	AATGGAAGTTGGAACAATAATGGTAATTCTGCATGGCTTATCAATAAATTTCTTGAAGGA
FprD3	M5	XM_644279	GATGATGGATTGGAACACATTTTCAGCTAAACAAGAAGTTCTTGACAAAGGAAAAAAGAC
SP7	M6	XM_643899	TTGCAGAGTCTATTGGAACAGCTCTTTCTGGTTATGTTCAATATAATTCTTCAAATTGGA
Metalloprotease	M7	XM_647071	ACTCCAGATGAAACTATGGATATTGAATCAGGAAATAAACTATTAGACCTTATTTGCACT
AmoebaporeA	M8	XM_648173	TTATTGAAGACAAAGTTGATGCCAATGCTATTTGTGCTAAGATTCATGCTTGCTAAGTTT
AmoebaporeB	M9	Q24824	CTGTTAGACAATATATCGACAACCTTTGTGGTAAAGCTAGTGGATTCCTTGGAACTCTTT
AmoebaporeC	M10	XM_650937	TACATCACTTGTTGGAAAGTTGATTGATTTAGTCCTTGGTGGAGCAGTTGATAAAGTAAC
Saplip1	N1	XM_650744	TTGTTTGCAACCTTTGTATTGGACTTGTTAATACCCTTGATGGCCTTATCGTAAATAAAG
Saplip2	N2	XM_650945	GAATCTGGGAATAGATCTGATCAATTTTGTGAATGGGCTAAAATGTGTCCTTCTTCAAAT

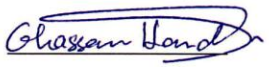
Proteinname	Spots-Position	Accession-Nr.	Oligonucleotidsequenz (5'—3'-Orientierung)
Saplip4	N4	XM_647067	TTGTAAATCTCAAGACTCAGAATCTCCTGCTGGAAAAGTATGTTCAGCTTTACTAGAAAA
Saplip5	N5	EAL50403	CTAGATGTATTGATGTTGCTATGAGATTAACTGAAGTTGCTGTTAAAGTGTTTGACCCAG
Saplip6	N6	XM_650728	GCACTTGCTGGTTCATGCATCAACTGTTTCCATATAATTGATGATTCAAGATACTATACT
Saplip7	N7	XM_651349	CTACAGAACTTTAATAGAACTTGGCTCTCGTGTGTTTGAAGAAGGAGTTACACCAATAAC
Saplip8	N8	XM_651821	TTCATACATTTGACCCATCAAAATTCGTTGTTAGTGAAGTGTGCTCTTCTCTTGGAAAAT
Saplip9	N9	XM_645284	TTGTGAGTCATTTGTAGGAATAGCACTTGATTATATAACTAAAGGAGAGACAGTAGAGAA
Saplip10 unvollständig	N10	-	ACAATGATGTTAGAATGTTGGAAATGGGAATAGAGGAGATTCGTAGATATGTTCAAGAGA
Saplip11ohne N-Terminus	O1	-	AGCTGAAAGCCAAAGAGATAAATTAGCAAAAGAAACATTATATACTATTGACAATACAGA
Saplip12	O2	XM_647629	ATTGCTGAACATGCACTAAAAGTAAGAGATCCTTTAACCTTTAATACTGAACAAACATGC
Saplip13	O3	XM_649997	GCACAATGTGTAGAAATCTTGCTGCATTCTTGTTACAAAAAGTTAGAAGAGATCGTATGT
Saplip14C-Terminus	O4	479.t00008	TGATCCACAAGAACTTAAAATAAAGAAAATGTGCGTTATGTTAAAGGGATGTGATACTCT
Saplip15C-Terminus	O5	XM_644284	AACTCATCGACTTATCACATGACTTACCTGCTTCTGGAAATCAACTTTGTACTTCATTAG
Saplip16C-Terminus	O6	219.t00022	TACTCTGCTGCTATGAGCTTAATGGCTAATTTTGATAGTAAAGTTATTGAAACTACCCCA
Grainin1	O7	XM_645280	ATATCAAGCTGATCCACTTATTCAAAGAGAATGGTGGTATCCACTTGCTACCTCAATTTC
Grainin2	O8	XM_645265	GGATCTAAGAAAGTTTCAAAGAACCAATTCATTAGTACTGCTGCTTATCTTGGACAATGC
Lysozym1	O9	XM_645284	TATGGTTCTAAATATTATTGGGGAAATCTCTTTGGATCATCTTACAAATATCGTTATCGA
Lysozym2	O10	XM_651841	GGTATTCAGATGTTGGATCAAATAGAGCTTTCTTCCAAGAACTTATTGATACTGCCCTTG
Asm1	P1	XM_644619	CATGAAAGGGTCATTGTACCGATGGAGAACTGTTGGATTATATGATCGTAATGAAGTCCA
Asm2	P2	XM_651421	AGCAGAGTATAATGATGTTATTCTTGGAGTATTATGTGGACATTTACATACAGATACTTT
Asm3	P3	XM_646365	ACAGAATGACGATGCTTTATGGAGAACATTTATGCAATATTTTAAAGACTCAAGTTATAA
Asm4	P4	XM_643770	GCATTCTAATAGAACTTTACATGATATTTGGTATGAACATATGAGAGCTGATTCTCACAT
Asm5	P5	XM_650953	TGTGAAGGTAATTGTCGAAAGGATTTATTATGTTCAATTCATTGTATGAAAGAGAGTGAA
Asm6	P6	XM_646625	TGTGTAATGACTTCTAATACAGAAGAAGAAGCCCGTGCATGTGTTAAACTGATGTTATAA
Asm7	P7	XM_651814	AAACTTTGTTCAATGGAAAATATGAGAGAGTCAGGGTATGCTGAATGCATTAAGAAGTAA
Adenylylcyclase assoziiertes Protein	P8	XM_650148	ATATCTTAAACCACAAGTTGAAGCTGCACAAGGATTTGATGAAATTCTTGGGAAATGTAT
G-Protein alpha UE	P9	XM_646645	GGGAGCAGTAAATGAGAAAGTATATACAAACCCAACAAATGCAACTGATGGTTCAAATAT
G-Protein beta UE	P10	XM_647655	GATGGTTATGCATTGTGTACTGGTTCATGGGATTCGACATTGAGAATTTGGGCAAATTAA
Sphingosin-1-Phosphat Lyase1deleted gene!	Q1	XM_645525	GACAGGTGGATTATATTGTTCACCATCAATTCCAGGTAGTAGAGCAGGAAATAATATTGC
Sphingosin-1-Phosphat Lyase2	Q2	XM_648678	GGGGAGAAGCAGCAGGATTTAATGTTCCAATATTTGATTTTAGAAATGAAGGAGTAATGA
Farnesyltransferase beta UE	Q3	XM_648349	AGACGGTGGATATAGAGATAAGCCTTCTAAAAAACCTGATTTGTACCATACTAACTACGC
Farnesyltransferase alpha UE	Q4	XM_644489	GAATTAGTTTTAGAATTACGTGATCGCTTAGACCTTGCTCATCAAAGTTATTGGGATTGG
Geranylgeranyltransferase beta UE	Q5	XM_649844	TTATGATTTTGCATTTGGTCAAATGCCAAAAAGAGAAAGTCATGGAGGATCAACATATTG
Rab- Geranylgeranyltransferase alpha UE	Q6	XM_650563	ACTCCAAAGGGTTGATAGTATGAGAAGTGGATATTATAAAGAACTTGAGAAAGATTTGCT
Rab-Geranylgeranyltransferase beta UE	Q7	XM_643143	AGTTTAATGAGGAAATATACAGATATTATTGGTGAAATTGACCCACGATTTGCTATGCCA
Ohne Namen: prenyl cysteine carboxyl methyltransferase2, putative	Q8	XM_648341	TGCAGCAAGTAATTTTAATCATGTTATCGAAACTTCTTCAAGAGACAGTCATAAATTGGT
Prenylcysteincarboxylmethyl-Transferase1	Q9	XM_652234	CACTACATCGAAACGTCACATCGTCAAGACCATGTATTAGTAACGAATGGAATATATCAT

Spot position = Position of Oligonucleotids on the Microarray

This work was done in the period from January 2007 to August 2010 in Bernhard Nocht institute for tropical medicine, in the department of molecular parasitology, and under the supervision of Professor Dr. Egbert Tannich.

I hereby certify that I have written this work independently and without assistance. No resource and tools, other than the mentioned ones were used in this work. Furthermore, I certify that I did not submit this dissertation at any other university in order to obtain a doctoral degree.

Hamburg, 10.08.2010



Ghassan Handal