THE UNIVERSITY OF CALGARY

Iron Superoxide Dismutase cDNAs of *Leishmania chagasi*: Characterization and Functional Studies

by

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ABSTRACT

Superoxide dismutases are a group of metalloenzymes which function to dismutate superoxide radicals into hydrogen peroxide and oxygen. This enzyme is thought to be involved in eleviating cells from toxic oxygen bursts arising from many cellular functions.

To determine the possible role superoxide dismutase genes play in *Leishmania* survival, two iron-containing superoxide cDNA from *Leishmania chagasi*, called L.c.FeSODA and L.c.FeSODB were characterized and functionally analyzed. Comparison of these two *L. chagasi* SOD deduced amino acid sequences with previously isolated MnSOD and FeSOD amino acid sequences revealed that they had higher homology to, and conservation of completely conserved invariant residues found in iron-containing SODs. Pulsed Field Gel Electrophoresis localized L.c.FeSODA and L.c.FeSODB to separate chromosomes. Southern analysis showed that L.c.FeSODA is comprised of a single copy gene, whereas, L.c.FeSODB belongs to a multi-gene family. Transcript levels and enzyme activity of L.c.FeSODA and L.c.FeSODB show differential stage expression, with higher levels present in the amastigote stage of the parasite compared to the promastigote stage. Overexpression assays using *L. chagasi* FeSODs overexpressed in an *E. coli* SOD null strain showed complete protection by these enzymes against free radical generating agents. As well, overexpression of these FeSODs in *L. chagasi* parasites showed protection against the free radical generating agents, paraquat and

nitroprusside. Initial attempts at the gene replacements of the L.c.FeSODA and L.c.FeSODB genes resulted in the successful one allele knock-out of the L.c.FeSODB gene. The characterization overexpression, and initial knock-out attempts of L.c.FeSODA and L.c.FeSODB from *Leishmania chagasi* contributed to the understanding of the possible role of SODs in *Leishmania* pathogenesis.

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Many people have contributed to the completion of this thesis, and without them I could not have made this possible. I would like to express my deepest gratitude to Dr. Lashitew Gedamu who was not only my constant supervisor but a good friend to talk to about anything. Without his encouragement at the onset of this project and his complete understanding at the completion of this project, I would have never reached this point. Thank you Lash. I also wish to thank my committee members, Dr. S. Lees-Miller and Dr. M. Lohka for their guidance and suggestions during this project. A special thank you also goes to Dr. Kostas Iatrou who agreed to be my external examiner.

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DEDICATION

To my best friend, Trevor, for his patience, understanding and constant encouragement no matter how long it took and to my mom and dad for their encouragement and belief in me to achieve anything I set my mind to.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

bisacrylamide	N,N'-methylene-bisacrylamide
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	disodium ethylenediaminetetra-acetate
EtBr	ethidium bromide
FCS	fetal calf serum
GFC	glass filter circles
GST	glutathione S-transferase
HCl	hydrochloric acid
HEPES	*N*-2-hydroxyethylpiperizine-*N'*-2-ethanesulphonic acid
HO-MEM	modified minimal essential medium
IPTG	isopropyl thio galactoside
Kb	kilobase
KV	kilovolts
KV/cm	kilovolts per centimeter
l	liter
LB	Luria Bertani (broth)
LMP	low melting point (agarose)

M	molar
mg	milligram (10^{-3} grams)
ml	millilter (10^{-3} liters)
mM	millimolar (10^{-3} moles/liter)
mmol	millimole (10^{-3} moles)
MOPS	3-(*N*-morpholino)-propanesulphonic acid
NaCl	sodium chloride
ng	nanogram (10^{-9} grams)
PABA	para-aminobenzoic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCI	phenol : chloroform : isoamyl alcohol
PCR	polymerase chain reaction
Pen/Strep	penicillin streptomycin
pg	picogram (10^{-12} grams)
PMA	phorbol myristic acid
pmol	picomoles (10^{-12} moles)
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SIN-1	3-Morpholino sydnonimine

SSC	sodium chloride-sodium citrate
SOD	superoxide dismutase
TAE	tris-acetate EDTA
Td	temperature of dissociation
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)aminomethane
UTR	untranslated region
UV	ultra-violet
V/cm	volts per centimeter
vol	volume
WCE	whole cell extract
μFd	microfarad (10^{-6} farad)
μg	microgram (10^{-6} gram)
μl	microliter (10^{-6} liter)
μM	micromolar (10^{-6} moles/liter)
μmol	micromole (10^{-6} moles)
%	percent

CHAPTER 1

INTRODUCTION

I. *Leishmania* spp.

A. Leishmaniasis

The disease leishmaniasis is one of the six major parasitic diseases recognized by the World Health Organization and accounts for an estimated 12 million cases worldwide (Wong, 1995). This high prevalence of the disease has posed a major health concern, especially in undeveloped countries. Leishmaniasis is classified into three different forms: cutaneous leishmaniasis (oriental sore), mucocutaneous leishmaniasis (espundia) and visceral leishmaniasis (kala-azar) (Liew and O'Donnell, 1993). Cutaneous leishmaniasis, which is by far the least life threatening, is identified through the presence of a skin ulcer which heals spontaneously, but leaves disfiguring scars. This form of the disease is caused by *L. major* and *L. tropica* but can become diffuse when affected by *L. aethiopica* and *L. mexicana*. Cutaneous leishmaniasis characteristically causes thickening of the skin with lesions and does not heal spontaneously due to its widespread nature. The second class of this disease is mucocutaneous leishmaniasis. It is caused by the metastasis of the parasites from the primary infection point to mucosal sites resulting in swelling and tissue destruction of the oronasal and pharynegeal mucosa. The main species to cause this form of the disease is *L. braziliensis*.The final form of this disease, visceral leishmaniasis, is caused by *L. donovani* and *L. chagasi*. This is the most fatal form of the disease characterized by fever, malaise, weight loss, coughing and

diarrhoea accompanied by anaemia, skin darkening and the most common clinical sign, hepatosplenomegaly.

The diagnosis of the various forms of leishmaniasis has generally relied on the presence of the parasite in biopsies (Liew and O'Donnell, 1993). Recently, more advanced techniques have been developed including immunofluorescence and counter-current electrophoresis. However, these require equipment and therefore are impractical for field work. The drug treatment for leishmaniasis has relied on antimony compounds such as the pentavalent antimonial compounds, sodium stibogluconate and N-methylglucaminine antimonate (Bryceson, 1987). Unfortunately, these drugs have very severe side effects and are often not strong enough to clear up visceral leishmaniasis. Also, these drugs still leave the patients susceptible to a second round of infection, so the only acceptable means of treatment for this disease ultimately will be a vaccine. Knowledge of molecular changes within the parasite upon infection and an understanding of the immune response to leishmanial infection will provide the background for vaccine development.

B. Life cycle

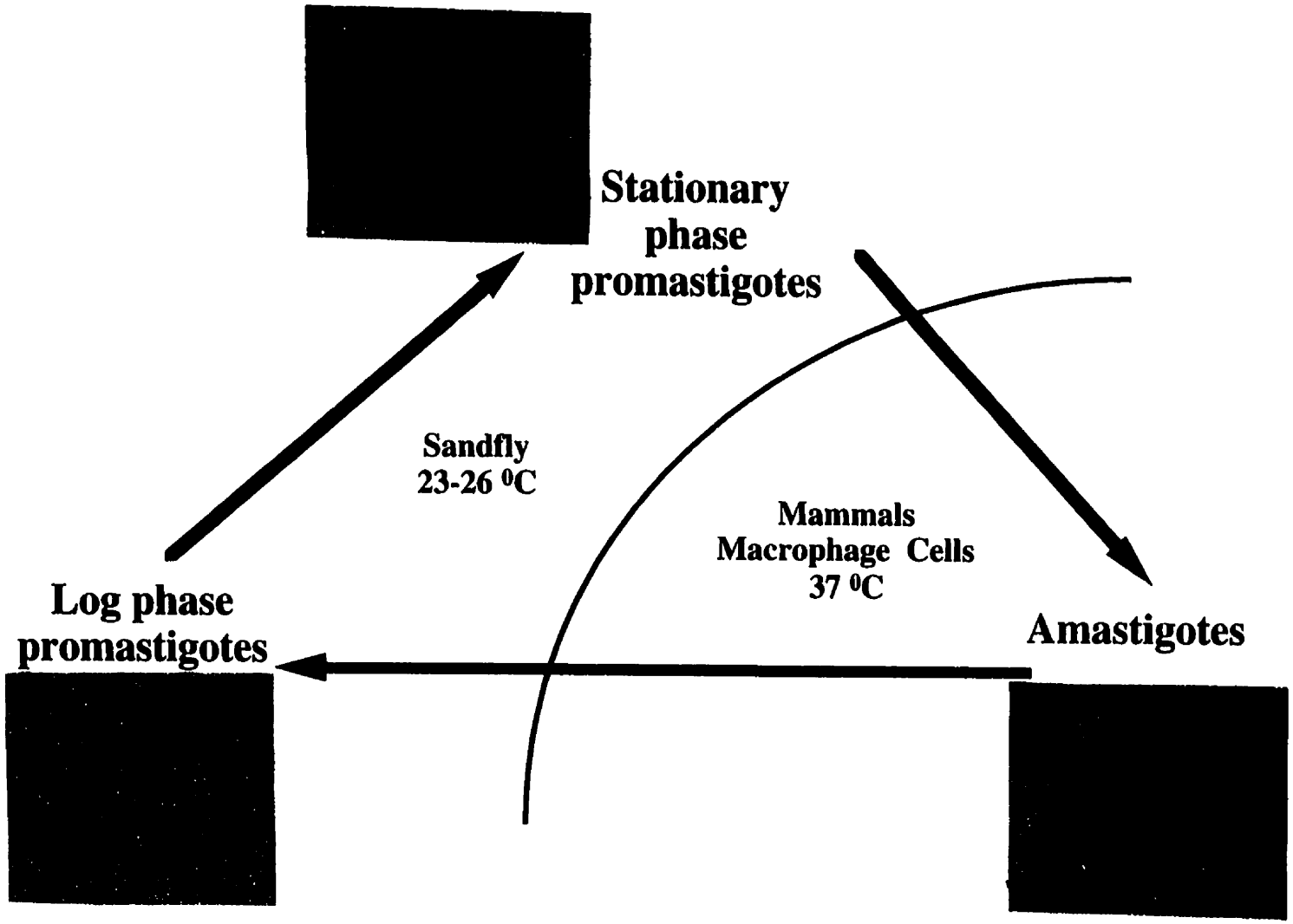
Leishmaniasis is caused by the protozoan *Leishmania* of the family Trypanosomatidae and the order Kinetoplastida. This parasite employs an elaborate life cycle, in which it goes through morphological changes from the sandfly vector to the human host. *Leishmania* grow and multiply, preparing for

infection in the female sandfly vector's alimentary tract. Parasites from the "Old World" (*L. donovani*) are transmitted by the sandfly of the genera *Phlebotomus* and those from the "New World" (*L. chagasi*), by the genera *Lutzomyia*.Within the sandfly gut, the parasites grow and divide as extracellular promastigotes (Fig. 1.1). Log phase promastigotes are rapidly dividing, uninfective, motile, spindly shaped organisms with a single anterior flagellum and measure 10 to 15 μm in length. As the parasites divide and grow, they begin to migrate to the salivary glands of the sandfly where they lose their motility and are considered stationary phase promastigotes. Upon a bite of the mammalian host by the infected sandfly, the parasites are transferred through the sandflies blood meal into the blood stream. Here, the parasites are readily phagocytosed by the host's macrophage cells and reside within the phagolysosomes. The promastigotes undergo a dramatic morphological change within the mammalian host by rounding up to 2 to 3 μm and retracting their flagellum becoming intracellular amastigotes. The life cycle is completed when a sandfly bites an infected host and ingests infected macrophages. The infected macrophages breakdown within the insect gut and release the amastigote form of the parasite which quickly transforms back to the extracellular promastigote form.

C. Genome organization and gene expression

The size of the *Leishmania* genome ranges from 10^7 to 10^8 bp of DNA, depending on the species being analyzed (Lighthall and Giannini, 1992). The chromosomal DNA consists of nuclear DNA and the kinetoplast (modified

Figure 1.1: Schematic of the life cycle of the *Leishmania* parasite. The parasite has a digenetic life cycle, alternating between an insect host (sandfly) and a mammalian host. Inset pictures display the *Leishmania* promastigote and amastigote stages morphology. Parasites shown in the photograph marked **L** are log phase promastigotes of *L. chagasi*, showing the spindle shape of the parasite with a monopolar flagellum; those in the photograph marked **S** are stationary phase promastigotes of *L. chagasi*, showing the less slender parasites with the absence of a flagellum; and parasites in the photograph marked **A** are purified *L. chagasi* amastigotes isolated from infected human macrophage cells (U937 cell line). Amastigotes round up and the monopolar flagellum is no longer visible.


Stationary phase promastigotes
Sandfly 23-26 °C
Mammals Macrophage Cells 37 °C
Log phase promastigotes
Amastigotes

mitochondrion) DNA, which exists as interlocked (concatenated) DNA molecules (Simpson and Braly, 1970). Karyotype analysis of *Leishmania* species has proven to be very arduous due to the difficulty in obtaining clear separation of chromosomes using Pulsed Field Gel Electrophoresis (PFGE). It has been estimated that the chromosome number ranges between 23 to 33 (Samaras and Spithill, 1987; Galindo and Ramirez, 1989) and chromosome sizes vary from 23 to 134 Mbp.

Recently, Wincker *et al.* (1996), developed a new method for identifing the molecular karyotype of *Leishmania infantum.* This group used a variety of known genes to hybridize separated chromosomes of this species. This resulted in the apppearance of 36 chromosomes ranging in size form 0.35 to ~3 Mbp. The comparison of this map with that of *L. major*, *L. tropica* and *L. aethiopica,* showed a very conserved linkage group between all species, suggesting that conservation of chromosome structure may be essential for the survival of this parasite. Although *Leishmania* is thought to be a diploid organism with no sexual cycle, the apparent copy number of genes at certain loci is indicative of aneuploidy. The ploidy of *Leishmania* still remains as open issue (Lighthall and Giannini, 1992).

Leishmania display some very interesting features of gene organization and expression. Most notably is the presence of genes in multi-copies, arranged in tandem arrays (Thomashow *et al.,* 1983; Landfear *et al.,* 1983). Two examples of this are the α-tubulin gene which is highly expressed and present in 30 copies

in *L. enriettii* (Curotto de Lafaille and Wirth, 1992) and the cysteine proteinase gene from *L. mexicana* which is present in a tandem array of 19 genes (Mottram, *et al.,* 1997). Recently, a very powerful technique has been established, called homologous gene replacement, which allows for analysis of gene function in *Leishmania* organisms (Tobin and Wirth, 1992; Tobin *et al.,* 1991). Homologous gene replacement has been demonstrated in various *Leishmania* species including the dihydrofolate reductase gene in *L. major* (Cruz and Beverley, 1990), the α-tubulin gene cluster in *L. enriettii* (Curotto de Lafaille and Wirth, 1992) and the cysteine proteinase gene cluster in *L. mexicana* (Mottram *et al.,* 1997).

Transcription of *Leishmania* genes is polycistronic (Borst, 1986), resulting in mature mRNA units being derived with the processing of pre-mRNAs by trans-splicing coupled to polyadenylation (Borst, 1986; Agabian, 1990). The production of single monocistronic messages from the initial polycistronic mRNA in trypanosomatids occurs through two cleavages, including trans-splicing of one message coupled to the polyadenylation of the immediate upstream message. A 39-nucleotide spliced leader sequence (SL RNA) arises from the SL gene which is found in about 200 tandemly arranged copies with a single unit size of 448 bp (Miller *et. al.,*1986). During the polyadenylation splicing event, the SL RNA is trans-spliced to the 5' end cistron on the polycistronic transcript and acts as a 5' cap. In this 5' region *Leishmania* and Trypanosomatidae contain an AG acceptor site and an adjacent upstream pyrimidine-rich region that are essential for this

splicing event (LeBowitz, *et al.*, 1993). Splicing at each 5' end is coupled to a second cleavage at a site within 1 kb upstream of the trans-splice acceptor site. Here the 3' polyA tract is added to the upstream cistronic message, coupling the polyadenylation of one message with the trans-splicing of the adjacent message. No conserved motifs have been identified in *Leishmania* species which participate in the polyadenylation process (Kapler *et al.*, 1990).

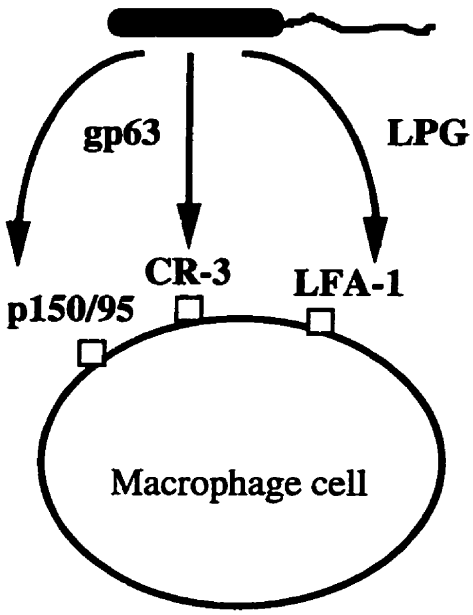
Interestingly introns are absent in *Leishmania* genes. RNA editing of mitochondrial transcripts is just one more processing mechanism which varies greatly from higher eukaryotes.

D.*Leishmania*-host interaction

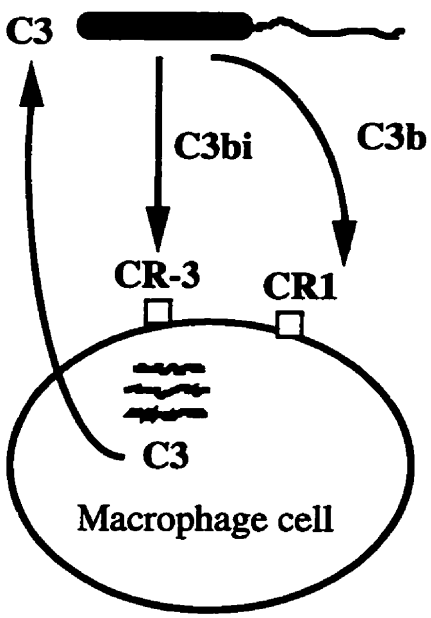
The mode through which *Leishmania* parasites attach and enter into the mammalian macrophage cell is of considerable interest. It is thought that the attachment mechanism of *Leishmania* promastigotes to macrophages is through parasite membrane ligands and the macrophage receptors to which they bind. Two of the most abundant ligands identified on all species of *Leishmania* are the glycoprotein lipophosphoglycan (LPG) and the surface protease glycolipid gp63 (Chang and Chaudhuri, 1990). The macrophage receptors to which these ligands bind to are not as clearly identified. Results of studies vary, depending on whether attachment takes place in the absence or presence of serum (Fig. 1.2). In the absence of serum (and thus complement), gp63 has been shown to associate directly with the macrophage integrin CR3 (Russell and Wilhelm,

Figure 1.2: Schematic showing the interaction of *Leishmaina* parasites with their host in the absence (A) and presence (B) of serum. (A) The *Leishmania*-macrophage interaction pathway in the absence of serum has been shown to be mediated through the glycoprotein surface protease, gp63 on the *Leishmania* surface with the integrin receptors CR3 and p150/95. The *Leishmania* LPG surface molecule interacts specifically with the macrophage LFA-1 receptor. **(B)** The *Leishmania*-macrophage interaction pathway in the presence of serum has been shown to be mediated through the activation of the complement pathway, specifically C3. C3b accumulates on the *Leishmania* surface and acts as a ligand for the CR1 macrophage receptor. (Figure adapted from Russell and Rohana, 1989).

A absence of serum



B presence of serum



1986; Russel and Wright, 1988), whereas LPG attaches via the lectin-like-LPS binding sites present on the CD18 dimers, LFA-1, CR3 and p150/95 (Talamas and Russell, 1989). In the presence serum, the infective metacyclic promastigotes have been shown to activate complement and accumulate C3b on their surface (Russell and Talamas-Rohana, 1989). C3b is known to be the ligand for the CR1 complement receptor, therefore, this is the major route for attachment in the presence of serum. As well, gp63 and LPG are able to bind the CR3 receptor for attachment in the presence of serum.

II. Superoxide dismutases

A. Classification, Distribution and Subcellular localization

Superoxide dismutases (SODs) are probably as old as aerobic respiration. The initial oxygenation of the earth by the earliest water-splitting photosynthetic organisms must have imposed selection pressure to develop a system to eliminate the toxic by-products of this reaction. SODs are a group of metalloenzymes that have been isolated from diverse organisms (Bannister *et al.*, 1987). Three isoforms of SODs have been identified, depending on their active site metal requirement: copper-zinc SOD (Cu/ZnSOD), manganese SOD (MnSOD) and iron SOD (FeSOD). As well, these different isoforms can be distinguished through their sensitivity to a number of agents (Beyer *et al.*, 1991). Cu/ZnSODs and FeSODs are inactivated by hydrogen peroxide, whereas, MnSOD is resistant to this compound. As well, Cu/ZnSODs are inhibited by

cyanide, but MnSODs and FeSODs are not. Finally, the three isoforms show varying sensitiviy to azide. FeSODs are the least sensitive to azide, followed by MnSODs and lastly Cu/ZnSODs which are the most sensitive to azide.

The isolation and distribution of the three isoforms of SODs has been studied extensively using the sensitivity of these enzymes to these various inhibitors. The first detailed study of SODs in obligate anaerobes was carried out by Hewitt and Morris (1975). SOD activity was reported in *Clostridium acetobutylicum* and *C. pastuerianum.* It was shown that in these species, SOD activity was insensitive to cyanide and inactivated by hydrogen peroxide, indicating that the enzyme was iron containing. Iron containing enzymes were subsequently isolated from the anaerobes *B. fragilis* (Gregory and Dapper, 1983) and *Desulfovibrio desulfuricans* (Hatchikan and Henry, 1977). Therefore, these isolations suggested that the most primitive form of SOD appeared to be the iron enzyme. In contrast to obligate anaerobes, many aerobic and facultative anaerobes have been shown to contain both Mn and Fe SODs. Most notably is the facultative anaerobe, *E. coli.* This bacterium contains an iron enzyme when grown anaerobically, as well as a manganese enzyme when grown in the presence of oxygen (Dougherty, *et al.,* 1978). Cu/ZnSODs have been shown to be mainly present in invertebrate species, such as the helminth *Ascaris lumbricoides* (Geerasimov, *et al.,* 1979) and the fruit fly *D. melanogaster* (Lee *et al.,* 1981), as well as in higher eukaryotes, although a few exceptions have been reported. Most recently, a Cu/ZnSOD was isolated from the bacterium *E. coli*

(Benov and Fridovich, 1994) and later, from *Legionella pneumophila* (St.John and Steinman, 1996). In higher eukaryotes, both Cu/Zn and MnSODs have been observed in all organisms studied to date (Reviewed in Bannister *et al.*, 1987).

A pattern for isoform distribution was deduced from the above studies. Cu/ZnSODs are essentially eukaryotic enzymes, while FeSODs are essentially prokaryotic enzymes. MnSODs are present in both prokaryotes and eukaryotes. This is generalization and with many exceptions. For instance, Cu/ZnSODs have been isolated from various bacterial species as mentioned above, as well, FeSODs have been isolated from several plant species such as *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* (Camp *et al.*, 1990). FeSODs have also been found in various parasitic protozoans such as *Entamoeba histolytica*, (Tannich *et al.*, 1991) *Trypanosoma cruzi* (Ismail *et al.*, 1997) and *Leishmania chagasi* (Ismail *et al.*, 1996). However, the majority of organisms seem to fit this pattern.

The subcellular distribution of SODs has been reviewed by Fridovich (1995). Cu/ZnSODs have been shown to be both cytosolic and extracellular in eukaryotes. All extracellular Cu/ZnSODs contain a hydrophobic signal peptide at their amino terminal end which aids in the secretion of the proteins. The Cu/ZnSOD isolated from *E. coli* shows a unique localization, namely in the periplasmic space (Benov and Fridovich, 1994). The significance of this localization is not clear, although it has been postulated that periplasmic Cu/ZnSODs may protect *E. coli* against extracellular sources of the superoxide

radical. In higher eukaryotes, MnSODs are found exclusively in the mitochondrial matrix, whereas FeSODs are found inside the chloroplasts of plants. The subcellular distribution of FeSODs has not yet been investigated in parasitic protozoa.

B.SOD structure

The structure of SODs has been studied using various biochemical techniques such as X-ray crystallography. Structural studies have shown that iron and manganese enzymes have very similar primary, secondary and tertiary structures, but have no resemblance to the copper/zinc enzyme (Stallings *et al.*, 1984). Cu/ZnSOD is thus considered to be an evolutionarily distinct isoform.

Mn and FeSODs have an individual subunit molecular weight between 20-24 kDa and exist as either dimers or tetramers in their active state depending on the species or conditions studied. Although the metal content of Mn and FeSODs varies between 1 and 2 atoms, in general there is one metal ion per individual subunit. Figure 1.3 shows a 3-D structure of an FeSOD from *Mycobacterium tuberculosis* in its quaternary form. As can be seen, each monomer is comprised mainly of α-helices, interspersed with β-sheets, which is very consistent with the 3-D structure of MnSODs. The metal in the active site of both Mn and FeSODs are bound to three histidine and one aspartate residue (green circle in Fig. 1.3), surrounded by hydrophobic residues, including three Tyr, three Trp, and two Phe all within 10 angstroms of the metal center (Parker

Figure 1.3: 3-D structure of an FeSOD from *Mycobacterium tuberculosis* in its quaternary form. Each monomer (represented by a different color) is comprised mainly of α-helices, interspersed with β-sheets. The metal in the active site appears as a green circle.

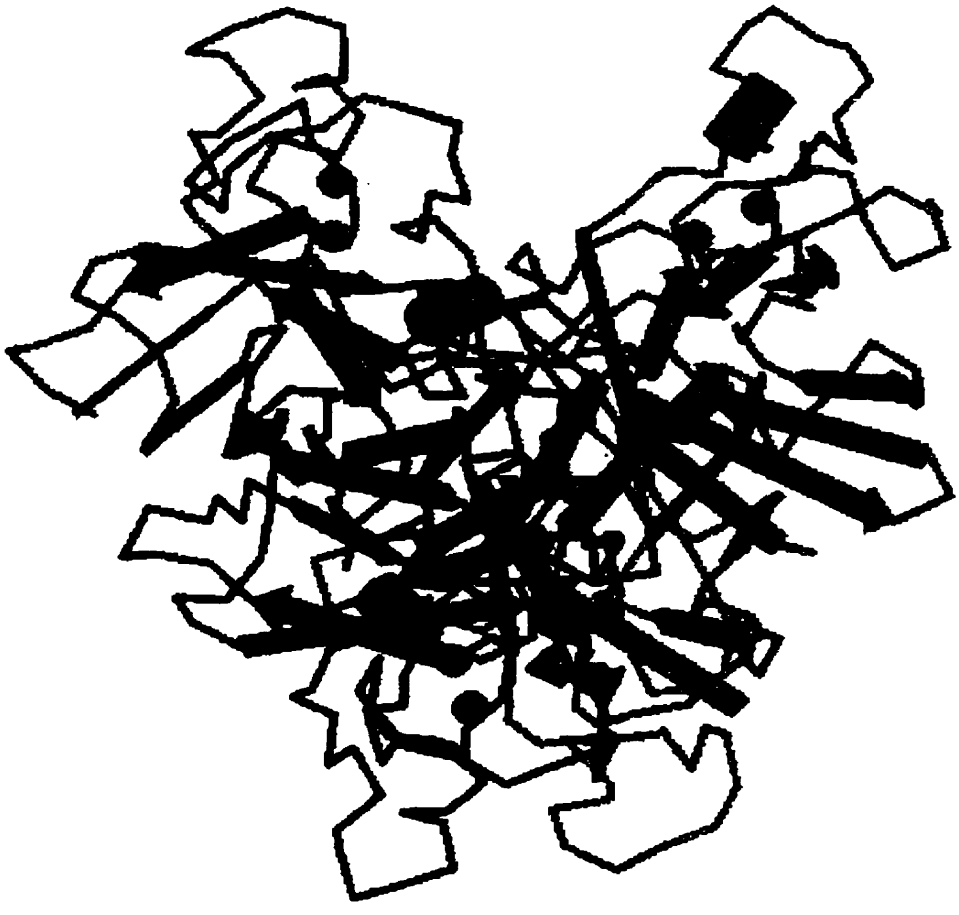
and Blake, 1988). Five major invariant residues have been identified which distinguish MnSODs from FeSODs (Parker and Blake,1988; DeShazer *et al.,* 1994). According to *E. coli* FeSOD, these residues are: Ala-76,Gln-77, Tyr-84, Ala-154, and Gly-155. The invariant residues which replace these residues in *E. coli* MnSOD are: Gly-76, Gly-77, Phe-84, Gln-154, and Asp-155.

Cu/ZnSODs are present in their active state as either dimers or tetramers with a monomer subunit weight between 15-18 kDa. Each monomer contains one Cu ion, which is specific to the enzyme and one Zn ion which is responsible for maintainance of enzyme structure and can be interchanged with a variety of other divalent metal ions without loss of enzyme activity. As seen in Fig. 1.4, the 3-D structure of bovine Cu/ZnSOD shows the quaternary structure which is characterized by eight anti-parallel β-sheets, giving the enzyme an overall barrel-like configuration (Tainer *et al.,* 1982). The Cu ion in the active site of this enzyme is bound by four histidine residues, whereas the Zn ion is bound by three histidine residues (one which is shared with the Cu ion) and one aspartate residue (Bannister *et al.,* 1987).

C. Enzyme function

SOD functions to dismutate O_2^- into H_2O_2 and O_2. Superoxide anion radicals are generated as a by-product in cells through a number of reactions which occur in aerobic organisms. O_2^- is generated through biological oxidations (eg. autooxidation of flavins and hydroquinones), mitochondrial respiration, NADPH-oxidase activation during the respiratory burst of macrophages and

Figure 1.4: The 3-D structure of bovine Cu/ZnSOD shows the quaternary structure of this enzyme. It is characterized by eight anti-parallel β-sheets, giving the enzyme an overall barrel-like configuration. The Cu ion in the active site of this enzyme is represented by a green circle, whereas the Zn ion is represented by a grey circle. There is one Cu ion and one Zn ion present in each monomer.



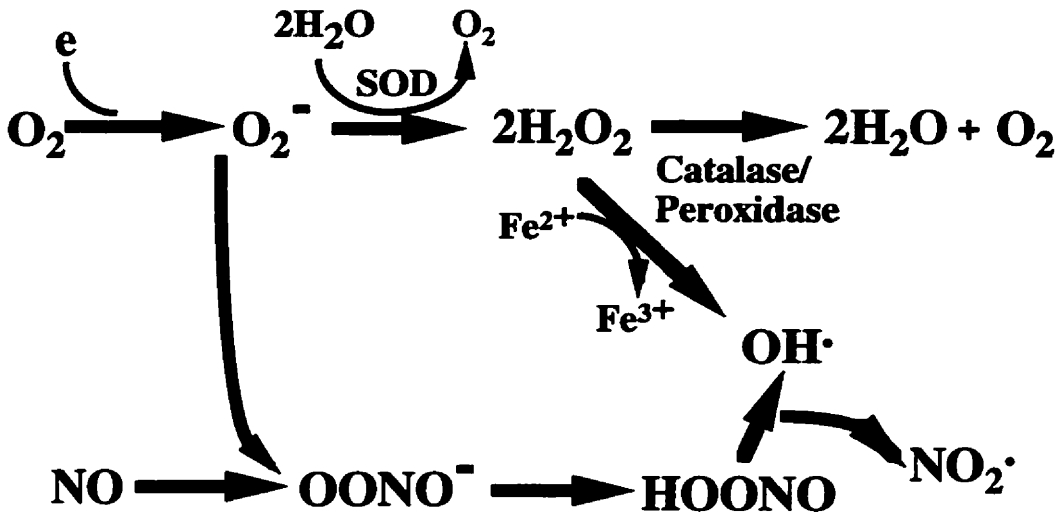
neutrophils during phagocytosis and through exposure to radiation or redox-cycling compounds such as paraquat (Bannister *et al.,* 1987). Given the continuous production of O_2^- in cells, it must find targets it can reduce or oxidize as it is a very unstable by-product. Without the elimination of the O_2^-, cells may be faced with the formation of many other potent reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) which are formed through the reduction of O_2^- (Fig. 1.5A). Most notably are the peroxynitrite anion and the hydroxy radical which are very toxic to all cells.

One main target is the family of dehydratases that contain [4Fe-4S] clusters at their active sites which include aconitase, β-phosphogluconate dehydrase, and Fumarase A and B. Following oxidation, these enzymes become inactive with the release of Fe^{2+} from the active site cluster. Fe^{2+} is then free to interact with H_2O_2 via the Fenton reaction (Fig. 1.5B) producing Fe^{3+} and toxic $OH^{\cdot}$. As well, O_2^- can react with H_2O_2 via the Haber-Weiss reaction (Fig.1.5C) which ultimately produces $OH^{\cdot}$. $OH^{\cdot}$ is a strong oxidant which is capable of damaging any cellular target, such as DNA. It is obvious that the Fenton and Haber-Weiss reactions are two reactions which need to be avoided for cell survival.

In vivo, O_2^- can also interact with NO, without metals, to produce the peroxynitrite anion (Huie and Padmaja, 1993). It has been shown to be responsible for the damage of many cellular components due to its ability to nitrate phenolic rings (Ischiropoulos *et al.,* 1992), hydroxylate aromatic rings

Figure 1.5: Reactions involved in the production of ROIs and RNIs. (A) Schematic showing the general overall reactions which ultimately produce toxic end products (ROIs and RNIs). **(B)** Fenton reaction showing how free Fe^{2+} react with H_2O_2 to produce the toxic end product $OH^{\cdot}$. **(C)** Haber-Weiss reaction showing the reaction between $O_2^{\cdot}$ and H_2O_2 to ultimately produce $OH^{\cdot}$.

A



B

Fenton Reaction

$$H_2O_2 + Fe^{2+} \text{-----> } HO^- + HO\cdot + Fe^{3+}$$

C

Haber-Weiss Reaction

$$O_2^- + Fe^{3+} \text{-----> } O_2 + Fe^{2+}$$

$$\underline{H_2O_2 + Fe^{2+}} \text{ } \underline{\text{-----> } HO^-} \text{ } \underline{+ HO\cdot + Fe^{3+}}$$

$$O_2^- + H_2O_2 \text{ -----> } HO^- + HO\cdot + O_2$$

(Hogg *et al.*, 1992), and oxidize lipids, proteins and DNA (Rubbo *et al.*, 1995).

D. Cellular Regulation of SOD

1. Mutant Phenotypes

The *in vivo* function of SOD has been characterized using modern genetic engineering techniques, such as gene replacements. Carlioz and Touati (1986) first reported the mutant *E. coli* strain which was unable to produce either MnSOD (*sodA*) or FeSOD (*sodB*). This mutant was unable to grow aerobically on a minimal glucose medium. Growth however, could be restored by removing O_2 and thus producing an anaerobic environment, or by restoring SOD production to the cells through the introduction of recombinant DNA containing a gene coding for SOD. This mutant *E. coli* strain also showed hypersensitivity to paraquat and enhanced mutation rates.

Mutant strains of eukaryotic organisms such as *Drosophilia*, which are homozygotes that lack the Cu/ZnSOD, are viable as larvae, although the larvae are extremely sensitive to paraquat treatment (Phillips, *et al.*, 1989). Adult flies resulting from these mutant larvae show a marked sensitivity to oxygen, living only an average of 12 days out of their normal 60 day life span.

The yeast *Saccharomyces cerevisiae* has also been mutated to produce a strain devoid of Cu/ZnSOD (Bilinski *et al.*, 1985). This mutant strain is unable to grow in the presence of air, unless the media is supplemented with the amino acids lysine and cysteine, indicating that SOD enzymes are participants in the biosynthesis of these amino acids. All growth is inhibited in the presence of

100% oxygen, even with lysine and cysteine supplements. The mutant phenotypes of SOD-deficient yeast can be reversed by the addition of SOD genes from various organisms.

2. Induction

It has been shown that the rate of SOD synthesis increases at high oxygen tension in many organisms, including bacteria, yeast, plants and mammals (Beyer *et al.,* 1991). The most common example of this type of induction is seen in the bacterium *E. coli* which is convenient to study because it is easy to expose to various growth conditions. The initial observation was that *E. coli* MnSOD levels appeared responsive to oxygen tension whereas, FeSOD levels did not (Gregory and Fridovich, 1973). Later, it was noted that the MnSOD levels were undetectable in extracts from anaerobically grown cells, but were induced in the presence of hyperbaric oxygen and drugs which stimulate O_2^- production (Hassan and Fridovich, 1977). In contrast, FeSOD levels were unresponsive to these environmental stimuli. Due to the inhibitory effect metal chelators had on MnSOD transcription, it was suspected that MnSOD levels must be controlled at the gene level through an iron-binding, redox-sensitive repressor molecule. The cloning of the MnSOD gene pomoter in E. coli (*sodA*) provided the identification of a consensus binding sequence for an iron-containing protein known to be a repressor of genes involved in iron uptake (Fur protein) (Hosni and Hui-Chan, 1992). This site was absent in the *E. coli* FeSOD (*sodB*) promoter region. Since the finding of the fur element, three other

transcriptional control elements have been identified in *sodA*. These include the aerobic respiration control gene products (Arc A, Arc B), the fumarate-nitrate reduction gene product (Fnr), and the proteins encoded by soxRS (superoxide response) and soxQ regulons. From the above studies, sodA production is dependent on intracellular iron concentrations and the redox state of the cell. The iron ions associated with the Fur and Fnr proteins act as sensors that respond to changes in the redox state of the cell. Therefore, in the absence of oxygen, both Fur and Fnr contain iron ions repress the expression of *sodA*. These studies show that, given the potency of its substrate (O_2^-), the tight regulation of SOD expression is one mechanism to allow the elimination of O_2^-.

E. Involvement of Oxidative Stress in Parasite Killing

The cells of the immune system, macrophages and monocytes, act in a variety of ways to up regulate immunity. These cells function by processing and presenting antigens to T cells, as well as cytokine dependent and independent killing of extracellular and intracellular invaders. The phagocytosis of microorganisms activates macrophage cells to use both oxygen-dependent and independent mechanisms to eliminate ingested microorganisms such as many bacteria and intracellular parasites such as *Leishmania* (Babior, 1984). Radicals produced by oxygen-dependent mechanisms (See Fig. 1.5 for examples) must be quickly oxidized or reduced by the appropriate enzymes of the invading organism for their survival. Failure to eliminate oxygen-derived radicals can lead

to the generation of highly toxic radicals which ultimately function to eliminate the invading microorganisms. Some examples of these toxic oxygen-derived molecules are the superoxide anion, hydrogen peroxide and the hydroxyl radical to name only a few. These radicals make up the group of reactants called reactive oxygen intermediates (ROIs).

Reactive nitrogen intermediates (RNIs) such as nitric oxide (NO) are another group of molecules produced by cytokine-activated macrophages. NO arises from the oxidation of the terminal guanidine nitrogen atom of L-arginine (Iyengar *et al.,* 1987) by a NADPH-independent enzyme called nitric oxide synthase (NOS). NOS levels can be induced through the presence of IFN-γ alone, in synergy with lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF-α) to produce high levels of NO (Nathan and Hibbs, 1991). Further, Wei *et al.,* (1995) showed that intramacrophage killing of *Leishmania* was induced by TNF-α and IFN-γ which was dependent upon the production of NO by NOS. Through the elimination of the mouse *nos* gene by knock-out techniques, it was demonstrated that without NOS, NO is not present and therefore cannot control the overproduction of IFN-γ and ultimately the overexpansion of the TH1 cells (Wei *et al.*, 1995). Without this control, the mice became susceptible to *L. major* infections. This shows that the presence of RNI species are important for macrophage cells to eliminate *Leishmania* infections. Therefore, if IFN-γ and TNF-α are induced through NO, then susceptibility of *Leishmania* elimination by macrophage cells may be influenced by the presence of RNIs. This mediation of

NO has only been shown in murine macrophage cells and has yet to be proven in human macrophage cells, so the exact mechanism of how NO affects the human host of *Leishmania* parasites is still not clear.

Recently, it has been reported that both TNF-α and IFN-γ induce the expression of SODs in various cell types, including macrophages (Harris *et al.*, 1991; Brune *et al.*, 1997). Taken together, the above studies indicate the importance of free radical species, and specifically O_2^- in the hosts defense against invading microorganisms. It would thus be beneficial that enzymes which neutralize the effects of the macrophage-produced O_2^- be expressed within the pathogen. These studies suggest a possible role for SODs as a virulence factor in the protection of the intracellular pathogen *Leishmania*.

Although the pathways which lead to the generation of ROIs and RNIs are independent within macrophage cells, a very toxic reaction occurs as a result of molecules being formed from both of these pathways. O_2^- has been shown to react three times faster with NO than with SOD, making NO the only molecule able to out compete endogenous SOD for its substrate. The superoxide anion and nitric oxide, can interact to form the peroxynitrite anion (ONOO-) which has proven to be a very toxic compound (Huie and Padmaja, 1993). The peroxynitrite anion has been shown to be harmful to many components of the cell, including proteins, lipids, sugars and DNA. Recent studies have revealed that *in vitro* generated peroxynitrite anions contribute to the elimination of many invading microorganisms such as *T. cruzi* (Denicola *et al.*, 1993) and *E. coli*

(Brunelli *et al.*, 1995). Assreuy *et al.* (1994) demonstrated that the killing of *L. major* by activated macrophages was primarily mediated by NO despite the simultaneous production of O_2^- and thus the generation of the peroxynitrite anion. These results are in contrast to the ones observed in *E. coli* and *T. cruzi*, but may be due to species-specific differences in sensitivity towards the radicals or differences in experimental conditions. Although the exact mechanism by which these various radicals induce microbial killing is not yet completely understood, these studies are a start to begin the elucidation of the pathways and mechanisms involved in the survival of intracellular microorganisms.

III. Objectives

Leishmania chagasi is an intracellular parasite which targets the macrophage cells of humans to cause visceral leishmaniasis. The parasite is able to survive and multiply within the very cells of the immune system which are supposed to protect the host against invading microorganisms. Therefore, it is crucial to understand the molecular mechanisms which enable this parasite's survival within the host's cells. This will allow a greater understanding of the pathogenicity and virulence of the *L. chagasi* parasites. The focus of this study was to characterize two iron-containing superoxide dismutase genes previously isolated from *L. chagasi* to try to understand their functional role in *Leishmania* survival. This was carried out by performing the following studies:

1. Characterization and sequencing of the FeSODB cDNA from *L. chagasi*.

2. Overexpression of L.c.FeSODA in *E. coli* to study the complementation potential of this protein to gain a better understanding of its functional role.

3. Overexpression of L.c.FeSODA and L.c.FeSODB cDNAs in *L. chagasi* parasites to study the effect of various free radical-producing agents on the growth of the parasites compared to wild-type growth.

4. Gene replacement studies using homologous recombination in *Leishmania* cells for knocking out the L.c.FeSODA and L.c.FeSODB genes in *L. chagasi* parasites.

CHAPTER 2

MATERIALS AND METHODS

I. Materials

A. Chemicals

Chemicals used to make buffers and solutions were from Fisher, BDH, Sigma, Gibco, BRL or ICN.

B. Enzymes and kits

All restriction enzymes, DNA or RNA modifying enzymes and kits used for DNA or RNA manipulations were purchased from Pharmacia unless otherwise mentioned.

C. Plasmids

The *E. coli* expression vector pGEX-2T was purchased from Pharmacia, the pGEM2 vector was obtained from Promega and the bluescript vector S/K- was obtained from Strategene. The *Leishmania* overexpression vector pX and the two knock-out vectors pX-63 Hyg and pX-63 Neo were generously provided by Dr. S. Beverley (Harvard Medical School, Boston, Mass.).

D. Culture media

Bacto agar, bacto tryptone and bacto yeast extact were obtained from Difco labs, while MEM, RPMI 1640, essential amino acids, non-essential amino acids and sodium pyruvate were all purchased from Gibco BRL.

E. Hybridization Materials

Hybond N^+, glass filter discs and Rapid hybridization buffer were obtained from Amersham.

F. Radioisotopes

[α-^{32}P] dCTP (3000 Ci/mmol) and [α-^{35}S] dATP (1000 Ci/mmol) were obtained from Amersham.

G. Autoradiographic and photographic materials

Kodak XAR-5 X-ray film, Dupont Intensification plus film and intensifying screens (DuPont Cronex) and cassettes were from Picker Canada Ltd.

II. Solutions and Buffers

The following is the list of working concentrations of solutions and buffers used in this study.

20% Acrylamide stock

96.5 g acrylamide, 3.35 g bis-acrylamide, 233.5 g urea, 100 ml 5X TBE in 500 ml

Annealing buffer

6.6 mM Tris-Cl pH 7.4, 6.6 mM $MgCl_2$, 0.05 M NaCl, 1 mM DTT

10X Agarose loading dye

50% glycerol, 0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)

Biotin

1 mg/ml in water at 60°C. Filter sterilize and store at -20°C

Calf intestinal alkaline phosphatase buffer

1 mM $ZnCl_2$, 1 mM $MgCl_2$, 10 mM Tris-Cl (pH 8.3)

Colony lysis buffer

10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 10% Triton X-100

Coomassie R-250 stain

2 g Coomassie R-250 dissolved in 500 ml methanol, 70 ml glacial acetic acid in 1 litre

Denaturation solution

1.5 M NaCl, 0.5 M NaOH

Depurination solution

0.25 M HCl

DNA elution buffer

0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA

Express destain solution

40% methanol, 10% glacial acetic acid in 1 litre

Formamide dye

0.5% bromophenol blue, 0.5% xylene cyanol FF, 1 mM EDTA in 95% formamide

Hemin

2 mg/ml in 1N NaOH. Filter sterilize and store at -20°C

HO-MEM medium for *Leishmania chagasi* culture

1 Litre incomplete HO-MEM prepared as follows, filter sterilized and stored at 4°C

Minimal essential medium 10 X with Hanks salts and L-glutamine 100 ml

MEM amino acids 50 X	10 ml
MEM non essential amino acids 100 X	10 ml
Sodium pyruvate 100 X (100 mM)	11 ml
Glucose (30%)	5 ml
Sodium bicarbonate (7.5%)	29.3 ml
Biotin (1 mg/ml)	0.1 ml
PABA (2 mg/ml)	0.5 ml
1M HEPES (sodium salt) pH 7.4	25 ml

Complete HO-MEM medium (100 ml) for viable parasites consists of 90 ml incomplete HO-MEM, 10 ml FCS, 2 ml Pen/Strep, 0.27 ml Hemin.

Isopropyl-β-D-thiogalactoside (IPTG)

Made up as a 0.1 M stock in ddH_2O and stored at -20°C

LB broth

10 g bacto tryptone, 10 g NaCl, 5 g yeast extract (pH 7.0)

1 X MOPS buffer

20 mM MOPS, 5 mM sodium acetate (pH 7.0), 1 mM EDTA

MTPBS

16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl

Neutralisation solution

1.5 mM NaCl, 0.5 mM Tris-Cl pH 7.2, 0.001 M EDTA

One-Phor-All buffer

10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate

PABA

2 mg/ml in water. Filter sterilize and store at -20°C

PBS

8.01 g NaCl/l, 101 mg KCl/l, 463 mg Na_2HPO_4/l, and 102 mg KH_2PO_4/l, pH to 7.2

PCI

phenol :chloroform :isoamyl alcohol (25:24:1)

PCR buffer

10 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$

PDC

0.625 gm/l Azure A, 0.625 gm/l methylene Blue

5 X running buffer for SDS-PAGE

2.5 mM Tris base, 192 mM glycine, 1% SDS pH 8.3

5 X SDS-gel loading buffer

250 mM Tris-HCl (pH 6.8), 500 mM DTT, 10% SDS, 0.5% bromophenol blue, 50% glycerol

20 X SSC

2 M NaCl, 300 mM Sodium citrate (pH 7.0)

STET

10 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5% Triton X-100

Solution D

4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl 0.1 M β-mercaptoethanol

T4 ligase buffer

66 mM Tris-Cl (pH 7.6), 6.6 mM $MgCl_2$, 66 mM ATP, 1 mM DTT, 50 μg/ml BSA

TAE

40 mM Tris-acetate, 1 mM EDTA

TBE

89 mM Tris-borate (pH 8.3), 89 mM boric acid, 2 mM EDTA

TE

10 mM Tris-Cl (pH 8.0), 1 mM EDTA

Urea mix

350.25 g urea, 150 ml 5 X TBE in 750 ml

III. Plasmid DNA isolation

A. Mini scale plasmid DNA Isolation

Mini scale plasmid DNA were isolated using the boiling prep method (Holmes and Quigley, 1981) with the following modifications. With a sterile loop, a single bacterial colony was inoculated in 5 ml of LB broth with the appropriate antibiotic. Cultures were grown to saturation with shaking at 37°C overnight . This overnight culture was then centrifuged at 6000 rpm for 10 min in an eppendorf microcentrifuge, the supernatant was decanted and the pellet was resuspended in 600 µl of STET buffer with vortexing. This cell suspension was transferred to a 1.5 ml microfuge tube and 60 µl of fresh lysozyme solution added. Contents were mixed by inverting the tubes and then were placed in a boiling water bath for 55 seconds. Tubes were centrifuged immediately in a microcentrifuge at 14,000 rpm for 15 min at room temperature. The gelatinous pellet was then removed with a sterile toothpick, 600 µl of PCI added and mixed vigorously. The tubes were then spun at 14,000 rpm for 10 min at room temperature and the aqueous phase was collected and mixed with equal volumes of cold isopropanol and kept at -20°C overnight for precipitation. The samples were centrifuged at 14,000 rpm for 10 min at 4°C, supernatant decanted and pellets air dried. Elimination of contaminating RNA was carried out by resuspending the pellets in 200 µl of sterile water, addition of RNase A to a final concentration of 10 µg/ml and incubating at 37°C for 2 hours. RNase A was

removed by a second PCI extraction and precipitating the aqueous phase with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. After 2 hours at -20°C, the plasmid DNA was spun down at 14,000 rpm for 15 min at 4°C and washed with 1 ml of 70% ethanol. DNA pellets were vacuum dried, resuspended in 50 µl of sterile water and quantitated using a spectrophotometer.

B. Large scale plasmid DNA isolation

Bulk plasmid DNA was isolated using the Qiagen plasmid maxi kit (Qiagen Inc. Chatsworth, CA.) according to the instruction supplied by the manufacturer and quantitated by Absorbance at 260 nm (Pye Unicam Pu 8600 UV/VIS spectrophotometer).

C. Oligonucleotide synthesis

Oligonucleotides required for DNA sequencing and PCR reactions were synthesized on a Pharmacia LKB Gene Assembler Plus DNA synthesizer, using a 0.2 µmole primer support. The primers were processed by placing the primer supports in 1 ml of 100% ammonia solution and incubating them at 55°C overnight to deprotect the primers. The ammonia solution was then dried to completion in a Savant speed vacuum, the pellet resuspended in 400 µl of ddH_2O, PCI extracted and the aqueous phase precipitated by adding 1/10 vol. 3 M NaAc and 2 volumes of 100% ethanol at -20°C overnight. The resulting pellet was washed with 70% ethanol and resuspended in an appropriate volume of

ddH_2O. The concentration was determined spectrophotometrically. As well, in later studies, oligonucleotides were synthesized through the University Core DNA Services Facility at the University of Calgary.

Oligonucleotides designed for sequencing, PCR, subcloning and gene replacement studies for L.c.FeSODA are listed in Table 2.1 and for L.c.FeSODB are listed in Table 2.2.

IV. Separation and isolation of DNA fragments

A. Agarose gel electrophoresis

Separation of DNA fragments was carried out on 0.7%-1.2% agarose gels. Agarose gels were prepared by dissolving the appropriate amount of ultra pure agarose (Gibco, BRL) in 1 X TAE buffer by boiling. Ethidium bromide was added to the dissolved agarose at a concentration of 0.5 mg/ml. DNA samples in a 1/10 vol. of DNA loading buffer were electrophoresed in 1 X TAE buffer at 7-10 volts/cm until DNA separation was attained. Photographs of the resolved DNA was obtained using the UVP Image Store 7500 system.

B. Separation and purification of DNA using low melting point agarose gels

PCR fragments or restriction products ranging from 200 bp to 7000 bp were purified after separation in low melting point agarose (Gibco, BRL). 1.0% gels, containing 1 X TAE were electrophoresed as in section IVA. The band was visualized with UV light and excised from the gels. The gel pieces were collected

in microfuge tubes and 1/10 vol. of 3 M NaAc was added. The agarose was melted by placing the tubes in a 68°C water bath for 10 minutes. Immediately upon removal of the tubes from the water bath, 1 vol. of Tris-HCl (pH 8.0) saturated ultra pure phenol (Gibco, BRL) was added to the tubes, mixed thoroughly and placed on ice for 10 minutes. Phases were separated by microcentrifugation at 14,000 rpm at room temperature for 10 minutes. 2 vol. of 100% ethanol was added to the aqueous phase and the samples were quick frozen in liquid nitrogen. The DNA was pelleted by spinning at 14,000 rpm at 4°C for 30 minutes, the pellet washed with 70% ethanol, dried, resuspended in the appropriate volume of ddH_2O and quantitated by Absorbance at 260 nm.

V. Digestion, dephosphorylation and deproteination of DNA

A. Digestion

Most plasmid digestions (10-40 μg) were routinely carried out in a final volume of 100 μl volume, containing 2-4 times units excess of restriction enzyme (Pharmacia) and the One Phor All restriction buffer (10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate pH 7.5) concentration as specified (Pharmacia). Digests were carried out at the specified temperature (usually 37°C) for 4-5 hours or overnight. Genomic DNA (40-60 μg) was digested with 4-5 times units excess restriction enzyme in a final volume of 150 μl at the specified temperatures overnight.

B. Dephosphorylation

To remove the 5' phosphates from the termini of restricted DNA products, 5 units of calf intestinal phophatase (Gibco, BRL) was added directly to the restriction digest reactions, with 1 X phosphatase buffer added and incubated at 37°C for 2-3 hours.

C. Deproteination

Proteins were removed from the restriction digests and dephosphorylation reactions was performed by adding equal volumes of PCI to the reaction tubes, mixing thoroughly and spinning at 14,000 rpm at room temperature for 10 minutes. The aqueous phase was then removed and the DNA fragments were ethanol precipitated from the aqueous phase.

VI. Cloning of DNA fragments

A. Ligation

All vectors and fragments used for ligation were LMP purified previous to the ligation reaction. The ligation reaction contained 150 ng of dephosphorylated vector, 4 fold molar excess of insert DNA, 1 X ligation buffer and 8-10 units of T4 DNA ligase (Pharmacia) in a total volume of 20 μl. Ligation reactions were incubated in a 12°C water bath for at least 16 hours.

B. Transformation

The *E. coli* host strain DH5α was used unless otherwise stated. The recipient host strain was made competent using the $CaCl_2$ method (Mandel and Higa, 1970) where a 5 ml overnight saturated culture was diluted 1/100 in 30 ml of LB broth and grown in a shaking incubator at 37°C until a density of 5 X 10^7 cells/ml was attained (~2hrs). The cells were collected by centrifiguation in a Sorval SS 34 rotor at 6,000 rpm for 10 minutes at 4°C. The cells were then resuspended in 10 ml of ice cold 50 mM $CaCl_2$ and incubated on ice for 30 minutes. At this time, the cells were re-pelleted in the above manner and the pellet was resuspended in 1 ml ice cold 50 mM $CaCl_2$ and incubated on ice for 1 hour. The competent cells were then used immediatedly or stored in the cold room (4°C) for up to 24 hours. Competent cells (100 μl) were added directly to the ligation reaction and mixed gently. This mixture was incubated on ice for 10 minutes, followed by a heat shock in a 45°C water bath for 2 minutes and finally, incubation at RT for 10 minutes to recover from the shock. 1 ml of LB broth was then added to the transformation mixture and incubated at 37°C for 45-60 minutes to allow the bacteria to express the drug resistance gene product of the transforming plasmid. After this time, the bacterial cells were gently pelleted through a quick spin and the supernatant decanted leaving ~ 200 μl of volume. The pellet was resuspended in the remaining media and spread on a pre-warmed LB agar plate containing the appropriate antibiotic. Plates were

incubated at 37°C for 16-18 hours to select for antibiotic resistant colonies and the resulting colonies were patched using a sterile loop onto a fresh pre-warmed LB plate containing the appropriate antibiotic. Screening of the colonies included colony lysis PCR (see Section VIII), mini plasmid isolation followed by restriction digests, as well as sequencing of the positive clones.

VII. Polymerase Chain Reaction

Polymerase chain reactions (PCR) were carried out in 100 µl volumes in a Techne PHC-2 thermal cycler. PCR reactions consisted of 1-2 ng of plasmid template DNA, 100 pmol of each sense and anti-sense primer, 1 X *Taq* DNA polymerase buffer (50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-Cl pH 9.0) (Pharmacia), 200 µM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP) and 1.25-2.5 units of *Taq* DNA polymerase (Pharmacia). PCR reactions were overlayed with 90 µl of sterile mineral oil and the reaction cycled according to appropriate cycling parameters. Generally, a 5 minute cycle of denaturation at 94°C was started followed by 30 cycles consisting of 3 steps: 1 minute denaturation at 94°C, 1 minute annealing at the annealing temperature, and 1 minute extension at 72°C. The reaction was completed with a 10 minute extension cycle. The annealing temperature of the PCR primers used were calculated at 12°C below the dissociation temperature (Td). The Td was calculated as follows: Td= 2(A+T) + 4(G+C).

VIII. Colony Lysis PCR

Bacterial colony lysis PCR was performed on patched colonies resulting from transformation to determine positive colonies and orientation of the integrated insert. Using a sterile toothpick, a small portion of the patched colony was removed and placed in 50 µl of colony lysis PCR buffer in a microfuge tube. The tubes were then boiled rapidly for 15 minutes, chilled on ice for 5 minutes and quick spun. A 100 µl PCR reaction was set up exactly as in section VII, except that a 10 µl aliquot of the boiled colony was used as the DNA template. A 15 µl aliquot of the PCR was analysed on a 1% agarose gel.

IX. Preparation of radiolabeled [α-^{32}P] DNA probes

A. Random prime radiolabeling of DNA using hexanucleotide primers

LMP purified DNA fragments (50 µg) of varying lengths were radiolabeled with 50 µCi [α-^{32}P] dCTP using the T7 Quick prime kit (Pharmacia) according to the instructions provided by the manufacturer. The procedure utilized is based on the method designed by Feinberg and Vogelstein (1984). Random labeled probes were separated from free unincorporated nucleotides using Sephadex G-50 medium columns in a 1 ml syringe. Incorporated radioactivity was determined by TCA precipitation and scintillation counting.

X. Southern blotting

Genomic DNA digests were loaded onto a 0.7% agarose gel and electrophoresed at 15 V/cm. Upon completion of resolution, the gel was visulalized under long wave ultraviolet light and photographed. The gel was then processed as follows: immersed in depurination solution (double the volume of the gel in each case) for 10 minutes, washed gently with water; immersed in denaturation solution for 30 minutes, washed gently with water; and finally immersed in neutralization solution for 20 minutes twice. At this time the gel was transferred through capillary action (Southern, 1975) onto a Hybond N^+ membrane (Amersham) for 18 -24 hours in 10-20 X SSC. The DNA was then fixed onto the nylon membrane through exposure to long wave ultraviolet light for exactly 5 minutes. Membranes were stored at -20°C until used for hybridization.

XI. Northern blotting

RNA samples were analyzed on formaldehyde-containing agarose gels as described by Alwine *et al.*, (1977). Briefly, RNA samples were dried to completion in a Savant vacuum drier, and resuspended in 4.5 µl ddH_2O, 2 µl 10 X MOPS buffer, 3.5 µl of 37% formaldehyde (final concentration 2.2 M) and 10 µl deionised formamide. The samples were then denatured by placing them in a 68°C water bath for 10 minutes and cooling briefly on ice. RNA loading dye was added to each sample and loaded onto a 1.2% formaldehyde-agarose gel (0.66

M formaldehyde) and electrophoresed at 7-8 V/cm in 1 X MOPS buffer. The gel was then washed in ddH_2O twice for 15 minutes each and equilibrated in 10 X SSC for 30 minutes. RNA was then transferred onto Hybond N^+ nylon membrane through capillary action as described above (Section X).

XII. DNA sequencing

The dideoxy sequencing method (Sanger *et al.*, 1977) was followed for sequencing DNA in this study using the T7 Sequencing kit (Pharmacia) and [^{35}S]α-dAT P (1000 Ci/mmol). 2-3 μg plasmid DNA (prepared by the mini-prep boiling method or QIAGEN as explained in section III) was used as the DNA template per sequencing reaction. Sequencing reactions were resolved on 8% acrylamide/8 M urea gels (0.4 mm thick) in 1 X TBE at 70 watts constant using the BRL S2 sequencing gel apparatus. Following electrophoresis, the gels were fixed in 10% methanol/10% acetic acid solution for 15 minutes and dried under vacuum for 1 hour and then exposed to X-ray film.

Some of the sequencing at the end of this study was performed at the Univeresity Core DNA services facility, University of Calgary.

XIII. Coupled T7 *in vitro* transcription/translation

In vitro transcription/translation reactions were carried out using the TNT coupled reticulocyte system (Promega) in the presence of [^{35}S] Methionine (Amersham). Qiagen prepared circular plasmid DNA (1 μg) was used for each

reaction and the procedure was followed as set out by the manufacturer. Reactions were frozen at -80°C in 5 μl aliquots until used. Translation products were analyzed by running 2 μl aliquots on a 10% SDS-polyacrylamide gel (procedure oulined in Section XVI). Gels were coomassie stained, destained and vacuum dried before autoradiography was performed.

XIV. *Leishmania* cell culture and techniques

A. *Leishmania* culture

The *Leishmania chagasi* strain MHOM/BR/74/PP75 was kindly provided by Dr. Richard D. Pearson, University of Virginia. Parasites were maintained at 23°C in the absence of CO_2 in modified minimal essential medium (HO-MEM). These conditions are ideal for the parasites to maintain their promastigote stage. The parasites were monitored daily and subcultured in their stationary stage (slender non-motile parasites). Parasites were subcultured at a density of 1 X 10^6 parasites/ml unless otherwise stated.

For treatment of parasites with various reactive oxygen and reactive nitrogen producing agents (Refer to Table 2.3 for chemical formulas of reagents and products produced from each reagent) parasites were seeded at a density of 1 X 10^6 parasites/ml in a total of 2 mls in 24 well microtiter plates (Costar). Parasites were allowed to recover overnight and then various concentrations of the reagents were added to the wells. After 6 days the viable parasites were counted using a hemocytometer and the growth curves determined. All

experiments were performed 3 times or more and each solution was prepared fresh on the day of treatment.

B. Parasite staining

Leishmania cells, at various stages, were stained using the Diff-Quick stain kit (Baxter). Parasites, at a concentration of 2 X 10^5 were gently pelleted in microfuge tubes, washed in 1 ml 1 X PBS and dissolved in 100 µl 1 X PBS. A 10 µl aliquot of cells was then spotted onto a microscope slide and allowed to air dry. Slides were then sequentially immersed 5 times each in fixative solution (1.8 mg/l Triarylmethane dye, 100% PDC in methyl alcohol), solution I (1 gm/l Xanthene dye mixture, 100% PDC and 0.1% sodium azide) and solution II (1.25 gm/l Thiazine dye mixture, 100% PDC and buffer) for one minute, rinsed in deionized water and allowed to air dry. The cell smear was mounted using permount and observed under oil immersion (100 X magnification).

C. Cryopreservation of parasites for long term storage

Aliquots of parasite cultures were frequently preserved in 10% DMSO in liquid nitrogen tanks for long term storage. Briefly, parasites from a 10 ml log phase culture (2-3 X 10^7 parasites) were pelleted at 3,000 rpm for 10 minutes at room temperature, washed twice with 1X PBS and resuspended in 5 ml complete HO-MEM, containing 10% DMSO. 1 ml aliquots were distributed into 1.5 ml cryovials and incubated on ice for 30 minutes. The vials were then incubated at -20°C for 3-4 hours, -80°C overnight and then transferred to liquid nitrogen for final preservation. To re-start a frozen culture, the cryovials and 10 ml of

complete HO-MEM were equilibrated in a 37°C water bath, and the thawed cells were carefully pipetted into the complete medium, drop by drop and then maintained as previously described (Section XIVA).

D. Pulsed Field gel electrophoresis

Chromosomal DNA from stationary phase promastigote cultures were isolated from 2 X 10^8 cells. Parasites were centrifuged at 6000 rpm for 10 minutes, cell pellet washed twice with PBS, resuspended in 500 ml of 10 mM Tris-Cl (pH 7.2), 20 mM NaCl, 100 mM EDTA and kept in a 70°C water bath. Concurrently, a 1.6% agarose solution in water was prepared and incubated at 70°C along with the cell pellet for 10 minutes. The *Leishmania* cell pellet was gently mixed with an equal volume of agarose solution and drawn into a 1 ml tuberculin syringe. The solution was allowed to harden at room temperature for 20 minutes. The needle adapter was removed, the solidified agarose rod pushed out gently and sliced into 1 mm thick pieces. These agarose slices were incubated at 70°C for 2 hours in 3 ml lysis solution (10 mM Tris-Cl, pH 7.2; 50 mM NaCl, 100 mM EDTA, 0.2% SDS, 0.5% sarkosyl) with gentle shaking. Following lysis, the slices were washed twice for 15 minutes at room temperature with washing solution (20 mM Tris-Cl, pH 8.0; 50 mM EDTA). Washing solution was replaced with 3 ml of proteinase K solution (1.0 mg/ml proteinase K, 100 mM EDTA, 0.2% SDS, 1% sarkosyl) and slices incubated at 42°C for 18 hours. After removing the proteinase K solution, slices were rinsed once with washing solution and incubated at room temperature for 2 hours in a

fresh washing solution containing 1mM PMSF. Slices were washed twice in washing solution and stored at 4°C in storage buffer (10-fold diluted washing solution).

High molecular weight DNA was separated using Tyler MB10 or a CHEF MAPPER electrophoresis system (BioRad). Pulse times of 150-120 seconds were routinely used. The total running time was usually between 36 to 48 hours. Electrophoresis was performed in 0.7% agarose gels containing 1.5 mg/ml ethidium bromide and 0.25 X TBE buffer. Southern transfer of pulsed field gels was carried out as for normal agarose gels.

E. Genomic DNA isolation

Stationary phase parasites (day 10-11) in a 100 ml culture were pelleted by centrifugation at 3,000 rpm for 10 minutes at room temperature. Cells were then washed twice with 1 X PBS and resuspended in 1 ml lysis buffer (10 mM Tris-Cl, pH 8.3; 50 mM EDTA; 1% sodium dodecyl sulfate (SDS)). Cells were resuspended by gentle tapping or pipetting to avoid shearing of high molecular weight DNA. The lysed parasites were then treated with 50 mg/ml of RNase A (Boehringer, Mannheim, Germany) and incubated at 37°C for 4 hours. Proteinase K (Boehringer, Mannheim) was then added at a final concentration of 100 mg/ml and incubation was continued overnight at 42°C. The DNA was then sequentially extracted with equal volumes of Tris-HCl (pH 8.0) saturated phenol, PCI and finally chloroform-isoamyl alcohol. The resulting aqueous phase was ethanol precipitated by addition of 1/10 vol. 3 M NaAc and 2 vol. absolute

ethanol. Following addition of ethanol, the tubes were gently inverted and the high molecular weight DNA precipitate was spooled out using a looped end pasteur pipette. The DNA was then washed with 70% ethanol and resuspended in an appropriate volume of ddH_2O and allowed to dissolve at 4°C overnight, at which point the concentration was analyzed spectrophotometrically. Genomic DNA was stored at -20°C for long term storage.

F. Total RNA isolation

Total RNA was isolated from *L. chagasi* cells using the guanidinium thiocyanate method as described by Chomczynski and Sacchi (1987) with minor changes. Briefly, a 100 ml culture was pelleted on day 5 for log phase parasites or on day 10-11 for stationary phase promastigotes by centrifugation at 3,000 rpm for 10 minutes at room temperature. The cells were then washed twice with 1 X PBS solution and dried to completion. The pellet was then resuspended in 600 µl of Solution D (See **Solutions and Buffers** section) by vigourous vortexing until all of the pellet was in suspension. This was followed by sequential addition of 60 µl 2 M NaAc (pH 4.0), 600 µl of water saturated phenol and 120 µl chloroform:isoamyl alcohol (49:1) with thorough mixing between each addition. Samples were then incubated on ice for 10 minutes followed by a 10 minute centrifuge at 14,000 rpm at room temperature. The aqueous phase was recovered and equal volumes of cold isopropanol was added, mixed well and incubated at -20°C overnight. The tubes were then spun at 14,000 rpm for 30

minutes at 4°C and allowed to air dry. The pellet was resuspended in a further 400 µl Solution D (the pellet was dissolved through vigorous vortexing and heating at 68°C for 5 minutes if necessary) and equal volumes of cold isopropanol was again added. The tubes were incubated at -20°C for 2-3 hours and then centrifuged at 14,000 rpm for 30 minutes at 4°C. The pellet was washed with 70% ethanol and then vacuum dried. The pellet was resuspended in 300 µl ddH_2O (in the manner explained above) and 20 units of Dnase I (Pharmacia) was added and incubated at 37°C for 3-4 hours. The mixture was PCI extracted and the aqueous phase ethanol precipitated. The resulting pure RNA was quantitated spectrophotometrically and analyzed on formaldehyde-agarose gels.

G. Whole cell extract preparation

100 ml *L. chagasi* cultures were allowed to grow to either log or stationary phase and then they were pelleted by centrifugation at 3,000 rpm for 10 minutes at room temperature. The pellet was washed twice with 1 X PBS, resuspended in 300 µl cold hypotonic buffer (5 mM Tris-HCl pH 7.8, 0.1 mM EDTA pH 8.0, 5 mM PMSF), incubated on ice for 20 minutes and lysed by sonication. Complete lysis was monitored by microscopy. The mixture was then centrifuged at 14,000 rpm for 30 minutes at room temperature and the clear supernatant was frozen in liquid nitrogen in 50 µl aliquots and stored at -80°C until used. Protein quantitation was performed with the Pierce BCA kit (Rockford, Illinois).

H. Stable transfection

The stable transfection protocol of electroporation used, described by Kapler *et al.,* (1990) used either plasmid DNA or linear DNA fragments. 4 X 10^7 late log *phase L. chagasi* parsites (day 8) were centrifuged at 3,000 rpm for 10 minutes at room temperature, washed with 1 X PBS and resuspended in 400 µl electroporation buffer (21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM Kcl, 0.7 mM sodium phosphate, 6 mM glucose). Resuspended cells were transferred to 0.2 cm BioRad gene pulser cuvettes, 30-40 µg DNA added and incubated on ice for 10 minutes. Electroporation took place in a BioRad Gene Pulser set at the following parameters: 0.45 kV (gives a 2.25 kV/cm voltage with the 0.2 cm electrode gap in the cuvette) and a capacitance of 25 mFd. Cuvettes were pulsed once and then incubated on ice for a further 10 minutes. Cells were then transferred to sterile culture flasks containing 10 ml of complete HO-MEM and incubated at 23°C for 48 hours at which point 10 µg/ml of either Geneticin (Gibco,BRL) or Hygromycin B (ICN) was added to select for resistant transfectants. Cultures were spun down and new media was added every seven days until cells appeared to have attained a normal growth pattern. The selectable antibiotic concentrations were then raised to the concentration desired.

I. Macrophage cell line (U937) infection with *Leishmania*

A human macrophage cell line-U937- was obtained from American Type Cell Collection and maintained in RPMI 1640 medium (Gibco,BRL)

supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine and 50 μg/ml Gentamicin (Gibco, BRL) at 37°C with 10% CO_2. Cells were subcultured every 3 days at a 1/10 dilution and grown as a suspension culture in untreated tissue culture flasks. For the infection procedure, U937 cells were expanded to a 100 ml culture, allowed to grow for 3 days and then split 1/2 in fresh media the day before infection. The day of infection, the cells were washed twice with 1 X PBS and seeded at 1 X 10^6 cells/ml in fresh media containing 7.5 ng/ml PMA in treated tissue culture flasks and allowed to grow for 48 hours. This allowed the cells to differentiate to macrophage cells and adhere to the bottom of the flasks in a monolayer.

When the macrophage cells were adhered, the cells were gently washed with incomplete RPMI media. A 100 ml flask of stationary phase (day 11) *Leishmania* parasites was collected through centrifugation, washed twice with 1 X PBS and resuspended at a concentration of 10-20 parasites/ macrophage cell in fresh complete RPMI media and added to the washed adherent cells. The parasites were allowed to infect for 24 hours, at which point the U937 cells were washed three times to remove free parasites and then re-incubated with fresh RPMI media. Macrophage cells were harvested after 3 days of infection by scraping the cells from the tissue culture flasks and centrifuging the suspension at 3,000 rpm for 10 minutes at 4°C. The infection was visualized using a cytospin procedure and stained by the Diff Quick stain kit.

XV. Purification of *E. coli* exprssed GST fusion protiens

The pGEX-2T vector, containing the glutathione S-transferase (GST) gene and a thrombin cleavage site was used for expression of recombinant GST fusion proteins. The *E.coli* host strain used for transformation was the QC779 null SOD strain, as well as the parental strain, GC4468. GST-fusion protein expression and purification was performed as described by Smith and Johnson (1988) with minor changes. Large scale cultures were prepared by inoculating recombinant pGEX-2T fusion vectors from a single colony in 100 ml of LB broth containing 100 μg/ml ampicillin and grown in a shaking 37°C incubator overnight. Cultures were then diluted 1/10 in a total of 1000 ml of LB broth and grown at room temperature until reaching an OD_{600} of 1. The cultures were then induced with 0.2 mM IPTG and grown for an additional 16-18 hours. The cells were then pelleted by centrifugation at 6000 rpm for 10 minutes and resuspended in 30 ml of 1 X MTPBS. Cell lysis was obtained through sonication of 5 ml aliquots of the cell suspension and addition of 1% Triton X-100 to the sonicate. The sonicate was then spun at 12,000 rpm in a Sorval SS 34 rotor for 10 minutes at 4°C. The clear, induced supernatant was then transferred to a clean tube and was ready for purification.

A 50% glutathione-agarose (Sigma) solution of beads was packed into a column. The supernatant was passed over the column three times, and washed three times with 1 X MTPBS buffer, followed by three washes with a 50 mM Tris-Cl (pH 8.0) solution. Fusion proteins were eluted from the glutathione beads by

adding aliquots of 50 mM Tris-Cl (pH 8.0) containing 10 mM reduced glutathione and collecting fractions of the run-through in clean microfuge tubes. Purity and amount of fusion protein obtained was monitored by running a 10 µl aliquot of the various fractions on 10% SDS-PAGE gels.

Pure protein was cleaved from GST by incubating a 50 µl aliquot of the fractions with 2.5 µg of thrombin enzyme at room temperature for 1 hour.

XVI. Denaturing polyacrylamide gels

SDS-acrylamide gels were set up using the Mini-Protean II electrophoretic cell (BioRad). Normally, protein samples were analyzed on a 10% resolving gel and a 5% stacking gel based on the method described by Laemmli (1970). Gels were electrophoresed at 150 V constant for a varying amount of time. Following electrophoresis, gels were generally stained for 30 minutes in Coomassie stain and then destained for 2 hours before being vacuum dried between two sheets of cellophane.

XVII. Superoxide dismutase non-denaturing gel assays

SOD gel assays were performed in 8% non-denaturing gels using *E. coli* MnSOD and FeSOD and Bovine Cu/ZnSOD as controls according to the procedure described by Beauchamp and Fridovich (1971). Briefly, samples were resolved as described in the above section and following electrophoresis were immersed in a solution containing 2 mg/ml nitroblue tetrazolium (Sigma) for 20

minutes in complete dark. This solution was removed and replaced with a solution containing 10 μg/ml riboflavin (Sigma) in 0.036 M phophate buffer (pH 7.8) for 20 minutes in complete dark. The gel was then illuminated until a uniform blue color developed in the gel. The areas containing active SOD proteins remained white.

XVIII. Densitometer scanning of autoradiographs

Quantitation of bands was performed through densitometer scanning using the LKB Ultrascan XL. Parameters were set for a Y-step = 2, X-width = 800 mM and a Smoothing = 1. The integration parameters dictated a peak width of 2, with the maximum number of peaks set at 50. Data from autoradiographic signal yielding an absorbance maximum >2.0 was not used and a lower exposure was scanned.

Table 2.1 Primers designed for sequencing and PCR using the L.c.FeSODA cDNA as a template.

Primer	Sequence	Range	Function
SODAS (Sense)	TCCTCCCGGATCCCGAATGTTCCGCCG CGTCTCG	108 to 140	PCR
SODAAS (Antisense)	CATCTAGGATCCTCTTACTTCGTGGCCT TCTC	799 to 830	PCR
SODA3S (Sense)	CACGACTACCACAGCCTC	1051 to 1068	PCR
HINDSODAKO (Sense)	CACTGAAAGCTTCGCACGACTCCAC	48 to 80	PCR (Knock-out)
XHOSODAKO (Antisense)	GGCATTCTCGAGCGTCTTCGGCATC	486 to 510	PCR (Knock-out)
SMASODAKO (Sense)	CGGAGGCCCGGGGCAGATGGCACAC	831 to 955	PCR (Knock-out)
BGLSODAKO (Antisense)	GTGGTAAGATCTATAAGGTACGGCG	1619 to 1643	PCR (Knock-out)

Table 2.2. Primers designed for sequencing and PCR using the L.c.FeSODB cDNA as a template.

Primer	Sequence	Range	Function
SODB1S (Sense)	GCGGAACTGTGCTGGCGCG	901 to 919	Sequencing
SODB1AS (Antisense)	CCTGGACGTCCGGCCCAC	2157 to 2174	Sequencing
SODB2S (Sense)	GCGGCGGCGTTCCTGCTTG	1153 to 1171	Sequencing
SODB2AS (Antisense)	AGGCAGGCATGCCATACG	1925 to 1942	Sequencing
SODB3S (Sense)	GGCGCGAAAAATGCGAACTG	2391 to 2410	Sequencing
SODB4S (Sense)	CCTCACGTTAATGGCACG	2688 to 2705	Sequencing
SODB4AS (Antisense)	GATCGCGGAACGTTGACGG	3564 to 3582	Sequencing
SODB5S (Sense)	CCGCAAAGATCGCCACAAG	3023 to 3041	Sequencing
SODB5AS (Antisense)	CGACCGTCCGTGTGTGTC	3231 to 3248	Sequencing
SODBATG (Sense)	TTTCCCGGGGGGATCCATGCCGTTCGCTGTT CAG	215 to 248	PCR
SODBTAA (Antisense)	CCCTCCCGGGGGATCCCCCGTTACTCGCTCG GC	862 to 895	PCR
HINDSODBKO (Sense)	CGCGTGAAGCTTCACCATCTTTGC	80 to 103	PCR (Knock-out)
XHOSODBKO (Antisense)	AAAACTCTCGAGGATCGCACTCGC	516 to 539	PCR (Knock-out)
SMASODBKO (Sense)	GGGGGCCCGGGAAGCGGAACTGTGC	888 to 913	PCR (Knock-out)
BGLSODBKO (Antisense)	CAAATGAGATCTCGTGCGGAAGAGAG	2192 to 2217	PCR (Knock-out)

Table 2.3. Chemical formulas and products formed from agents used to produce reactive oxygen intermediates and reactive nitrogen intermediates.

Reagent	Chemical Formula	Products formed
Paraquat (Methyl viologen)	$(N_2(CH_3)_2)^{+2}$	O_2^-
Sodium Nitroprusside	$Na_2[Fe(CN)_5NO]$	NO and O_2^-
Hydrogen Peroxide	H_2O_2	H_2O_2
3-Morpholino sydnonimine (Sin-1)	$C_6H_{12}N_4O_2Cl$	NO and O_2^-

CHAPTER 3

CHARACTERIZATION OF TWO IRON-CONTAINING SUPEROXIDE DISMUTASE CDNAS

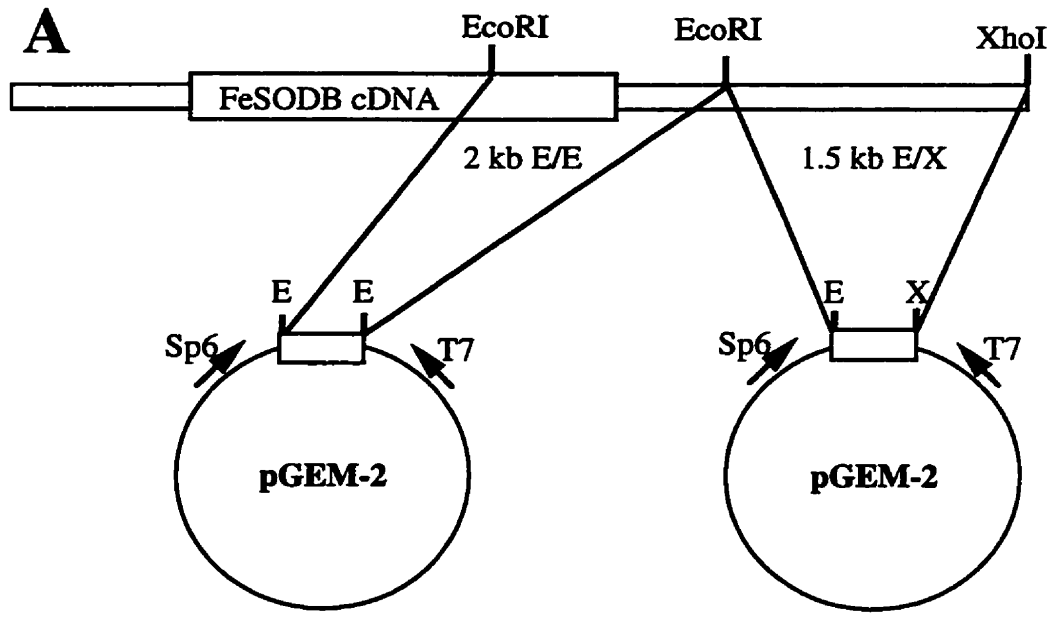
II. Results and Discussion

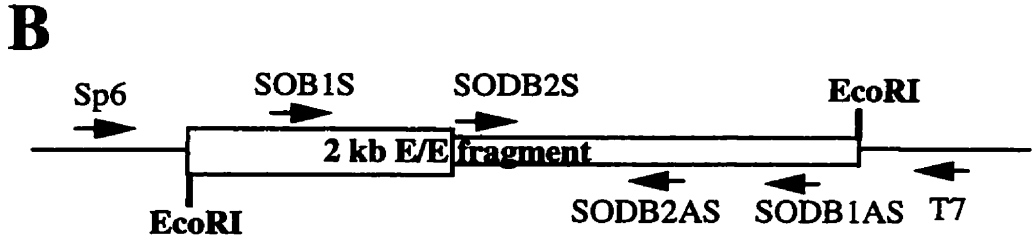
A. Sequencing strategy of L.c.FeSODB cDNA and comparison of L.c.FeSODA and L.c.FeSODB with SOD amino acid sequences from various organisms.

The isolation of two cDNA clones from a *Leishmania chagasi* promastigote cDNA library, called L.c.FeSODA and L.c.FeSODB was performed previously in our lab (Ismail, 1996). The complete sequencing and partial characterization of the 1.7 kb L.c.FeSODA cDNA has been presented previously (Ismail, 1996). Prior to the start of this project, sequencing of the 3.8 kb L.c.FeSODB cDNA from the 5' UTR to the first internal *Eco*RI site was initiated in collarboration with Dr. Said Ismail. The sequence of the remainder of the L.c.FeSODB cDNA was completed during this project as outlined in Fig.3.1.

The L.c.FeSODB cDNA contains two internal *Eco*RI sites. An *Xho*I site at the very 3' end of the cDNA was created using linkers. These sites were used for insertion in the pBluescript vector (Fig. 3.1A). These sites were determined by restriction digestion patterns of the L.c.FeSODB cDNA. To reduce the number of primers required for sequencing, the 2 kb *Eco*RI/*Eco*RI fragment was subcloned into the pGEM-2 vector (Fig 3.1A) and the SP6 and T7 primers were used to sequence the extreme ends of this 2 kb fragment. Primers were then designed to complete the sequencing of this fragment (Fig. 3.1B). A similar strategy was employed for the 1.5 kb *Eco*RI/*Xho*I fragment which was subcloned into the pGEM-2 vector (Fig. 3.1A and C).

Figure 3.1: Schematic summarizing the strategy used for sequencing the L.c.FeSODB cDNA. (A) Schematic showing restrictions of L.c.FeSODB cDNA with EcoRI and XhoI which releases a 2 kb EcoRI/EcoRI fragment and a 1.5 kb EcoRI/XhoI fragment. These fragments were then cloned into the pGEM-2 vector and the vector based primers SP6 and T7 were used to initiate sequencing of the 5' and 3' ends these two fragments. **(B)** and **(C)** Schematics showing the positions and orientation of the internal primers used to complete the sequencing of the L.c.FeSODB cDNA.


A
EcoRI
EcoRI
XhoI
FeSODB cDNA
2 kb E/E
1.5 kb E/X
E
E
E
X
Sp6
T7
Sp6
T7
pGEM-2
pGEM-2


B
Sp6
SOB1S
SODB2S
EcoRI
2 kb E/E fragment
EcoRI
SODB2AS
SODB1AS
T7

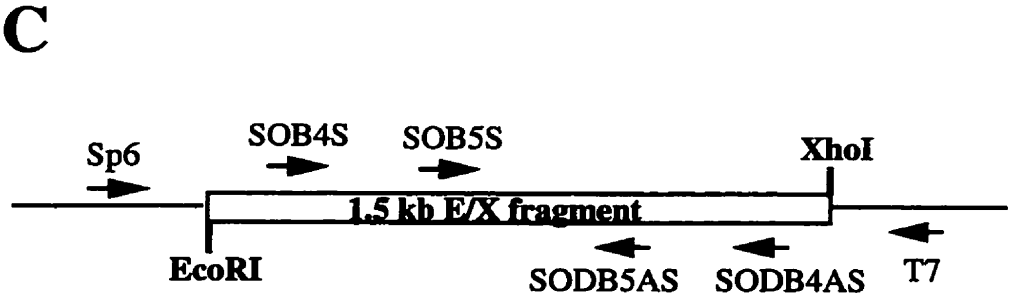

C
Sp6
SOB4S
SOB5S
XhoI
1.5 kb E/X fragment
EcoRI
SODB5AS
SODB4AS
T7

Fig. 3.2 shows the complete nucleotide sequence of the L.c.FeSODB cDNA. The FeSODB cDNA consists of a total of 3822 bps with a 230 nucleotide 5'UTR, a 588 nucleotide coding region and a 3004 nucleotide 3' UTR. The open reading frame starts at nucleotide 231 and ends at the termination codon TAA at position 818. The coding region encodes a 195 amino acid protein with a predicted molecular weight of 21,617 Daltons.

The predicted amino acid sequence of L.c.FeSODA (Ismail, 1996) is shown in Fig. 3.3 in comparison with that of L.c.FeSODB and SOD amino acid sequences from other organisms. Analysis of the predicted amino acid sequences of L.c.FeSODA and L.c.FeSODB reveals the presence of the four invariant residues (three histidine and one aspartate) postulated to be involved in Fe binding at the active site (Parker and Blake, 1988). Four additional residues are present also which are considered primary indicators of an Fe-containing enzyme. These are Ala-103;71, Gln-104;72, Ala-177;144, and Gly-178;145 for L.c.FeSODA and L.c.FeSODB. Thus, L.c.FeSODA and L.c.FeSOB have an overall homology to Fe-containing SODs.

An alignment of the amino acid sequences of L.c.FeSODA and L.c.FeSODB was performed using published amino acid sequences from various Fe and Mn containing SODs using the CLUSTAL/SeqVu programs (Fig. 3.3). Percent identities were calculated from pairwise alignments of the two *Leishmania* SODS with iron-containing SODs from *Entamoeba histolytica* (Bruchhaus andTannich, 1994), *E. coli* (Carlioz *et al.,* 1988) and *Coxiella burnetii*

Figure 3.2: The complete nucleotide sequence of the L.c.FeSODB cDNA. The complete nucleotide sequence and the corresponding one letter amino acid abbreviation for each triplet codon are shown. The L.c.FeSODB cDNA is 3822 bp in length, including a 230 nucleotide 5' UTR, a 588 nucleotide coding region (195 amino acids), and a 3004 nucleotide 3' UTR. Residues marked in red are the amino acids which are involved in coordinating the Fe at the active site of the enzyme. Residues shown in purple are the four invariant residues thought to be completely conserved through all iron-containing SODs.

1963 tcattccttcctgtcttgccccccccccacctcgtctcgcggctctctctctctcggttgtgtctttgctgttgttg

2043 ttgttgtgtctgtgtgcgtgtgatagcacgagcaagtgaccgaaaccatagttggaacagtaacaacagagcgtgatgga

2123 catgatggacgggtggggcggggtgggggaaggagtgggccggacgtccaggaaatcatacagaaagagctctcttccgc

2203 acgtggtctcatttgggtgtaacccccgccctcttcccccccactttgtttttacttcccctttctttcctgtgcttttcat

2283 ccccgccaacggtggtccatgacgagatggtattgacacgactatcgaaaaaaaagcgggaaagaaggagcacgcacaac

2363 aactgcaaagagaacaacgtggaaaataggcgcgaaaaatgcgaactgacgtacagaggaatgttgaagaattcaagaat

2443 cgttccttttgcgcacatgtaacgatccttcaggatcgaggaaagatgagctggagaaaaagcgtctgcgcgtgcactca

2523 aattgttgaaggaatcggaaagttactcatgcgcttctcagcgtctgtgacttttcgttttcccctcttcttgtgtctcca

2603 gcctgcgtgcgtctggctgctgtgacctttcctcacccctcctttgccgcccccgctgtgccgtccgtctcccctctccta

2683 ttcagcctcacgttaatggcacgttgagcgcaggtatacctctgcctcactgttagccagtgtgaaaggcggtgagctgt

2763 aaagacaacggtagggtcgccagagagggtgaagggtacaccgttttgcaactctctatgctttaggtctacagagtagt

2843 agcaggtgcgccttttatttcttttgccgcactgtttctccgtttttttttttttcgtctgtgaatcgctatcttctgcg

2923 acccctccgtccccctcacttttacatctgccagtcgttgatgtgtggtacggttcttgtactcttcttctctccccagc

3003 gtttctttggtgctcaagaaccgcaaagatcgccacaagagaaggagcaaacgaaaacaaaaaaagaacaccataaagac

3083 gacgagaaggcggtaagaggagtgtggtttcagcgcacgtacatacatagacacacacatgtgtatgtattctgcagcaa

3163 gacgatgccaaacgagtggcggtacggatgaggggagaaatacagggaaaaaagacgtgcccgcgtacgacacacacgga

3243 cggtcgtttgaaaacaaggtgggagcagggaaagtaaagtgactgcaagagttttcgctttcccgcaagtgcacttcagt

3323 gaccctgctgcgcatctgctctcctcctcgtgcccagccagtctcgcttgtgtgccgggctagtgtaaagtgccacgtaa

3403 gcgcctgtatccgccggtgtactgtgctagttaccactctttctctatgcgtatctgccgctctgtcactcgttgtggct

3483 gctcagcggtctctctccttcaccgtgtcctctttgcgcgtgtacgtgtgccctatgtgcgtggaatgcaatatcctccc

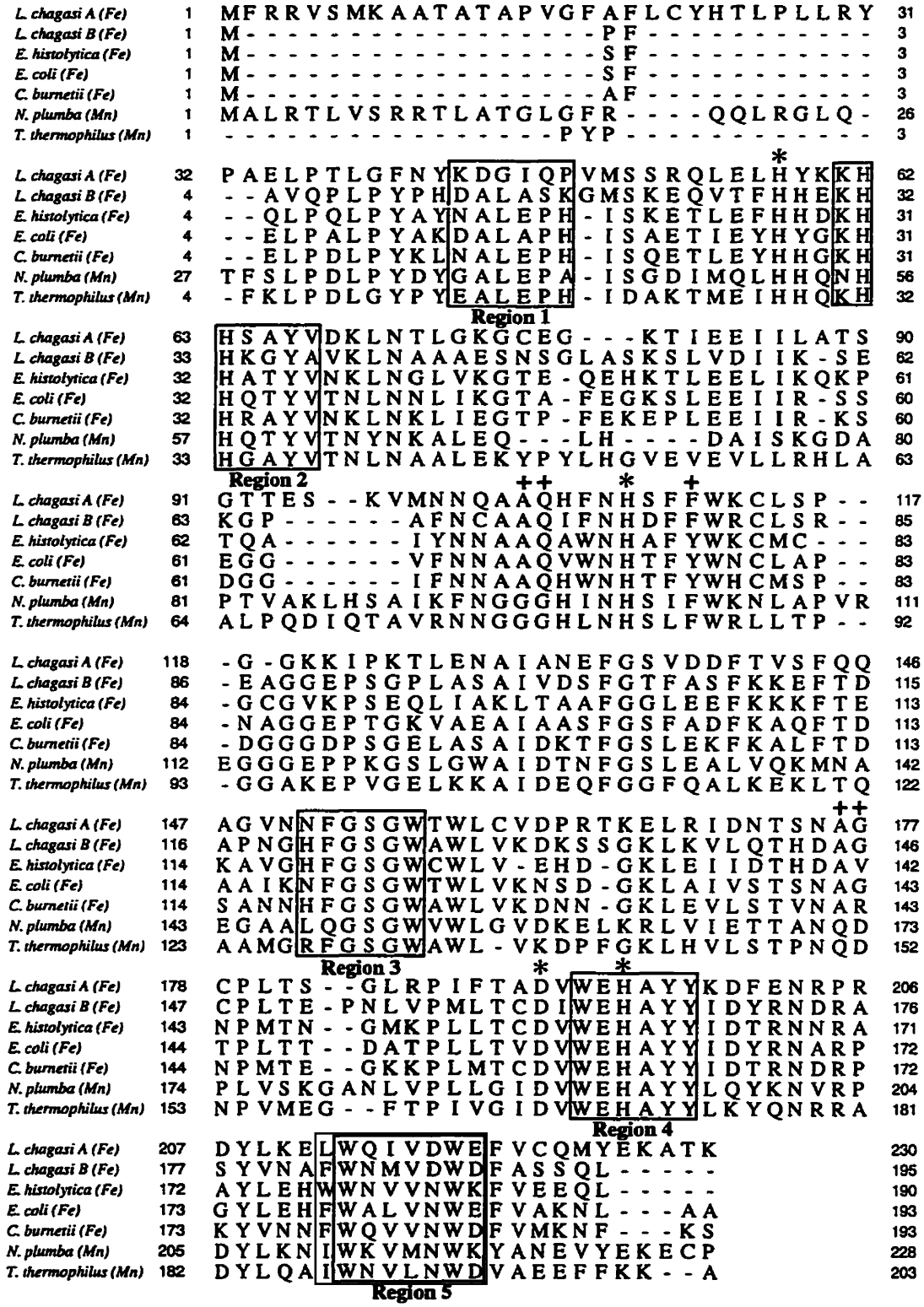
3563 tccgtcaacgttccgcgatctcttcgtcttgtttttttttcctctgcatgtattgttgccgtttctcctccagagtgtga

3643 acgctacctctctcctctctcctgttcttctttgcttgcttgcttggctctgcgtgggggttttatgccttacacagtta

3723 tgactaagcactcaaaacatatatgcggtctatcaacggcagtgaagggaggccgaaaagtgtgctgccaaaaaaaaaaa

3803 aaaaaaaaaaaaaaaaaaaa

Figure 3.3: Alignment of the predicted amino acid sequences of FeSODA and FeSODB from *Leishmania chagasi* with Fe and MnSOD sequences from phylogenetically diverse organisms. The iron-containing SODs used in the alignment are from *Entamoeba histolytica* (Bruchhaus and Tannich, 1994), *Escherichia coli* (Carlioz *et al.*, 1988), and *Coxiella burnetii* (Heinzen *et al.*, 1992). The *Nicotinia plumba* (Bowler *et al.*, 1989) and *Thermus thermophilus* (Sato *et al.*, 1987) manganese-containing SODs were also used in the alignment. The five conserved regions of Fe and MnSODs are boxed and the amino acids used to distinguish between Fe and MnSODs are marked with crosses (+). The invariant amino acids thought to be involved in coordinating the metal ions in the active sites of these enzymes are marked with asterisks (*).


L. chagasi A (Fe) 1 MFRRVSMKAATATAPVGFAFLCYHTLPLLRY 31
L. chagasi B (Fe) 1 M-----------------PF---------- 3
E. histolytica (Fe) 1 M-----------------SF---------- 3
E. coli (Fe) 1 M-----------------SF---------- 3
C. burnetii (Fe) 1 M-----------------AF---------- 3
N. plumba (Mn) 1 MALRTLVSRRTLATGLGFR----QQLRGLQ- 26
T. thermophilus (Mn) 1 ----------------PYP---------- 3
L. chagasi A (Fe) 32 PAELPTLGFNYKDGIQPVMSSRQLELHYKKH 62
L. chagasi B (Fe) 4 --AVQPLPYPHDALASKGMSKEQVTFHHEKH 32
E. histolytica (Fe) 4 --QLPQLPYAYNALEPH-ISKETLEFHHDKH 31
E. coli (Fe) 4 --ELPALPYAKDALAPH-ISAETIEYHYGKH 31
C. burnetii (Fe) 4 --ELPDLPYKLNALEPH-ISQETLEYHHGKH 31
N. plumba (Mn) 27 TFSLPDLPYDYGALEPA-ISGDIMQLHHQNH 56
T. thermophilus (Mn) 4 -FKLPDLGYPYEALEPH-IDAKTMEIHHQKH 32
Region 1
L. chagasi A (Fe) 63 HSAYVDKLNTLGKGCEG---KTIEEIILATS 90
L. chagasi B (Fe) 33 HKGYAVKLNAAAESNSGLASKSLVDIIK-SE 62
E. histolytica (Fe) 32 HATYVNKLNGLVKGTE-QEHKTLEELIKQKP 61
E. coli (Fe) 32 HQTYVTNLNNLIKGTA-FEGKSLEEIIR-SS 60
C. burnetii (Fe) 32 HRAYVNKLNKLIEGTP-FEKEPLEEIIR-KS 60
N. plumba (Mn) 57 HQTYVTNYNKALEQ---LH----DAISKGDA 80
T. thermophilus (Mn) 33 HGAYVTNLNAALEKYPYLHGVEVEVLLRHLA 63
Region 2
L. chagasi A (Fe) 91 GTTES--KVMNNQAAQHFNHSFFWKCLSP-- 117
L. chagasi B (Fe) 63 KGP-------AFNCAAQIFNHDFFWRCLSR-- 85
E. histolytica (Fe) 62 TQA-------IYNNAAQAWNHAFYWKCMC--- 83
E. coli (Fe) 61 EGG-------VFNNAAQVWNHTFYWNCLAP-- 83
C. burnetii (Fe) 61 DGG-------IFNNAAQHWNHTFYWHCMSP-- 83
N. plumba (Mn) 81 PTVAKLHSAIKFNGGGHINHSIFWKNLAPVR 111
T. thermophilus (Mn) 64 ALPQDIQTAVRNNGGGHLNHSLFWRLLTP-- 92
L. chagasi A (Fe) 118 -G-GKKIPKTLENAIANEFGSVDDFTVSFQQ 146
L. chagasi B (Fe) 86 -EAGGEPSGPLASAIVDSFGTFASFKKEFTD 115
E. histolytica (Fe) 84 -GCGVKPSEQLIAKLTAAFGGLEEFKKKFTE 113
E. coli (Fe) 84 -NAGGEPTGKVAEAIAASFGSFADFKAQFTD 113
C. burnetii (Fe) 84 -DGGGDPSGELASAIDKTFGSLEKFKALFTD 113
N. plumba (Mn) 112 EGGGEPPKGSLGWAIDTNFGSLEALVQKMNA 142
T. thermophilus (Mn) 93 -GGAKEPVGELKKAIDEQFGGFQALKEKLTQ 122
L. chagasi A (Fe) 147 AGVNNFGSGWTWLCVDPRTKELRIDNTSNAG 177
L. chagasi B (Fe) 116 APNGHFGSGWAWLVKDKSSGKLKVLQTHDAG 146
E. histolytica (Fe) 114 KAVGHFGSGWCWLV-EHD-GKLEIIDTHDAV 142
E. coli (Fe) 114 AAIKNFGSGWTWLVKNSD-GKLAIVSTSNAG 143
C. burnetii (Fe) 114 SANNHFGSGWAWLVKDNN-GKLEVLSTVNAR 143
N. plumba (Mn) 143 EGAALQGSGWVWLGVDKELKRLVIETTANQD 173
T. thermophilus (Mn) 123 AAMGRFGSGWAWL-VKDPFGKLHVLSTPNQD 152
Region 3
L. chagasi A (Fe) 178 CPLTS--GLRPIFTADVWEHAYYKDFENRPR 206
L. chagasi B (Fe) 147 CPLTE-PNLVPMLTCDIWEHAYYIDYRNDRA 176
E. histolytica (Fe) 143 NPMTN--GMKPLLTCDVWEHAYYIDTRNNRA 171
E. coli (Fe) 144 TPLTT--DATPLLTVDVWEHAYYIDYRNARP 172
C. burnetii (Fe) 144 NPMTE--GKKPLMTCDVWEHAYYIDTRNDRP 172
N. plumba (Mn) 174 PLVSKGANLVPLLGIDVWEHAYYLQYKNVRP 204
T. thermophilus (Mn) 153 NPVMEG--FTPIVGIDVWEHAYYLKYQNRRA 181
Region 4
L. chagasi A (Fe) 207 DYLKELWQIVDWEFVCQMYEKATK 230
L. chagasi B (Fe) 177 SYVNAFWNMVDWDFASSQL----- 195
E. histolytica (Fe) 172 AYLEHWWNVVNWKFVEEQL----- 190
E. coli (Fe) 173 GYLEHFWALVNWEFVAKNL---AA 193
C. burnetii (Fe) 173 KYVNNFWQVVNWDFVMKNF---KS 193
N. plumba (Mn) 205 DYLKNIWKVMNWKYANEVYEKECP 228
T. thermophilus (Mn) 182 DYLQAIWNVLNWDVAEEFFKK--A 203
Region 5

(Heinzen, *et al.*, 1992) , and the manganese-containing SODs from *Nicotina plumbaginifolia* (Bowler *et al.*, 1989), and *Thermus thermophilus* (Sato *et al.*, 1987). The five conserved regions of Fe and MnSODs defined by Heinzen *et al.* (1992) are boxed in Fig. 3.3. Interestingly, the two *L. chagasi* SODs showed less identity to each other (37%) than to those of other organisms. *L. chagasi* FeSODB has a closer identity to the Fe-containing SODs of *E. histolytica*, *E. coli*, and *C. burnetii* (52%, 54% and 56% respectively) compared to *L. chagasi* FeSODA (43%, 48% and 45% respectively). No MnSOD displayed greater than 39% identity with either of the *L. chagasi* SODs.

L. chagasi FeSODA had a higher identity with *N. plumba* MnSOD than *L. chagasi* FeSODB. One reason for this difference in identity could be that *L. chagasi* FeSODA contains a 31 amino acid hydrophobic extension at the amino terminus, which is absent in *L. chagasi* FeSODB. A similar amino terminus extension of 26 amino acids is seen in *N. plumba*. This extension has been shown to resemble a transit peptide for mitochondrial targetting in *N. plumba* (Bowler *et al.*, 1989).

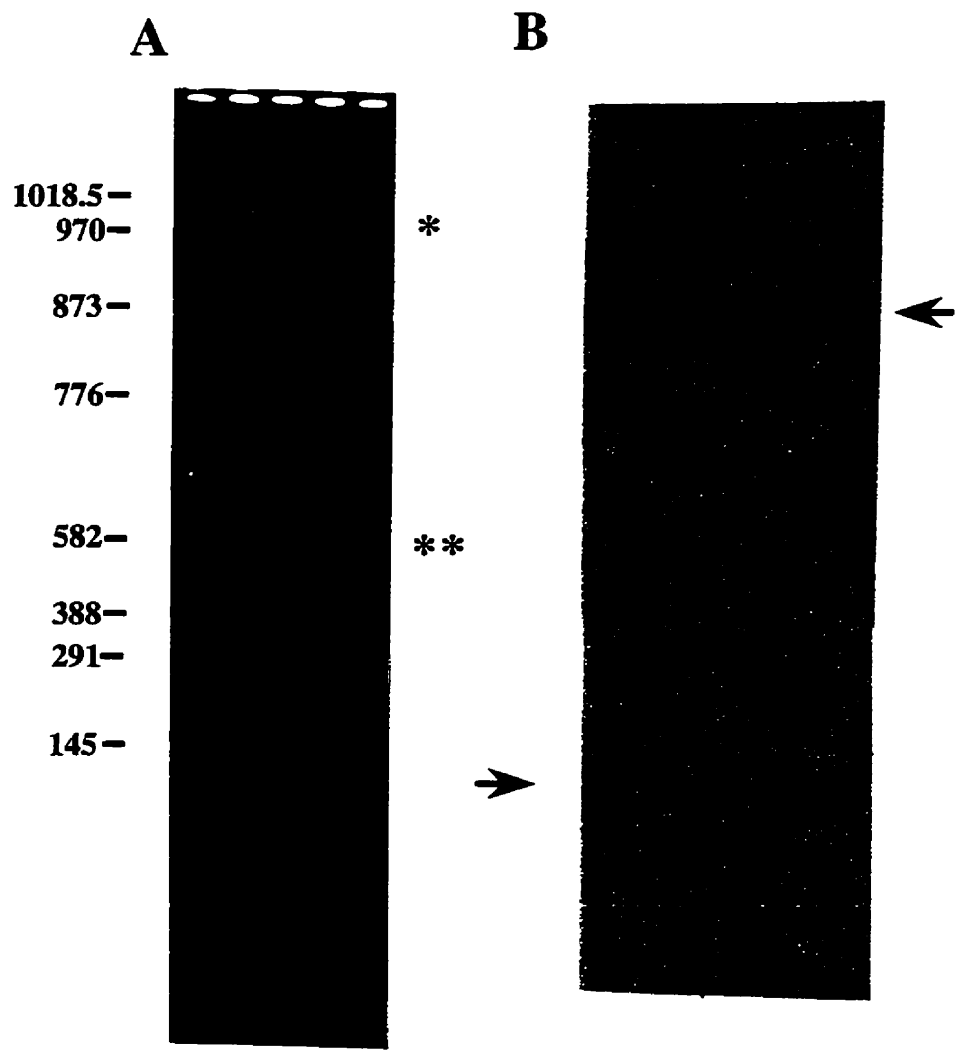
Recently, two different FeSOD cDNAs have been isolated from *Trypanosoma cruzi* (Ismail *et al.*, 1997) which belongs to the same family as *Leishmania* spp. Cross-species comparison of the deduced amino acid sequences from *L. chagasi* and *T. cruzi* resulted in higher identity scores than intra-species comparison. L.c.FeSODA and T.c.FeSODA show a 55% identity, whereas L.c.FeSODB and T.c.FeSODB showed a very high identity score of

62%. Surprisingly, T.c.FeSODA contains a 31 amino acid amino terminal end extension, very similar to the L.c.FeSODA enzyme. Both T.c.FeSODB and L.c.FeSODB enzymes are devoid of this extension, suggesting that this pattern may be unique to trypanosomatids. This amino acid extension suggests that L.c.FeSODA may have a putative signalling sequence which targets the protein to a separate organelle. Perhaps L.c.FeSODA and L.c.FeSODB perform similar detoxifying functions, but in a different subcellular location such as the mitochondria. This may explain why the organism has evolved to have two iron containing enzymes. Differing functions relating to distinct localization of the enzymes within the individual organisms has been suggested but has found little support to date (Britton and Fridovich, 1977). If the FeSODA enzyme of *L. chagasi* is later shown to localize to a specific organelle, this will provide some support for this idea.

B. Chromosomal localization of L.c.FeSODA and L.c.FeSODB genes

In order to localize the position of the L.c.FeSODA and FeSODB genes on *Leishmania chagasi* chromosomes, *L. chagasi* total genomic DNA was subjected to chromosomal separation using pulsed field gel electrophoresis. The resulting gel was transferred by Southern blotting and analysed using the complete cDNA coding region from L.c.FeSODA and L.c.FeSODB probes. The chromosomal separation is shown in an ethidium bromide stained gel (Fig. 3.4A). As shown in Fig. 3.4B, bottom arrow (L.c.FeSODA) and Fig. 3.4B, top

Figure 3.4: Chromosomal localization of *Leishmania chagasi* FeSODA and FeSODB genes. (A) Ethidium bromide stained gel showing the separation of *L. chagasi* chromosomes using pulsed field gel electrophoresis. Asterisk(s) (*) indicate the chromosomes where the two genes are localized. **(B)** Southern blot of the PFGE hybridized with the entire cDNA of L.c.FeSODA (at bottom arrow) and the entire cDNA of L.c.FeSODB (at top arrow). The molecular size markers were the lambda PFG ladder (New England Biolabs).


A
B
1018.5
970
873
776
582
388
291
145
*
**

arrow (L.c.FeSODB), each gene is localized to a separate chromosome. L.c.FeSODA shows localization to a single band at a lower molecular weight chromosome of approximately 582 kb, whereas, L.c.FeSODB is localized to a single higher molecular weight chromosome of approximately 920 kb. Many of the housekeeping genes which contain highly conserved sequences of *Leishmania* have been localized to very high molecular weight chromosomes (where L.c.FeSODB is located) (Lighthall and Giannini, 1992) using pulsed field gel electrophoresis, such as genes for ribosomal RNA and β-tubulin. Genes localized to lower sized chromosomes (L.c.FeSODA) have been shown to perform more specialized functions (Lighthall and Giannini, 1992).

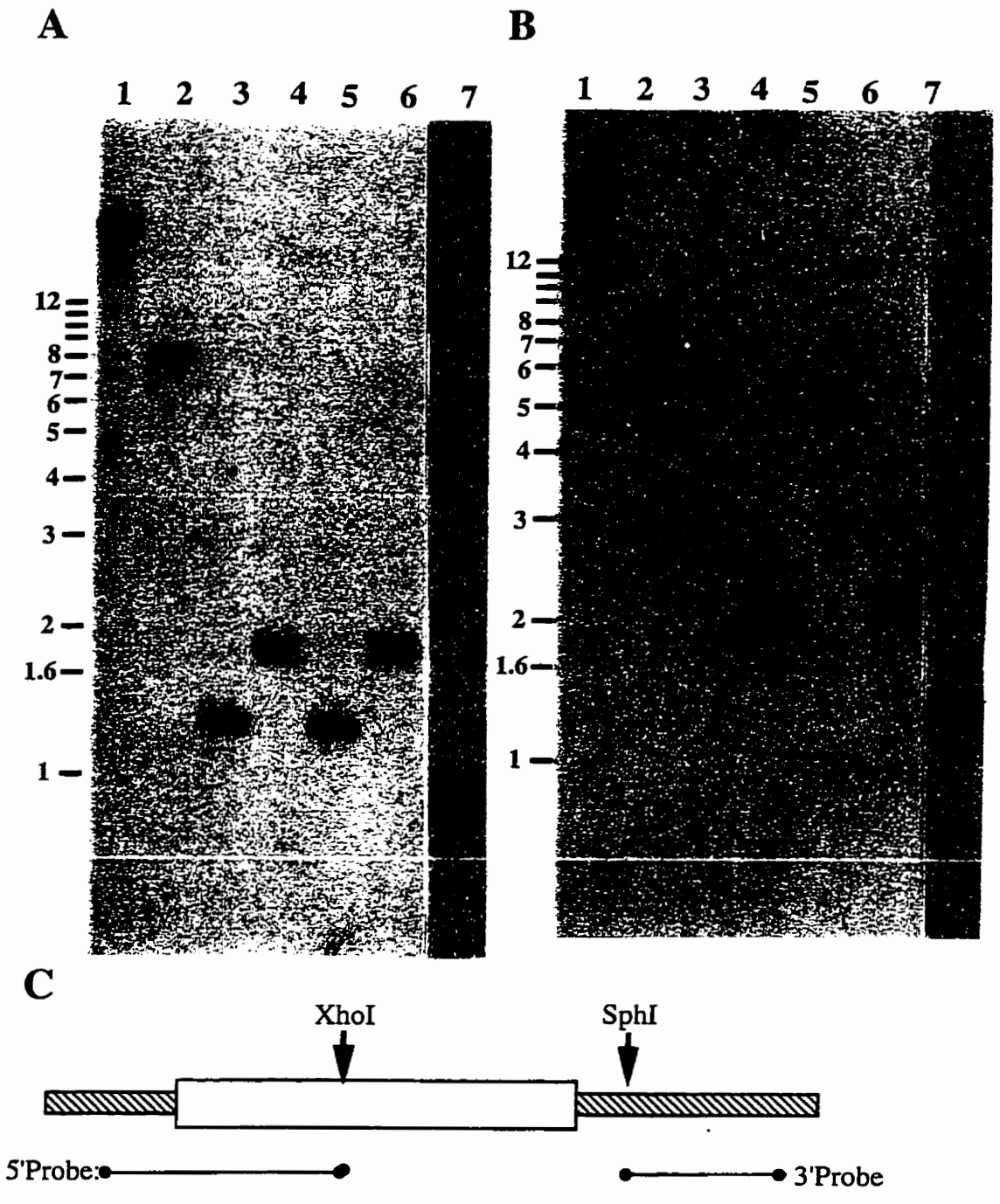
C. Genomic organization of L.c.FeSODA and L.c.FeSODB

The genomic organization of the L.c.FeSODA and FeSODB genes was analyzed by Southern blot analysis. Total genomic *L. chagasi* DNA was digested with several different endonucleases, separated on an agarose gel, and hybridized with probes derived from two separate cDNA fragments (Fig.3.5C). These probes were designed to reveal the copy number of the L.c.FeSODA gene. To determine if the L.c.FeSODA sequence is a single copy gene, total *L. chagasi* genomic DNA was digested with *Bam*HI, *Pst*I, *Sph*I, *Xho*I, *Pst*I / *Sph*I, *Pst*I / *Xho*I and *Sph*I / *Xho*I. The L.c.FeSODA cDNA sequence contains single *Sph*I and *Xho*I sites but no *Bam*HI and *Pst*I sites. Thus, if L.c.FeSODA is present as a single copy, then all of the above single and double restriction digests

should result in a single hybridizing band, regardless of which probe is employed. This was observed in Fig.3A-C. With each of these digests, only one hybridizing band was observed, indicating that L.c.FeSODA is a single copy gene. Results of the Southern blot hybridization with the 5' probe are shown in Fig.3.5A with a schematic diagram of my interpretation in Fig.3.6. With the 5' cDNA probe, digestion with *Bam*HI resulted in one hybridizing band greater than 12 kb in size (Fig.3.5A, lane1) and an 8 kb *Pst*I band (lane 2). Therefore, these two enzymes cut outside of the L.c.FeSODA gene within the upstream and downstream regions. Also, *Sph*I and *Xho*I digests released 1.25 kb (lane 3) and 2 kb (lane 4) bands respectively, suggesting that there is a single cut within the gene and a single site for each of these enzymes upstream of the gene, with the *Sph*I site at closer proximity than the *Xho*I. A double digest with either *Pst*I / *Sph*I (lane 5) or *Pst*I / *Xho*I (lane 6) resulted in identical sized bands as seen with the *Sph*I and *Xho*I single digests respectively, at about 1.25 kb and 2 kb. This confirms the presence of a single *Sph*I and *Xho*I site within the gene and suggests that the *Sph*I site is closer to the L.c.FeSODA gene than *Pst*I in the upstream region. As well, *Xho*I has a cut site closer than *Pst*I. Finally, a double digest with *Sph*I / *Xho*I (lane 7) resulted in a band at approximately 700 bp which confirms the presence of the predicted internal *Xho*I site as well as the intergenic *Sph*I site and upstream *Xho*I site. Fig.3.6A summarizes these interpretations.

This same Southern blot hybridized with the L.c.FeSODA 3'probe

Figure 3.5: Southern blot analysis of the L.c.FeSODA and L.c.FeSODB genes.(A) and **(B)** Lane 1-*Bam*HI, Lane 2-*Pst*I, Lane 3-*Sph*I, Lane 4-*Xho*I, Lane 5-*Pst*I / *Sph*I, Lane 6-*Pst*I / *Xho*I, Lane 7-*Sph*I / *Xho*I. **(A)** Autoradiogram of southern blot hybridized with a 463 bp probe amplified using the primers 5'HindSODA/3'XhoSODA which span the 5'UTR and approximately half of the coding region of the L.c.FeSODA cDNA. **(B)** Autoradiogram of southern blot hybridized with a 592 bp probe amplified using the primers 5'SODA3S/3'BglSODA which spans the region downstream from the *Sph*I restriction site within the 3'UTR of the L.c.FeSODA cDNA to near the end of the 3'UTR. The schematic in **(C)** marks the position of these probes. **(D)** and **(E)** Lane 1-*Bam*HI, Lane 2-*Pst*I, Lane 3-*Sal*I, Lane 4-*Xho*I. **(D)** Autoradiograph of southern blot hybridized with a 320 bp probe amplified with 5'SODBATG/3'XhoSODB which spans the beginning of the L.c.FeSODB coding region to approximately the middle of the coding region. **(E)** Autoradiograph of southern blot hybridized with a 1.5 kb region released with the internal *Xho*I and *EcoR*I restriction sites within the 3'UTR of the L.c.FeSODB cDNA which lie downstream from the 3'UTR *Sal*I site and at the extreme end of the 3'UTR (The *EcoR*I site comes from the MCS of the bluescript vector the L.c.FeSODB cDNA was recovered in during cloning). The schematic in **(F)** marks the positions of these probes. The positions of the 1 kb molecular weight markers (Gibco, BRL)are shown to the left of **(A)**, **(B)**, **(D)** and **(E)**.


A
B
1 2 3 4 5 6 7
12
8
7
6
5
4
3
2
1.6
1
C
XhoI
SphI
5'Probe:
3'Probe

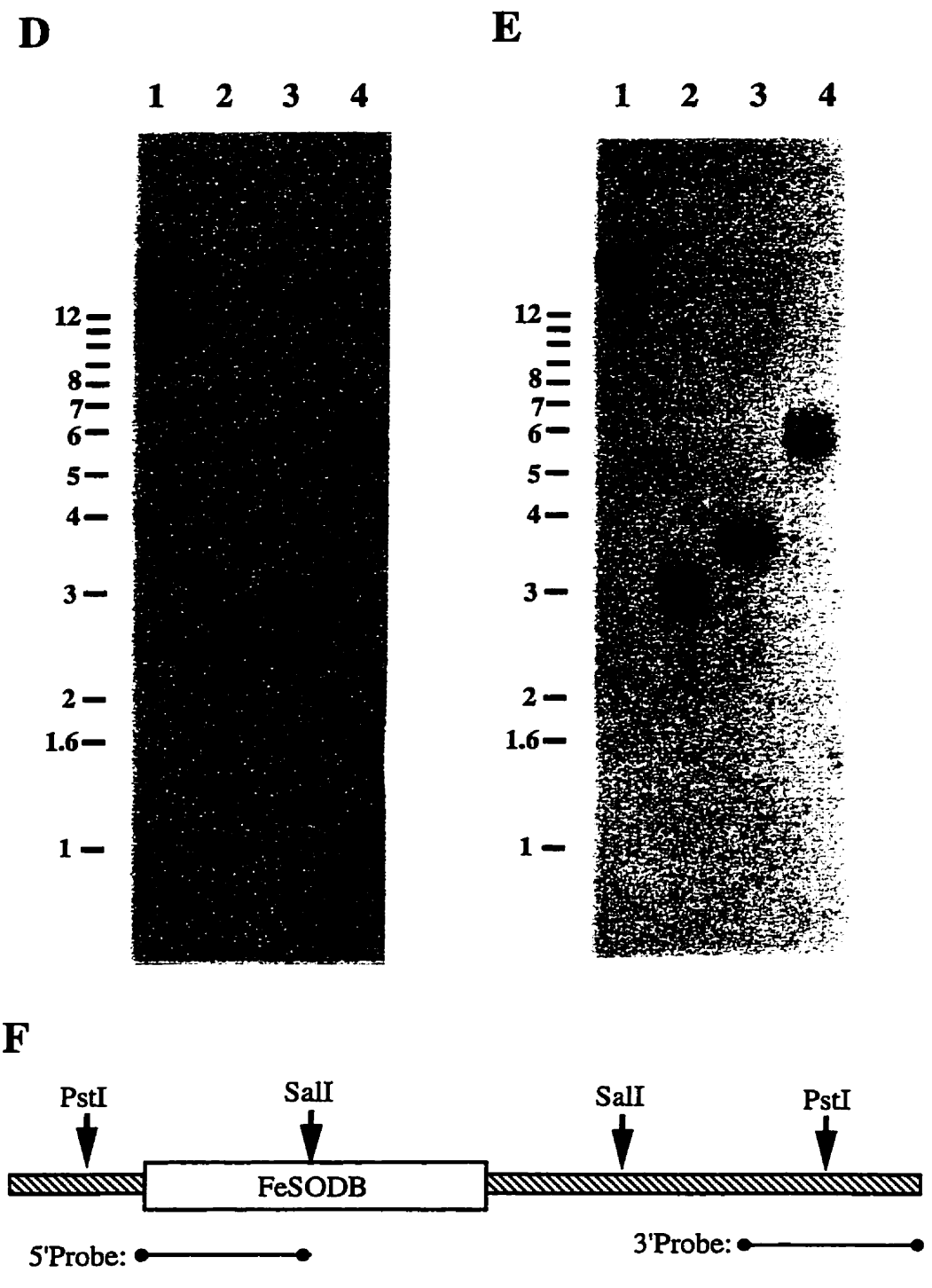
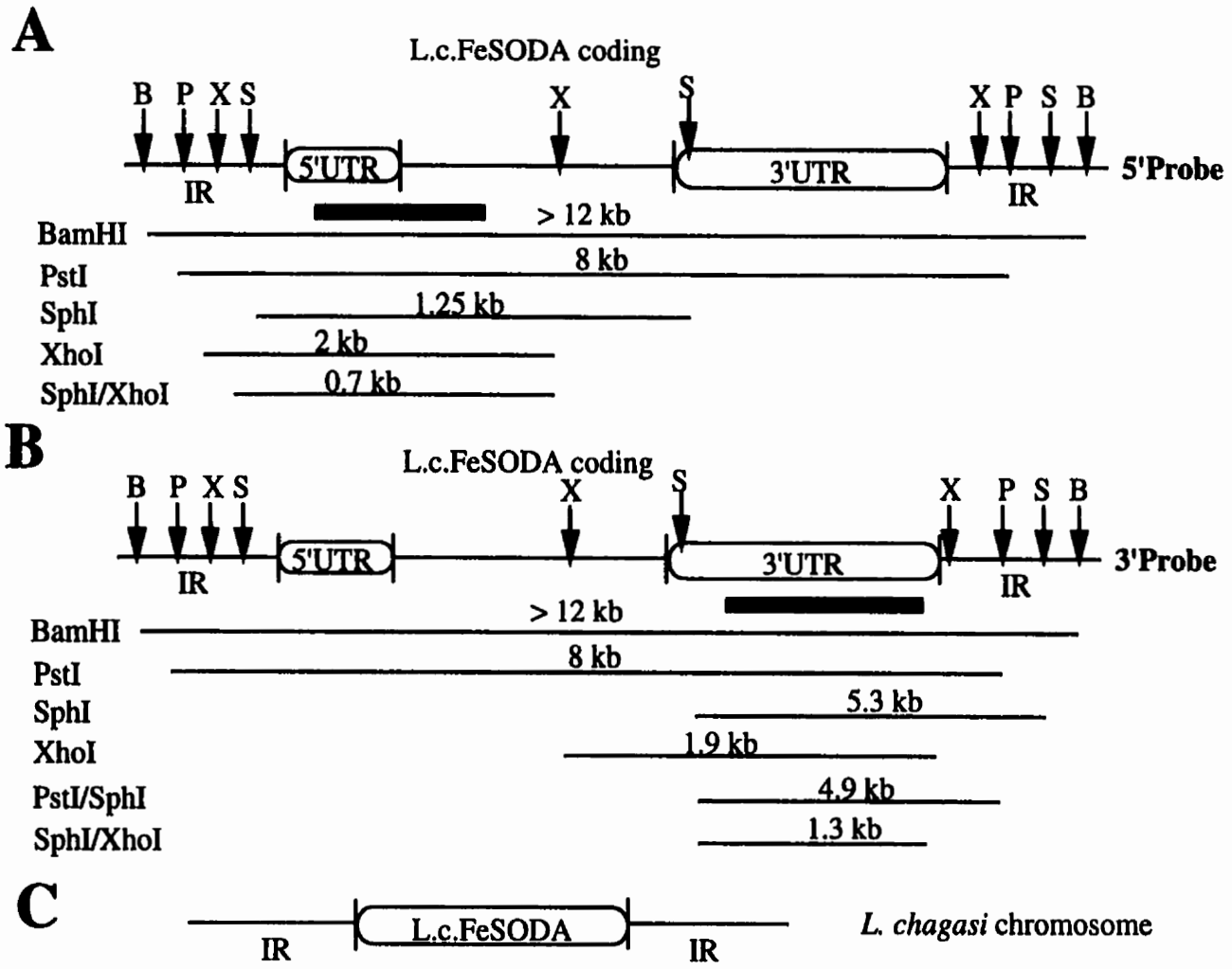

D
1 2 3 4
12
8
7
6
5
4
3
2
1.6
1
E
1 2 3 4
12
8
7
6
5
4
3
2
1.6
1
F
PstI
SalI
SalI
PstI
FeSODB
5'Probe:
3'Probe:

Figure 3.6: Summary of the results of the L.c.FeSODA Southern blot analysis of Fig. 3.5A and B. Results of the Southern blot hybridized with **(A)** 5'probe and **(B)** 3'probe of L.c.FeSODA. The restriction fragments hybridizing to each of the probes are shown with thin lines and labeled with the sizes corresponding to the bands seen in the autoradiograms in Fig. 3.5A and B. IR=intergenic region, B=*Bam*HI, P=*Pst*I, S=*Sph*I and X=*Xho*I. **(C)** Model hypothesizing the arrangement of the L.c.FeSODA gene within the *L. chagasi* genome.


A
L.c.FeSODA coding
B P X S
X
S
X P S B
5'UTR
3'UTR
IR
IR
5'Probe
> 12 kb
BamHI
8 kb
PstI
1.25 kb
SphI
2 kb
XhoI
0.7 kb
SphI/XhoI
B
L.c.FeSODA coding
B P X S
X
S
X P S B
5'UTR
3'UTR
IR
IR
3'Probe
> 12 kb
BamHI
8 kb
PstI
5.3 kb
SphI
1.9 kb
XhoI
4.9 kb
PstI/SphI
1.3 kb
SphI/XhoI
C
IR
L.c.FeSODA
IR
L. chagasi chromosome

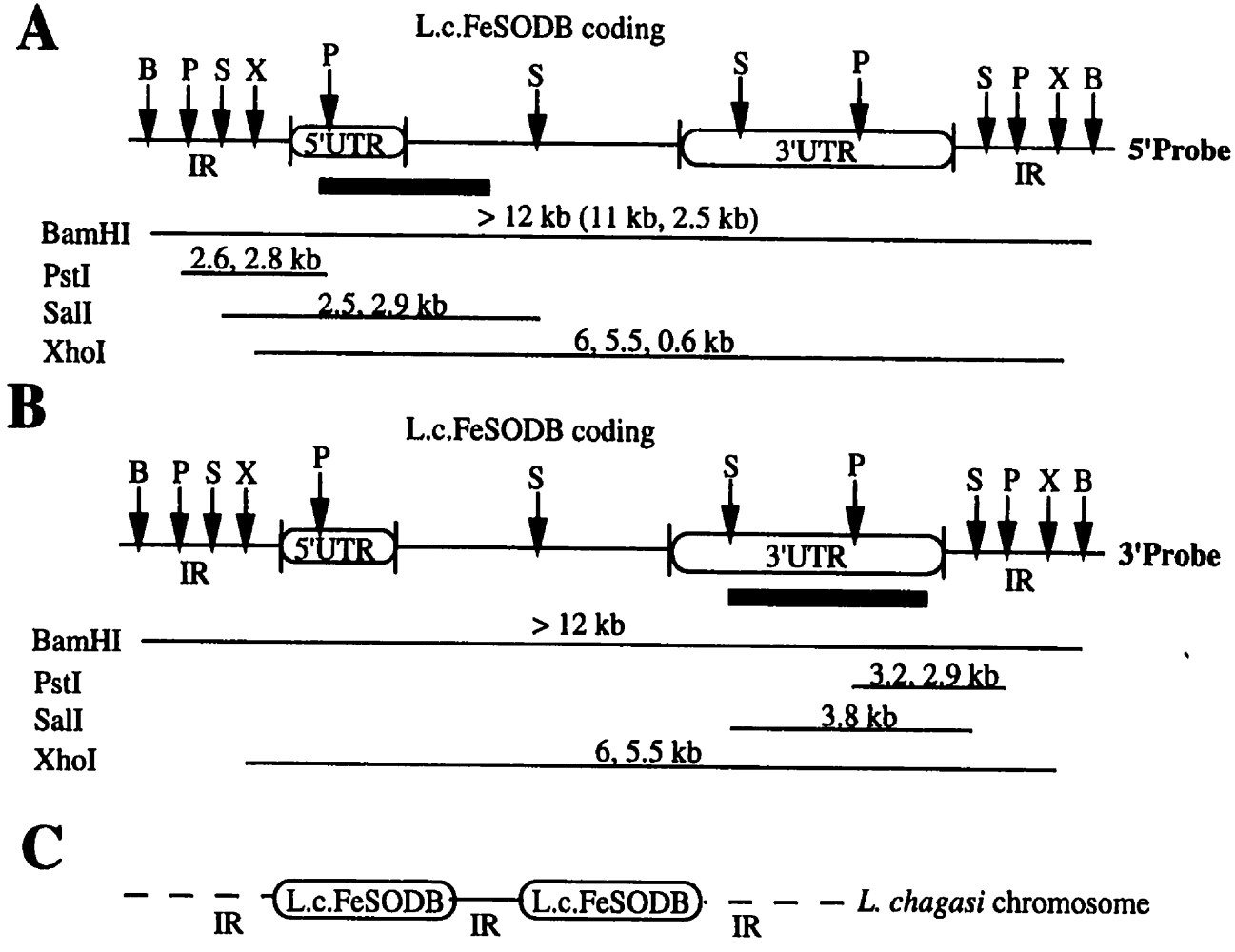
(Fig.3.5C) confirmed the single copy nature of the L.c.FeSODA gene. Single bands were seen with *Bam*HI, *Pst*I, *Sph*I and *Xho*I digests at >12 kb (Fig.3.5B, lane1), ~8 kb(lane2), ~5.3 kb (lane3) and ~1.9 kb (lane4) respectively. This confirms the presence of *Bam*HI and *Pst*I cut sites outside of the L.c.FeSODA gene within the downstream and upstream regions. Further, the single band seen with the *Sph*I and *Xho*I digests suggests that there is a single site for each of these enzymes within the L.c.FeSODA gene and a single site in the downstream region of the gene. The double digest with *Pst*I / *Sph*I resulted in a band of ~4.9 kb (Fig.3.5B, lane 5) which migrated faster than the *Sph*I band, suggesting that the external *Pst*I site within the downstream region is closer to the gene than the external *Sph*I site. The double digest with *Pst*I/*Xho*I revealed a band at ~1.9 kb (Fig.3.5B, lane 6), a similar band seen with the *Xho*I single digest, suggesting that the external *Xho*I site within the downstream region is closer to the gene than the external *Pst*I site. Finally, the double digest with *Sph*I / *Xho*I resulted in a band of ~1.3 kb, again suggesting that the external *Xho*I in the downstream region is closer to the gene than the external *Sph*I site. Fig.3.6B summarizes the interpretation of these results.

Taken together, these results of the Southern blots suggested that the L.c.FeSODA gene is present within the *L. chagasi* genome as a single copy gene. The model presented in Fig.3.6C shows a putative position of the L.c.FeSODA gene with its internal and external cut sites as determined from the above results.

Fig. 3.5D and E show the results of the Southern blot hybridized with probes from L.c.FeSODB cDNA. Total *L. chagasi* genomic DNA was digested with *Bam*HI, *Pst*I, *Sal*I and *Xho*I. The L.c.FeSODB cDNA contains two internal cut sites for both *Sal*I and *Pst*I but no internal sites for *Bam*HI and *Xho*I (See schematic in Fig.3.5F for positions of sites within the cDNA). The following observations should be made if L.c.FeSODB is present as a single copy gene. Use of the 5' or 3' cDNA probes with the *Bam*HI and *Xho*I digested DNA should result in the same hybridizable band in each case. However, hybridization of the *Pst*I digest with the 5' probe should result in one band while use of the 3' probe will result in two bands. Because of an internal site, digestion with *Sal*I should result in the presence of one band when hybridized with the 5'probe and a different band when hybridized with the 3'probe.

Results of the Southern blot hybridized with the L.c.FeSODB 5'probe (Refer to Fig.3.5F for position) revealed a single hybridizing band with a *Bam*HI digest (Fig.3.5D, lane1) >12 kb in size, plus two bands of lower molecular weight at~11 kb and 2.5 kb (Refer to Fig.3.7A for a schematic showing a summary of these results). The intensely hybridizing band suggests that there are *Bam*HI sites surrounding the L.c.FeSODB gene region. Because a *Bam*HI site is not present within the L.c.FeSODB cDNA, the 2.5 kb less intense band may be a result of cross-hybridization with another FeSOD gene or L.c.FeSODB may be arranged in tandem as a multi-copy gene family. It is unlikedly that this band is derived from the L.c.FeSODA gene because of no cross hybridization of the

Figure 3.7: Summary of the results of the L.c.FeSODB Southern blot analysis of Fig. 3.5D and E. Results of the Southern blot hybridized with **(A)** 5'probe and **(B)** 3'probe of L.c.FeSODB. The restriction fragments hybridizing to each of the probes are shown with thin lines and labeled with the sizes corresponding to the bands seen in the autoradiograms in Fig. 3.5D and E. IR=intergenic region, B=*Bam*HI, P=*Pst*I, S=*Sal*I and X=*Xho*I. **(C)** Model hypothesizing the arrangement of the L.c.FeSODB gene within the *L. chagasi* genome.


A
L.c.FeSODB coding
B P S X
P
S
S
P
S P X B
5'UTR
3'UTR
IR
IR
5'Probe
BamHI
> 12 kb (11 kb, 2.5 kb)
PstI
2.6, 2.8 kb
SalI
2.5, 2.9 kb
XhoI
6, 5.5, 0.6 kb
B
L.c.FeSODB coding
B P S X
P
S
S
P
S P X B
5'UTR
3'UTR
IR
IR
3'Probe
BamHI
> 12 kb
PstI
3.2, 2.9 kb
SalI
3.8 kb
XhoI
6, 5.5 kb
C
IR
L.c.FeSODB
IR
L.c.FeSODB
IR
L. chagasi chromosome

probes in the PFGE studies and different bands appeared with the two probes using the same enzymes on numerous Southern blots. Digestion with *Xho*I (Fig.3.5D, lane 4) resulted in the hybridization of three bands of low intensity at ~6, 5.5 and 0.6 kb. Again this hybridization pattern cannot be explained by the presence of only a single copy of L.c.FeSODB. Restriction with *Pst*I (Fig.3.5D, lane2) resulted in the hybridization of two bands at ~2.6 and 2.8 kb, suggesting that more than one gene is present. Finally, *Sal*I digestion (Fig.3.5D, lane3) resulted in the hybridization of two bands at ~2.5 and 2.9 kb, again suggesting that more than one gene is present.

The results of the same Southern blot hybridized with a L.c.FeSODB 3'probe (see schematic in Fig.3.5F for position) is shown in Fig.3.5E. Digestion with *Bam*HI (Fig.3.5E, lane1) revealed a single band of >12 kb, suggesting that *Bam*HI has cut sites on either side of the L.c.FeSODB (Refer to Fig.3.7B for a schematic summary of the results of this Southern hybridization). An *Xho*I digest (lane4) resulted in two hybridizing bands at ~6 and 5.5 kb, suggesting that more than one gene is present. Restriction with *Pst*I (lane 2) resulted in two bands at ~2.9 and 3.2 kb. The 3.2 kb band is consistent with the expected digestion product at the two internal *Pst*I sites within the L.c.FeSODB cDNA and the 2.9 kb band must arise from the internal *Pst*I cut site at the 3' end of the L.c.FeSODB gene and an additional external *Pst*I site downstream of the gene. Digestion with *Sal*I (Fig.3.5E, lane 3) revealed a single hybridizing band at ~3.8 kb which must

arise from the internal *Sal*I site within the L.c.FeSODB 3'UTR and an external *Sal*I site within the downstream region.

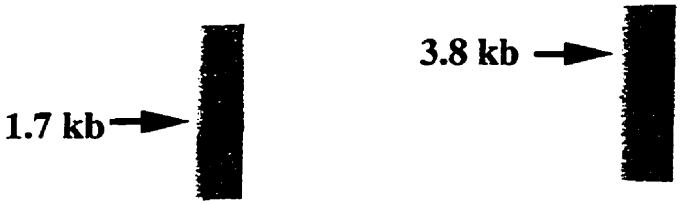
Based on the results of the Southern blot analysis, it is concluded that the L.c.FeSODB gene is not present as a single copy gene within the *L. chagasi* genome and, therefore, may be arranged as a multi-copy gene family. The model in Fig.3.7C suggests a possible tandem arrangement of the L.c.FeSODB gene in multi-copies within the genome.

D. Transcript size of L.c.FeSODA and L.c.FeSODB

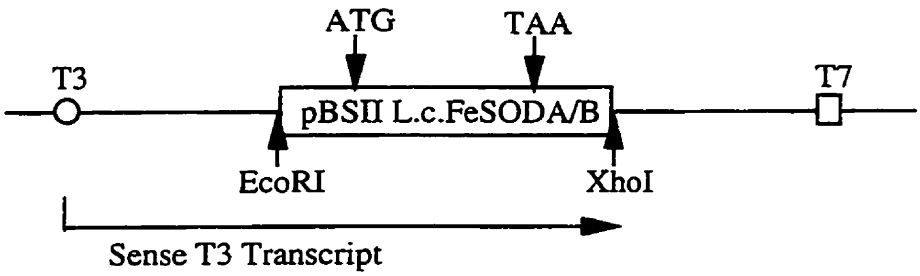
In order to analyze if the size of the transcript produced by L.c.FeSODA and L.c.FeSODB are similar to the predicted size from the cDNAs, 5 μg of total RNA from the stationary stage of the parasite was resolved on a formaldehyde-agarose gel and subjected to Northern blot analysis. The blot was hybridized with 3' UTR specific probes from L.c.FeSODA (the fragment amplified with the primers SODA3S/SODA3AS) and L.c.FeSODB (the 1.5 kb fragment restricted from the cDNA using *Eco*RI/*Xho*I). The results are shown in Fig.3.8A. The sizes of L.c.FeSODA and L.c.FeSODB were 1.7 kb and 3.8 kb respectively in accordance with the predicted transcript size. This confirms that complete full length cDNAs were isolated from the cDNA library.

Figure 3.8: Northern blot analysis of the L.c.FeSODA and L.c.FeSODB transcript sizes and *in vitro* translation of the L.c.FeSODA and L.c.FeSODB cDNAs.(A) Autoradiogram of a Northern blot of stationary phase total RNA of *L. chagasi* (5 μg) hybridized with a 3'UTR specific probe from L.c.FeSODA resulting in a 1.7 kb single transcript and a 3'UTR specific probe from L.c.FeSODB cDNA resulting in a 3.8 kb single transcript. **(B)** Schematic of the L.c.FeSODA and L.c.FeSODB cDNAs in pBluescript II SK (+/-) plasmid. The positions of the T3 and T7 phage promoters are shown. **(C)** Autoradiogram of *in vitro* translation reactions (2 μl of a 25 μl reaction loaded in each lane). Lane 1: T3 reaction carried out in the absence of plasmid template; Lane2: T3 reaction carried out with the Luciferase plasmid as the template (as supplied by the manufacturer); Lane3: T3 reaction carried out in the presence of pBS II L.c.FeSODA plasmid revealing a 26.4 kDa product; and Lane4: T3 reaction carried out in the presence of pBS II L.c.FeSODB plamsid revealing a 21.6 kDA product. Low molecular weight markers are shown on the left of the autoradiogram (Pharmacia).

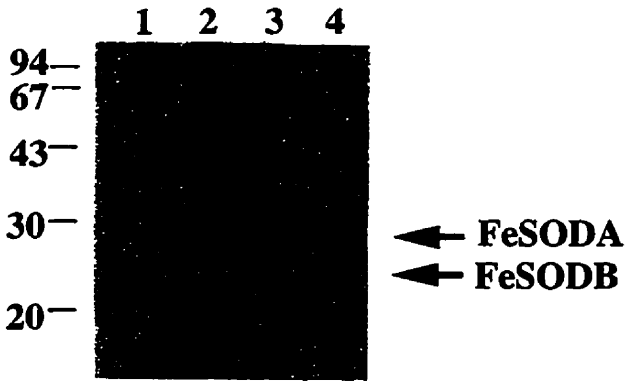
A


1.7 kb
3.8 kb

B


ATG
TAA
T3
T7
pBSII L.c.FeSODA/B
EcoRI
XhoI
Sense T3 Transcript

C


1 2 3 4
94
67
43
30
20
FeSODA
FeSODB

E. *In vitro* translation of L.c.FeSODA and L.c.FeSODB cDNA clones

The L.c.FeSODA and L.c.FeSODB isolated clones are present within the pBluescript II SK (+/-) vector as an EcoRI/XhoI fragment. T3 and T7 promoters are present within the pBluescript vector, with the T3 promoter residing upstream of the *Eco*RI cloning site and the FeSOD coding regions (See Fig.3.8B for a schematic of the vector). These pBluescript constructs were used to determine the size of the L.c.FeSODA and L.c.FeSODB proteins using a TNT coupled reticulocyte lysate system. 1 μg of Qiagen purified circular plasmid (pBSII L.c.FeSODA and pBSII L.c.FeSODB) was used for translation in this lysate system, using T3 RNA polymerase. The results of the *in vitro* translation reactions are seen in Fig. 3.8C. Lane 3 reveals a major band at ~26.4 kDa which agrees with the predicted protein size of L.c.FeSODA. Lane 4 reveals a major band at ~21.6 kDa which also agrees with the predicted protein size of L.c.FeSODB. As a negative control, the rabbit reticulocyte was translated with T3 RNA polymerase in absence of any template (Fig.3.8C, lane 1) which shows the absence of any major bands. As a positive control, a Luciferase plasmid was added to the rabbit reticulocyte lysate (which is provided by the manufacturer) and a major band was seen at ~94 kDa (Fig.3.8C, lane 2) which confirmed that the system was working.

III. Summary

1. The FeSODB cDNA clone from *L. chagasi* was sequenced in its entirety and found to contain a 230 nucleotide 5'UTR, a 588 nucleotide coding region and a very long 3004 nucleotide 3'UTR for a total cDNA length of 3822 bp.

2. The previously isolated clone L.c.FeSODA and L.c.FeSODB showed complete conservation of the four invariant residues completely conserved in all iron-containing SODs, comprising the acitve site of the enzyme. As well, the four reported invariant residues which differentiate FeSODs from MnSODs were conserved within both of these enzymes.

3. Southern blot analysis suggested that L.c.FeSODA is a single copy gene within the *L. chagasi* genome and is localized to a lower molecular weight chromosome. L.c.FeSODB was shown to be arranged as a multi-copy gene within the *L. chagasi* genome and localized to a higher molecular weight chromosome.

4. Northern analysis revealed that L.c.FeSODA showed a single transcript of 1.7 kb in size, whereas, L.c.FeSODB showed a single transcript size of 3.8 kb.

5. L.c.FeSODA and L.c.FeSODB cDNAs encode for proteins of 26.4 and 21.6 kDa respectively as determined by *in vitro* coupled transcription/translation reactions.

CHAPTER 4

FUNCTIONAL ANALYSIS OF L.c.FeSODA AND L.c.FeSODB cDNAs

I. Introduction

Chapter 3 involved the characterization of the L.c.FeSODA and L.c.FeSODB cDNAs which showed that the isolated cDNA sequences code for the iron-containing superoxide dismutase enzymes of *L. chagasi*. This chapter presents results of experiments designed to understand the possible functional role these enzymes have in the parasite life cycle.

II. Results and Discussion

A. Differential expression of L.c.FeSODA and L.c.FeSODB in *L. chagasi*

1. Differential gene regulation

It was important to determine the regulation of L.c.FeSODA and L.c.FeSODB gene expression during the different growth phases and life cycle stages. To this end, total RNA was isolated from both the log and stationary phase of the promastigote stage of the parasite. As well, total RNA was isolated from the infective form of the parasite, in the amastigote stage. This was achieved by the infection of a human macrophage cell line (U937) with stationary phase promastigotes. It has been established that upon entry of the stationary phase promastigote into the macrophage cell through phagocytosis, the parasite goes through a drastic morphological change, including rounding up and loss of motility, and resides within the phagolysosome of the macrophage cell (Streit *et al.,* 1996). Fig.4.1A and 4.1B can be compared to observe the

Figure 4.1. Uninfected and *L. chagasi* infected human macrophage U937 cells. (A) Photograph of an uninfected human macrophage cell. The nucleus (N) is stained a dark purple in the center of the cell, surrounded by the lighter purple stained cytoplasm which contains many empty vacuoles (V). **(B)** Photograph of a *L. chagasi* infected human macrophage cell, three days after infection. Leishmania parasites in the amastigote stage appear as darkly stained spherical particles within the cytoplasm (Am). **(C)** Photograph of a *L. chagasi* infected human macrophage cell, six days after infection. The darkly stained nucleus is being extruded from the cell.

A

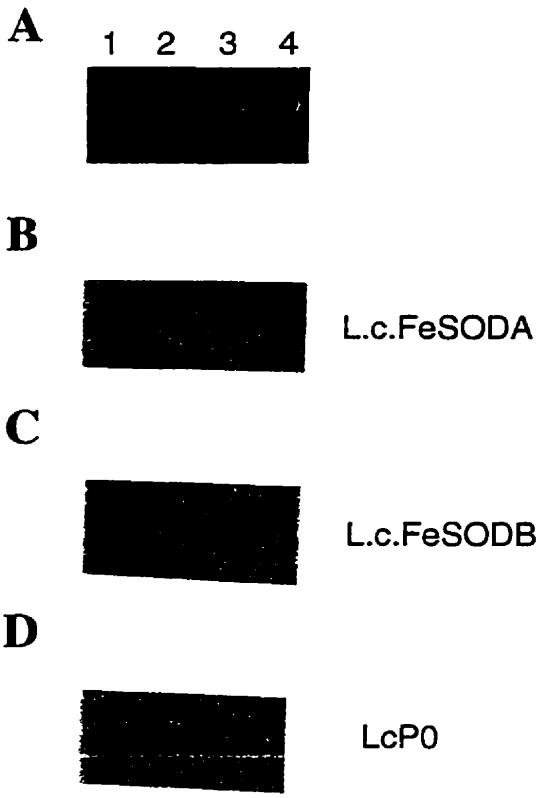
B

C

differences between non-infected and infected U937 cells. The amastigote (Am) forms are visible as darkly stained circular structures within the cytoplasm of the macrophage cell (Fig.4.1B and C). The morphological change of the parasite is believed to be accompanied by changes in gene expression.

Varying amounts of RNA from the log, stationary and amastigote stage (infected U937 macrophages) of the parasite were blotted and studied by Northern analysis. Fig.4.2A shows the ethidium bromide stained gel of log phase RNA (lane 1), stationary phase RNA (lane 2), amastigote-infected U937 RNA (lane 3) and U937 RNA alone (lane 4). Because the majority of the total RNA arises from the U937 macrophage cells, an increased amount of amastigote total RNA was loaded as compared to the promastigote RNA. This RNA was blotted to a nylon membrane and probed with either a L.c.FeSODA 3'UTR specific probe (SODA3S/SODA3AS) (Fig.4.2B) or a L.c.FeSODB 3'UTR specific probe(1.5 kb EcoRI/XhoI fragment (Fig.4.2C). The amount of RNA loaded was normalized to the intensity of the LcP0 transcript signal (Fig.4.2D), which is a constituitively expressed ribosomal binding protein in *L. chagasi* (Dr. Y. Skieky, personal communication). After normalizing to the intensity of the LcP0 mRNA signal using densitometry scanning, the levels of L.c.FeSODA mRNA in stationary phase promastigotes were 1.5 fold higher than levels in log phase promastigotes. A further 1.5 fold increase in L.c.FeSODA mRNA was seen in the amastigote stage as compared to the stationary phase promastigtoes, resulting in an overal 2.25 fold increase from log phase promastigotes to the amastigote

Figure 4.2. Differential expression of L.c.FeSODA and L.c.FeSODB transcript levels. (A) Ethidium bromide stained gel of total RNA isolated from: lane 1-log phase promastigote parasites (3 μg), lane 2-stationary phase promastigote parasites (3 μg), lane 3-amastigote stage parasites from U937 infected cells (20 μg), and lane 4-uninfected U937 cells (20 μg). The three *Leishmania* ribosomal bands seen in lanes 1 and 2 are faint in lane 3. The middle ribosomal RNA band of *Leishmania* migrates with the 18S band of U937 RNA which explains the more prominent middle band. **(B)** Autoradiograph of northern blot hybridized with a 3'UTR probe from L.c.FeSODA. **(C)** Autoradiograph of northern blot hybridized with a 3'UTR probe from L.c.FeSODB. **(D)** Autoradiograph of northern blot hybridized with a LcP0 probe which is constitutively expressed in *L. chagasi.* This was used as a control for densitometry scanning using three separate trials.


A
1 2 3 4
B
L.c.FeSODA
C
L.c.FeSODB
D
LcP0

stage.

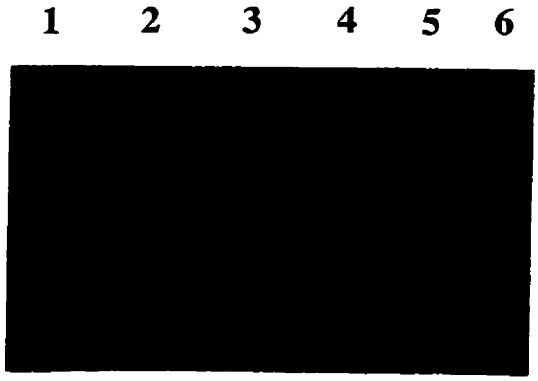
The level of L.c.FeSODB mRNA was about 3 fold higher in stationary phase promastigotes as compared to log phase promastigotes. A further 3 fold increase in message was seen in amastigote stage parasites as compared to stationary phase promastigotes.

To study if there was an increase in SOD protein activity from the log to stationary phase, SOD assays were performed on non-denaturing protein gels using whole cell extracts from each one of these stages. As seen in Fig. 4.3A (lanes 3 and 4) an increase in SOD activity was observed from log to stationary phase. Identical gels were incubated with either 5 mM H_2O_2 or 2 mM KCN. In the presence of H_2O_2 (Fig.4.3B), which inactivates FeSOD enzymes, the activity bands in log phase (lane3) and stationary phase (lane 4) completely disappeared, as did the positive control FeSOD band from *E. coli* (lane 2, bottom band). Further, in the presence of 2 mM KCN (Fig.4.3C), the activity bands in the various phases were not inactivated, while the Cu/ZnSOD band from bovine was completely inactivated (Fig.4.3C, lane 1). These data indicate that the activity band in the whole cell extracts (WCEs) from *L. chagasi* are iron-containing enzymes.

Taken together, the increase in transcript levels of both L.c.FeSODA and L.c.FeSODB from log phase to stationary phase promastigotes correlates with the increase in enymatic activity seen from log phase to stationary phase promastigotes. Because only stationary phase promastigotes are able to infect

Figure 4.3. Enzymatic assay of SOD activity using a 8% non-denaturing polyacrylamide gel, showing differential protein activity from log to stationary phase. (A) Samples loaded are as follows: lane 1: Bovine Cu/ZnSOD (2 μg; Sigma Chemicals), lane 2: *E. coli* MnSOD (upper band) and FeSOD (lower band) (2 μg each; Sigma Chemicals), lane 3: log-phase protein extract (120 μg), lane 4: stationary-phase protein extract (120 μg), lane 5: purified FeSODA-GST fusion protein (5 μg), and lane 6: GST protein alone (5 μg). **(B)** Identical gel as shown in (A) except that it was treated with 5 mM H_2O_2. **(C)** Identical gel as shown in (A) except that it was treated with 2 mM KCN.

A



B

C

macrophage cells, this increase in FeSOD mRNA levels from log to stationary phase may be adaptive and reflect an increased requirement for SOD to successfully infect the macrophage. Once a stable infection is attained within the phagolysosome of the macrophage cell, steady state levels of L.c.FeSODA and L.c.FeSODB mRNA increase further, possibly because the L.c.FeSODA and L.c.FeSODB protein may play a role in survival of the parasite inside the host.

Recently, SODs have been implicated in the stationary-phase survival of *Legionella pneumophila*, an intracellular bacteria. St. John and Steinman (1996) showed that the activity of a Cu/ZnSOD was increased during the transition from log to stationary-phase growth. In this study, we have shown a similar increase in both L.c.FeSODA and L.c.FeSODB transcript levels, as well as enzymatic activity from log phase to stationary phase of the promastigote stage of *L. chagasi.* A further increase in transcript level was also observed from the stationary phase to the amastigote stage of the parasite. Activated macrophages have been shown to kill intracellular pathogens via two separate oxidative pathways involving ROIs and reactive nitrogen intermediates (RNIs) (Babior, 1984; Denis, 1994). Because of their simultaneous generation, products of the two pathways such as O_2^- and nitric oxide, have been shown to interact to form peroxynitrite, which is a stronger oxidant than the reactants (Liew *et al.,* 1990). Therefore, elimination of O_2^- could alleviate this component from this deadly reaction. This could suggest a reason for the increase in both SODs of *L. chagasi* during the various developmental stages. The differential expression of

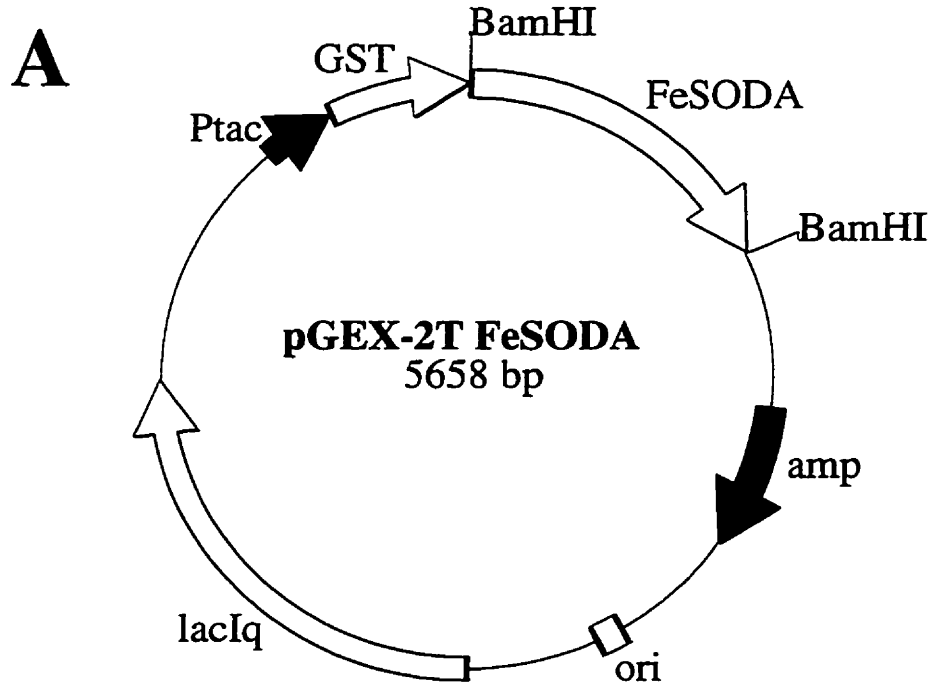
L.c.FeSODA and L.c.FeSODB in the stationary phase and further in the amastigote stage could implicate these enzymes in the preparation and ultimately the protection of these parasites upon infection into the macrophage cell.

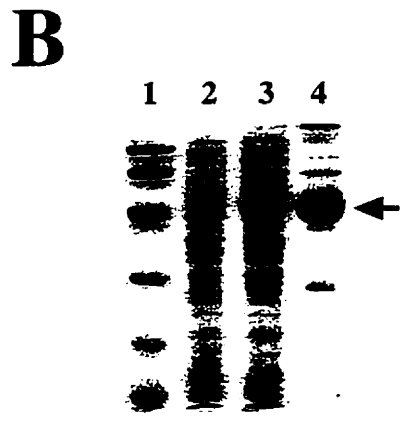
B. Overexpression, Complementation and enzymatic activity of L.c.FeSODA in *E. coli*

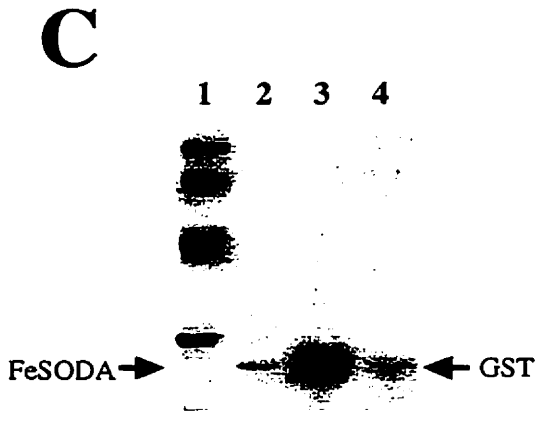
1. pGEX-2T L.c.FeSODA activity and expression

The L.c.FeSODA protein was overexpressed in *E. coli* by cloning the coding region of the L.c.FeSODA cDNA in an *E. coli* expression vector pGEX-2T (Smith and Johnson, 1988). This expression vector works by directing synthesis of foreign polypeptides in *E. coli* as fusions with the carboxy terminus of the 27.5 kDa glutathione S-transferase (GST) protein. As seen in Fig. 4.4A, the construction of the recombinant vector was performed by amplifying the entire coding region of the L.c.FeSODA cDNA using the two primers SODAS and SODAAS (see Table 2.1). The resulting product was digested with *Bam*HI and ligated into the *Bam*HI site of the pGEX-2T vector. The resulting vector is referred to as pGEX-2T FeSODA. Little expression is seen in untreated culture (Fig.4.4B, lane 2), but when induced, the GST-FeSODA fusion protein is expressed as a 53.9 kDa protein, with part of the protein in the soluble fraction (Fig.4.4B, lane 4) and part partially insoluble (lane 3). The GST-FeSODA fusion protein was purified on glutathione agarose beads and subsequently cleaved

Figure 4.4. pGEX-2T FeSODA vector and coomassie stained gels showing *E. coli* expressed FeSODA-GST fusion protein and thrombin cleaved FeSODA-GST proteins. (A) Circular map of pGEX-2T FeSODA (5658 bp) constructed by cloning the coding region of the L.c.FeSODA cDNA (amplified with SODAS and SODAAS) into the *Bam*HI site of the plasmid. The L.c.FeSODA coding region is cloned in frame and downstream of the GST sequence. **(B)** Coomassie stained gel showing uninduced FeSODA-GST extract (lane 2), pellet fraction of induced FeSODA-GST extract (lane 3) and aqueous fraction (supernatant) of induced FeSODA-GST extract (lane 4) (Arrow shows the position of the fusion product at ~ 53.9 kDa). 10 µl of each extract was loaded in each lane. **(C)** Coomassie stained gel of thrombin cleavage products of FeSODA-GST. Purified cleaved FeSODA expressed protein (lane 2) running at 26.4 kDa, cleaved FeSODA and GST proteins (lane 3) running at 26.4 kDa and 27.5 kDa respectively, and purified cleaved GST expressed protein (lane 4) running at 27.5 kDa. Protein molecular weight standards are shown in lane 1 of **(B)** and **(C).**


A
BamHI
GST
FeSODA
Ptac
BamHI
pGEX-2T FeSODA
5658 bp
amp
lacIq
ori

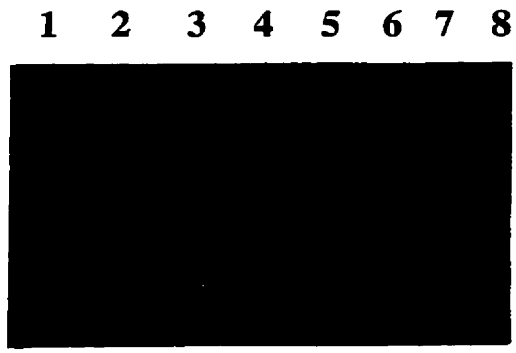

B
1 2 3 4


C
1 2 3 4
FeSODA
GST

with thrombin resulting in a 27.5 kDa GST band (Fig.4.4C, lane 4 and lane 3, upper band) and a 26.4 kDa L.c.FeSODA band (lane 2 and lane3, lower band).

The pGEX-2T FeSODA recombinant plasmid was transformed into two different strains of *E. coli* for further complementation experiments. One *E. coli* mutant strain, QC779, is devoid of both the *E. coli* MnSOD (*sodA*) gene and the *E. coli* FeSOD (*sodB*) gene which provides a null SOD background. It is important to note than when this null SOD *E. coli* strain was obtained (Carlioz and Touati, 1986), the presence of an *E. coli* Cu/ZnSOD was not discovered until 6 years later. However, in all of the activity assays performed, Carlioz and Touati (1986) never observed the presence of a Cu/ZnSOD band which suggests it is present at very low levels and does not influence the activity or complementation assays that were performed. As a control, the pGEX-2T FeSODA plasmid also was transformed into GC4468, which is the parental strain of QC779 containing both the Mn and FeSODs of *E. coli*. In order to assay for activity of the overexpressed recombinant protein in these two strains, whole cell extracts were prepared from both the untransformed *E. coli* strains, GC4468 and QC779, as well as from cells transformed with the recombinant FeSODA-GST plasmid and original plasmid without insert. As shown using an activity assay (Fig.4.5), the GC4468 cells possess both and MnSOD and FeSOD band (lane 3) as expected and the QC779 cells possess no detectable activity (lane 6) as expected. Assay of the two strains transformed with the pGEX-2T vector showed no difference from the untransformed cells (lanes 4 and 7 respectively).

Figure 4.5. Enzymatic assay of SOD activity using a 8% non-denaturing polyacrylamide gel, showing activity of expressed recombinant protein, FeSODA-GST, in GC4468 and QC779 cells. (A) Samples loaded are as follows: lane 1: Bovine Cu/ZnSOD (2 μg; Sigma Chemicals), lane 2: *E. coli* MnSOD (upper band) and FeSOD (lower band) (2 μg each; Sigma Chemicals), lane 3: protein extract from GC4468 cells (10 μg), lane 4: protein extract from GC4468 cells transformed with pGEX-2T vector alone (10 μg), lane 5: protein extract from GC4468 cells transformed with pGEX-2T FeSODA recombinant vector (10 μg), lane 6: protein extract from QC779 cells (10 μg), lane 7: protein extract from QC779 cells transformed with pGEX-2T vector alone (10 μg), and lane 8: protein extract from QC779 cells transformed with pGEX-2T FeSODA recombinant vector (10 μg).


1 2 3 4 5 6 7 8

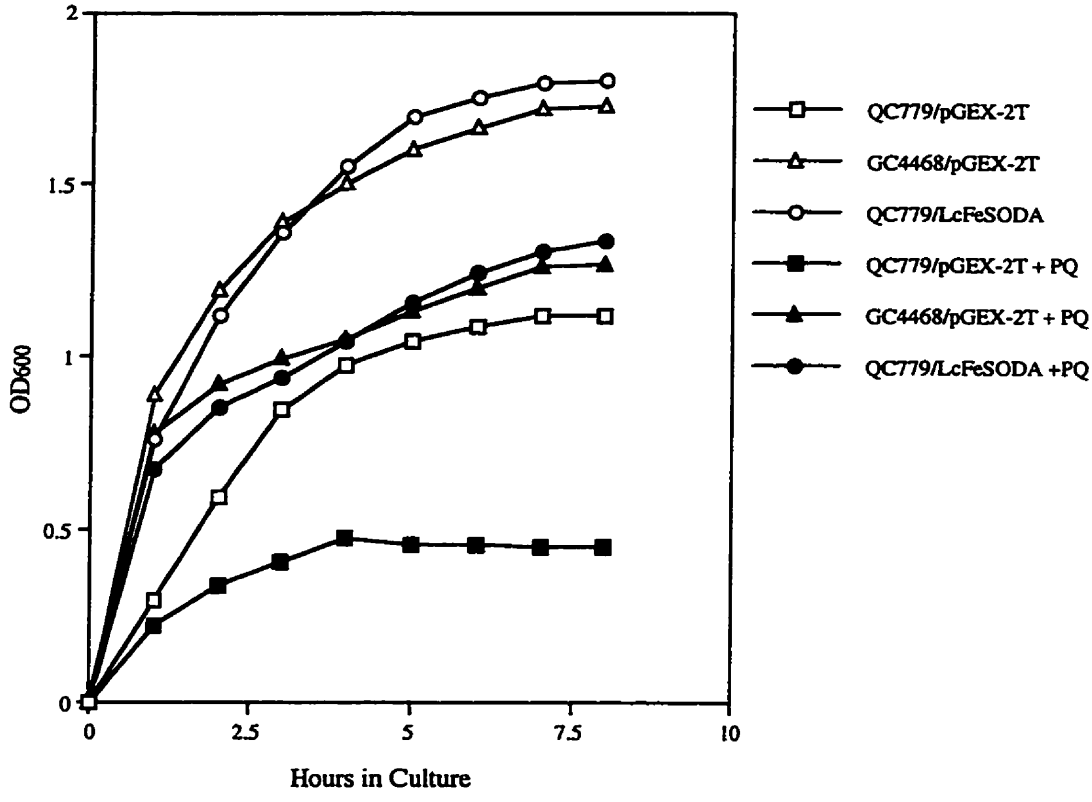
When the two strains were transformed with the recombinant FeSOD expression vector, two extra bands appeared in the GC4468 strain (lane 5) and two bands appeared in the QC779 strain (lane 8). These results suggest that the recombinant protein, GST-FeSODA is active within these cells. The presence of two bands in the QC779 strain transformed with the recombinant vector may be due to dimer or tetramer formation.

2. Complementation of null SOD *E. coli* strain (QC779) with L.c.FeSODA

In order to study the functional role of the L.c.FeSODA protein, the null SOD strain, QC779 was transformed with the pGEX-2T FeSODA recombinant plasmid and challenged with paraquat, a herbicide that has been shown to produce superoxide radicals *in vivo* (See Table 2.3 for compound formula and products produced). The *E. coli* strain, QC779 has previously been shown by Carlioz and Touati (1986) to be highly sensitive to paraquat toxicity. As shown in Fig.4.6, L.c.FeSODA restored the growth of QC779 cells in the presence of 0.05 mM paraquat to the level of the parental strain, GC4468 in the presence of 0.05 mM paraquat, thus suggesting *L. chagasi* FeSODA is able to complement the function of *E.coli* SODs.

Repeated attempts to clone the coding region of L.c.FeSODB into the pGEX-2T vector, in a variety of strains, were unsuccessful. Possibly overexpression of L.c.FeSODB is lethal to the cells. This prevented complementation experiments in *E. coli* with L.c.FeSODB to be performed, but

Figure 4.6. Effect of paraquat on the growth of *E. coli* transformants. Sensitivity to paraquat was determined by growth inhibition of liquid cultures. Paraquat (0.05 mM) was added to log phase cultures and the OD was recorded hourly for 8 hours. The *E. coli* transformants tested for complementation were GC4468/pGEX-2T, QC779/pGEX-2T and QC779/pGEX-2TFeSODA. Lines connecting open points represent growth curves of cultures grown in the absence of paraquat. Lines connecting solid points represent growth curves of cultures grown in the presence of paraquat. Growth curves were perfomed in triplicate and the average was graphed.


2
1.5
1
0.5
0
OD600
0
2.5
5
7.5
10
Hours in Culture
QC779/pGEX-2T
GC4468/pGEX-2T
QC779/LcFeSODA
QC779/pGEX-2T + PQ
GC4468/pGEX-2T + PQ
QC779/LcFeSODA +PQ

previous studies with *Trypanosoma cruzi* FeSODB (which showed high identity with L.c.FeSODB) revealed that T.c.FeSODB was able to complement *E. coli* in the same manner as L.c.FeSODA (Ismail *et al.*, 1997).

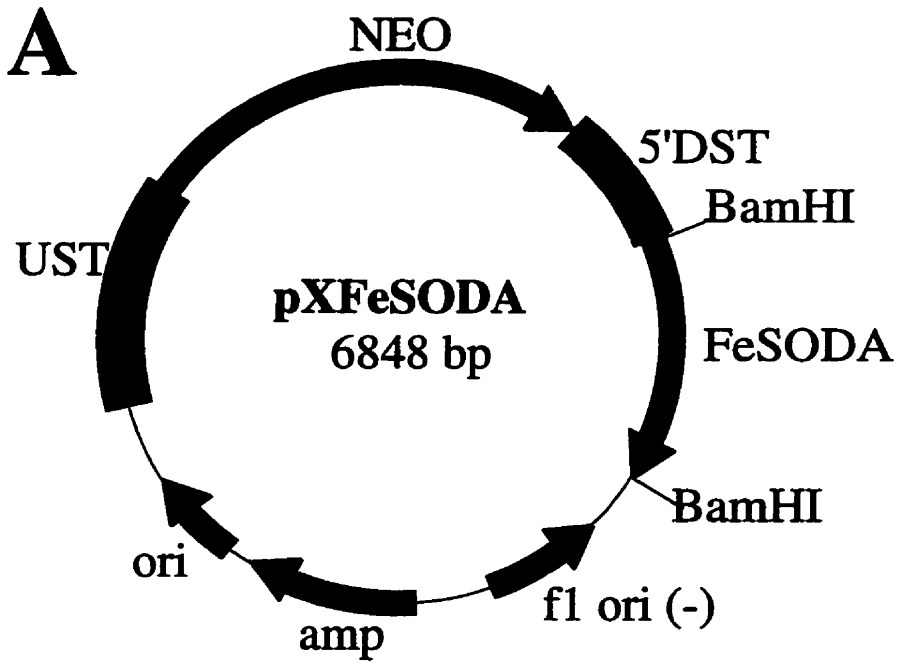
C. Overexpression of L.c.FeSODA and L.c.FeSODB in *L. chagasi*

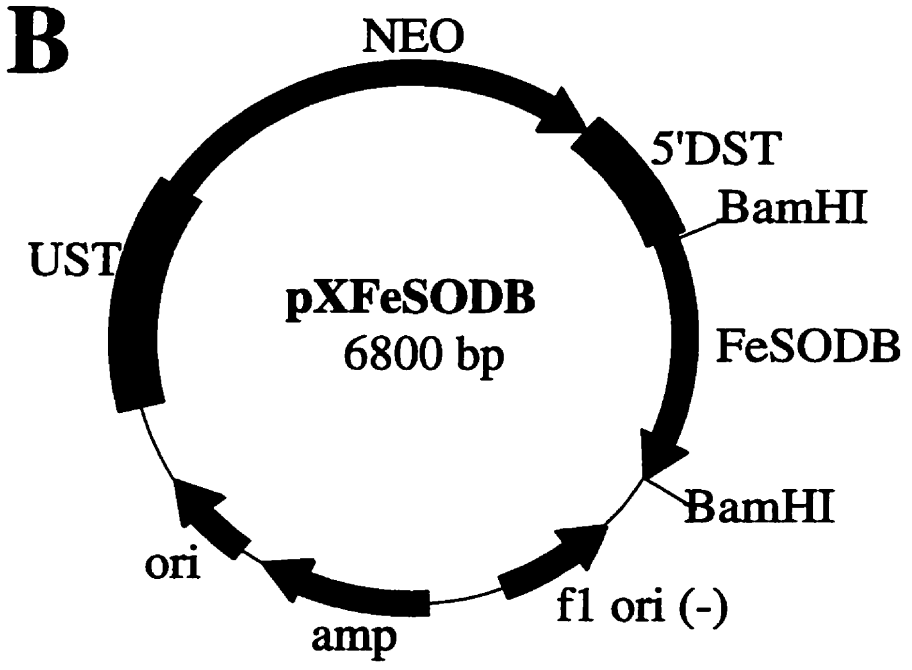
1. Cloning of L.c.FeSODA and L.c.FeSODB in the pX plasmid

To study the effects of overexpressing L.c.FeSODA and L.c.FeSODB in *L. chagasi*, the entire coding region of both of these cDNAs were cloned into the *Bam*HI site of the pX plasmid, as shown in Fig.4.7A (pXFeSODA) and Fig.4.7B (pXFeSODB). The pX plasmid is a specially designed *Leishmania* specific expression vector (LeBowitz *et al.*, 1990) which contains the neomycin (NEO) gene (selectable marker) in conjunction with part of the dihydrofolate reductase gene (DHFR) of *L. major* producing a fusion hybrid NEO/DHFR mRNA. As well, the NEO/DHFR region is flanked by the intergenic regions of the DHFR gene called the UST and 5'DST regions (Fig. 4.7). When this vector is transfected into *Leishmania* cells, it is maintained extrachromosomally (LeBowitz *et al.*, 1990) and directs the synthesis of a polycistronic transcript of the NEO/DHFR region and the cloned fragment within the multiple cloning site of the vector. The UST and 5'DST regions contain the appropriate signals for proper trans-splicing and polyadenylation of the transcripts.

The recombinant plasmids, pXFeSODA and pXFeSODB, were transfected into *L. chagasi* cells and selected in liquid medium to a concentration of 400

Figure 4.7. Circular maps of pXFeSODA and pXFeSODB plasmids. (A) The coding region of L.c.FeSODA (amplified with SODAS and SODAAS) was cloned into the *Bam*HI site of the pX vector to obtain the recombinant vector pXFeSODA (6848 bp). **(B)** The coding region of L.c.FeSODB (amplified with SODBATG and SODBTAA) was cloned into the *Bam*HI site of the pX vector to obtain the recombinant vector pXFeSODB (6800 bp).


A
NEO
5'DST
BamHI
FeSODA
BamHI
f1 ori (-)
amp
ori
UST
pXFeSODA
6848 bp


B
NEO
5'DST
BamHI
FeSODB
BamHI
f1 ori (-)
amp
ori
UST
pXFeSODB
6800 bp

μg/ml G418. This was the concentration used for the following experiments unless otherwise noted. No differences in morphology or growth rates were observed for these two transfectants as compared to wild type *L. chagasi* parasites (data not shown).

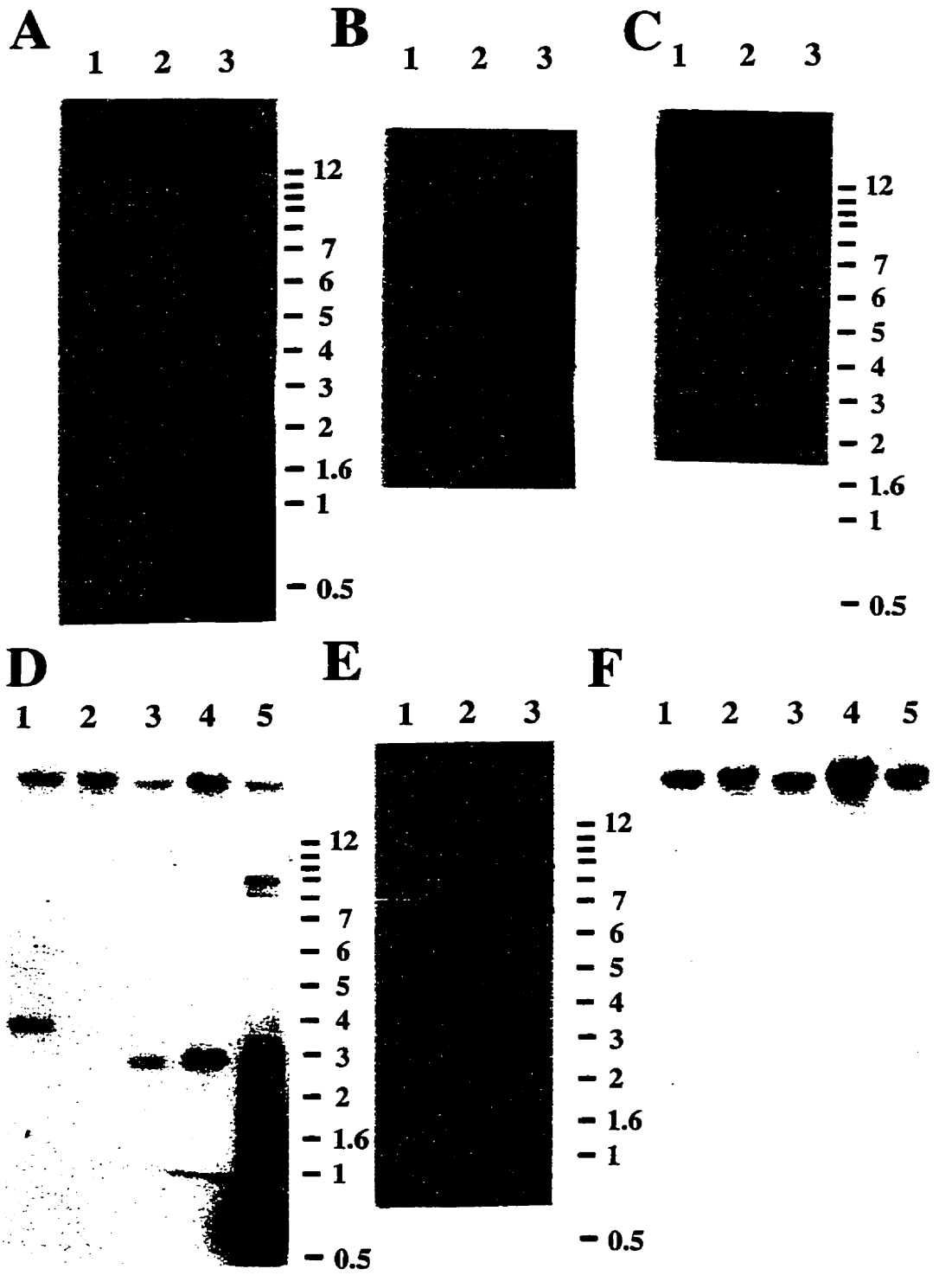
2. Southern and Northern blot analysis of pX FeSODA and pX FeSODB.

Genomic DNA from wild type, pX transfected cells and pXFeSODA and pXFeSODB cells was isolated and digested with *Bam*HI. This DNA was then subjected to Southern blot analysis with three different probes. The results of the Southern analysis with pXFeSODA is shown in Fig.4.8A, B and C. Fig.4.8A is probed with a coding region specific probe from L.c.FeSODA. As can be seen in lane 1 and 2, a high molecular weight band (>12 kb) is seen in the wild type DNA and cells transfected with the pX plasmid alone (identical to the result seen in Fig.3.5A, lane1). Lane 3 (pXFeSODA transfected cells) contains three bands: one >12 kb, one at ~ 7 kb and one band at approximately 700 bp. These data suggest that the coding region (700 bp band) was from the plasmid and is present in large amounts. This insert is not seen in the two control lanes. The extra band at ~7 kb in lane 3 is probably due to linearized plasmid because it is running at the expected molecular weight.

A blot with similar digests, was hybridized with a probe from the neomycin gene coding region as seen in Fig.4.8B. The NEO probe hybridized to both the pX transfected cells, as well as the pXFeSODA transfected cells (lanes 2 and 3)

Figure 4.8. Southern blot analysis of wild type, pX, pXFeSODA and pXFeSODB transfected cells. (A), (B) and (C) Lane 1: wild type DNA digested with *Bam*HI; lane 2: DNA from pX transfected cells digested with *Bam*HI and lane 3: DNA from pXFeSODA transfected cells digested with *Bam*HI. **(A)** Autoradiogram of Southern blot hybridized with a L.c.FeSODA coding region specific probe (SODAS/SODAAS), **(B)** NEO specific probe and **(C)** L.c.FeSODA 3' UTR specific probe (SODA3S/BGLSODAKO).

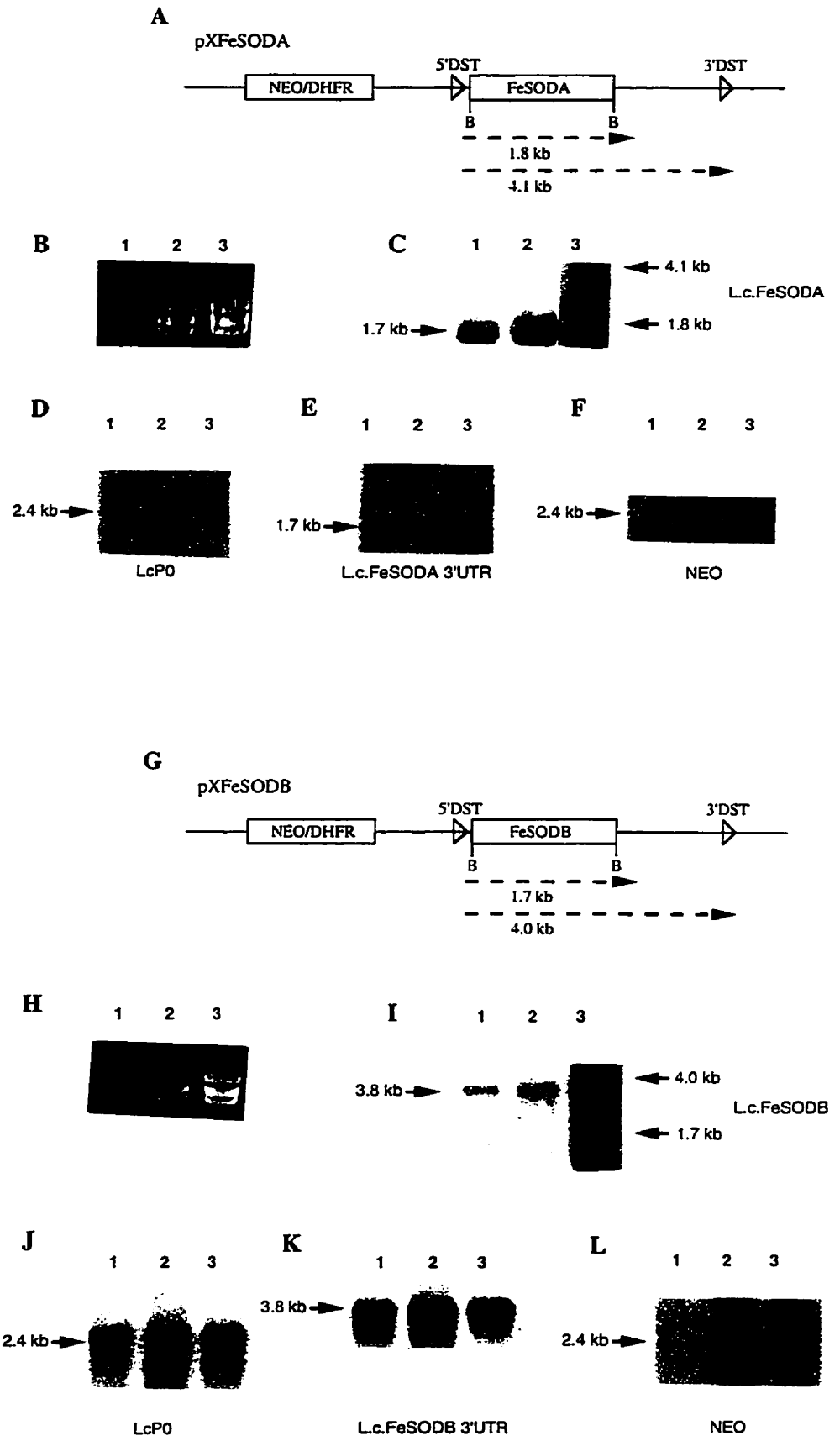
(D) and **(F)** Lane 1: wild type DNA digested with *Bam*HI; lane 2: wild type DNA digested with *Xho*I; lane 3: wild type DNA digested with *Bam*HI / *Xho*I; lane 4: DNA from pX transfected cells digested with *Bam*HI / *Xho*I and lane 5: DNA from pXFeSODB transfected cells digested with *Bam*HI / *Xho*I. **(E)** Lane 1: wild type DNA digested with *Bam*HI/*Xho*I; lane 2: DNA from pX transfected cells digested with *Bam*HI / *Xho*I and lane 3: DNA from pXFeSODB transfected cells digested with *Bam*HI / *Xho*I. **(D)** Autoradiogram hybridized with a L.c.FeSODB coding specific probe (SODBATG/SODBTAA), **(E)** Neo specific probe, and **(F)** a L.c.FeSODB 3'UTR specific probe (*Eco*RI / *Xho*I fragment).


A
1 2 3
12
7
6
5
4
3
2
1.6
1
0.5
B
1 2 3
C
1 2 3
12
7
6
5
4
3
2
1.6
1
0.5
D
1 2 3 4 5
12
7
6
5
4
3
2
1.6
1
0.5
E
1 2 3
12
7
6
5
4
3
2
1.6
1
0.5
F
1 2 3 4 5

two primers that were used for cloning, that the entire L.c.FeSODB coding region was present.

Fig.4.8D is probed with a coding region specific probe from L.c.FeSODB. As can be seen in lanes 1, 2 and 3, a high molecular weight band (>12 kb) is seen in the wild type DNA and cells transfected with the pX plasmid alone (identical to the result seen in Fig.3.5E, lane1). Lane 3 and 4 show two identical bands, one >12 kb and one band at approximately 2 kb indicating that the wild type cells digested with *Bam*HI and *Xho*I give two bands. In lane 5, two hybridizable bands are observed in the the pXFeSODB transfected cells, as was observed in the wild type cells. As well, there is an extra very strong band at ~ 800 bp, the insert released from the pX vector and is not present in the two control lanes. A similar blot was hybridized with a probe from the neomycin gene coding region as seen in Fig.4.8E. The NEO probe hybridized to the pX transfected cells as well as the pXFeSODA transfected cells (lanes 2 and 3) but not to the wild type cells (lane 1). This suggests that the untransfected cells do not carry a copy of the neomycin transferase gene, whereas the two transfected strains do provided by pX. The sizes of the bands seen in the two transfected strains agrees with the linearized size of the pX plasmid. Thus, the transfected plasmids again have remained extrachromosomal. As a further control, a similar blot to the two above was hybridized with a 3'UTR specific probe from L.c.FeSODB (Fig.4.8F). An endogenous hybridizing band of >12 kb was observed to be of equal intensity in each case.

Figure 4.9. Northern blot analysis of overexpressing pXFeSODA and pXFeSODB in *L. chagasi* cells and selected for with 400 μg/ml G418. (A) and **(G)** Schematics showing the probable origin of the transcripts from the overexpressing vector during post-transcriptional processing for pXFeSODA and pXFeSODB respectively. **(B)** and **(H)** Ethidium bromide stained gel.10 μg of total RNA was loaded in each lane from control untransfected cells (lane 1), control cells transfected with the pX vector alone (lane 2) and cells transfected with pXFeSODA **(B)** or cells transfected with pXFeSODB **(H).** The entire coding region of the L.c.FeSODA cDNA **(C)** or the L.c.FeSODB cDNA **(I)** was used as a probe. **(D)** and **(J)** are identical blots probed with LcP0 as a loading control. **(E)** and **(K)** are identical blots probed with L.c.FeSODA or L.c.FeSODB 3'UTR specific probes respectively. **(F)** and **(L)** are identical blots probed with a NEO specific probe. Arrows on the right side indicate the major and minor transcripts observed in overexpressing cells and their sizes. Arrows on the left side indicate the size and position of endogenous transcripts.


A
pXFeSODA
NEO/DHFR
5'DST
FeSODA
3'DST
B
B
1.8 kb
4.1 kb
B
1 2 3
C
1 2 3
4.1 kb
L.c.FeSODA
1.7 kb
1.8 kb
D
1 2 3
2.4 kb
LcP0
E
1 2 3
1.7 kb
L.c.FeSODA 3'UTR
F
1 2 3
2.4 kb
NEO
G
pXFeSODB
NEO/DHFR
5'DST
FeSODB
3'DST
B
B
1.7 kb
4.0 kb
H
1 2 3
I
1 2 3
3.8 kb
4.0 kb
L.c.FeSODB
1.7 kb
J
1 2 3
2.4 kb
LcP0
K
1 2 3
3.8 kb
L.c.FeSODB 3'UTR
L
1 2 3
2.4 kb
NEO

Total RNA from wild-type, pX transfected, pXFeSODA and pXFeSODB transfected parasites was isolated and analyzed using Northern blotting analysis. Fig.4.9B shows the ethidium bromide stained gel of the isolated RNA. The blot probed with a L.c.FeSODA coding specific probe revealed an endogenous transcript size of 1.7 kb in the wild type RNA (Fig4.9C, lane 1). A similar size transcript was observed in total RNA isolated from pX transfected cells (lane 2). The apparent increase in intensity from untransfected to pX-transfected parasites was eliminated after normalization with the LcP0 probe (Fig.4.9D). pXFeSODA transfected RNA (Fig.4.9C, lane 3) showed the hybridization of two major bands at 1.8 kb and 4.1 kb when hybridized with a L.c.FeSODA coding specific probe. The 4.1 kb band is not present in the lanes containing untransfected or pX transfected RNA. These results are consistent with polyadenylation occuring in the pXFeSODA transfected cells at the adventitious processing site (Fig.4.9A) within the pX plasmid backbone resulting in the smaller transcript, and with the larger transcript arising from polyadenylation at the regular site within the UST region (See schematic in Fig.4.9A) (LeBowitz *et al.*, 1993). As well, 1.7 kb may not be separable from the 1.8 kb band as there are no other bands present which could represent the endogenous band within the overexpressing RNA (lane 3). The 1.8 kb band is probably comprised of both endogenous and overexpressing L.c.FeSODA. To confirm the presence of an endogenous band within the overexpressing cells, this blot was hybridized with a 3'UTR L.c.FeSODA specific probe which would

not hybridize to the overexpressing RNA. An endogenous band was observed at 1.7 kb in each lane at equal intensity when normalized to the LcP0 probe (Fig.4.9E). Hybridization of this same blot with a *Neo*-specific probe resulted in the expected 2.4 kb *Neo*/DHFR hybrid transcript only in RNA from the transfectants (Fig.4.9F). After normalization to the constitutively expressed LcP0 transcript level (Fig.4.9D) it was concluded that pXFeSODA showed a much higher level of expression of transcript level compared to the endogenous L.c.FeSODA level.

Similar results were seen with L.c.FeSODB. Total RNA isolated from wild-type parasites (Fig.4.9H) and probed with a L.c.FeSODB coding specific probe detected the endogenous transcript (3.8 kb) (Fig.4.9I, lane 1). A similar sized transcript was seen in total RNA isolated from parasites transfected with the pX vector alone (Fig.4.9I, lane 2). Again the apparent increase in transcript level was eliminated when normalized to the LcP0 probe (Fig.4.9J). RNA from pXFeSODB transfected cells showed a minor transcript at about 1.7 kb and a major transcript at 4.0 kb when hybridized with a L.c.FeSODB coding specific probe (Fig.4.9I, lane 3). The 1.7 kb band was not observed in wild-type or pX transfected total RNA and can be explained as with pXFeSODA above with the presence of an adventitious processing site (See schematic Fig.4.9G). The absence of an exact 3.8 kb endogenous band suggested that it may not have separated from the 4.0 kb expression product. Thus the 4.0 kb overexpressing band contains both the endogenous and overexpressing RNA. The presence of

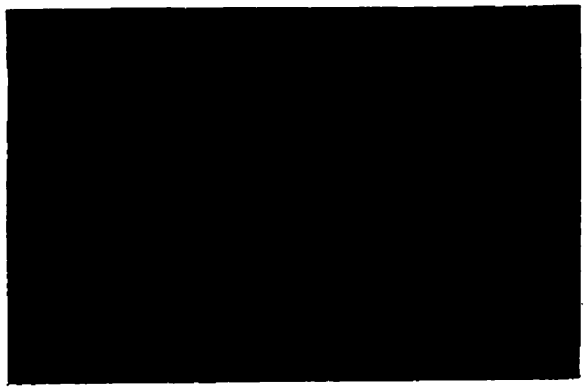
endogenous L.c.FeSODB RNA in the overexpressing cells was confirmed with the hybridization of this same blot with a L.c.FeSODB 3'UTR specific probe (Fig.4.9K). Hybridization of this same blot with a *Neo*-specific probe resulted in the expected 2.4 kb *Neo*/DHFR hybrid transcript only from the transfectants (Fig.4.9L). After normalization to the constitutively expressed LcP0 transcript level (Fig.4.9J) it was concluded that parasites transfected with pXFeSODB contained a higher level of L.c.FeSODB transcript compared to the control cells. It should be noted that no difference in parasite growth rate was detected in the transfected parasites as compared to the wild type (data not shown).

3. Enzymatic activity of whole cell extract protein from overexpressing parasites

In order to see if there was a correlation between the increase in FeSOD transcript levels and enzymatic activity, we performed SOD activity assays in non-denaturing cells using WCEs isolated from control and overexpressing parasites. As shown in Fig.4.10, the SOD activity was lower in untransfected and pX transfected parasites (lanes 3 and 4) compared to the pXFeSODA and pXFeSODB overexpressing parasites (lanes 5 and 6). This suggests that there is an increase in protein activity in overexpressing cells.

Figure 4.10. Enzymatic assay of SOD activity using a 8% non-denaturing polyacrylamide gel, showing greater protein activity in overexpressing cells as compared to wild type cells. Samples loaded are as follows: lane 1: Bovine Cu/ZnSOD (2 μg; Sigma Chemicals), lane 2: *E. coli* MnSOD (upper band) and FeSOD (lower band) (2 μg each; Sigma Chemicals), lane 3: log-phase protein extract (120 μg), lane 4: stationary-phase protein extract (120 μg), lane 5: pX transfected stationary cells (120 μg), lane 6: pXFeSODA transfected stationary cells (120 μg) and lane 7: pXFeSODB transfected stationary cells (120 μg).

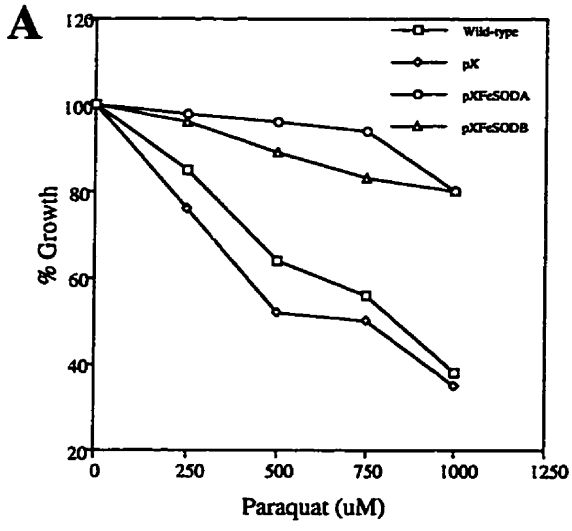
1 2 3 4 5 6 7

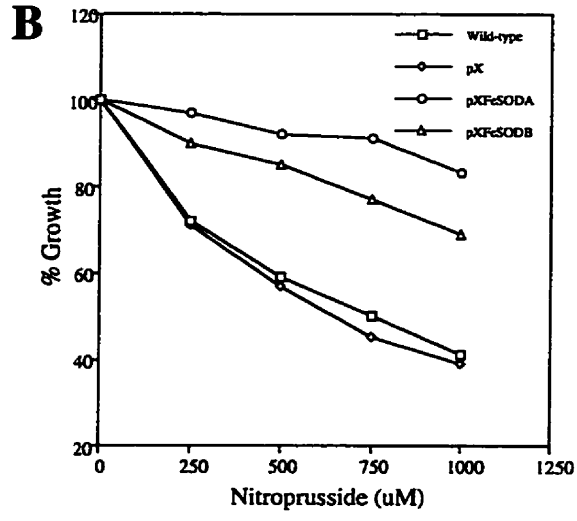


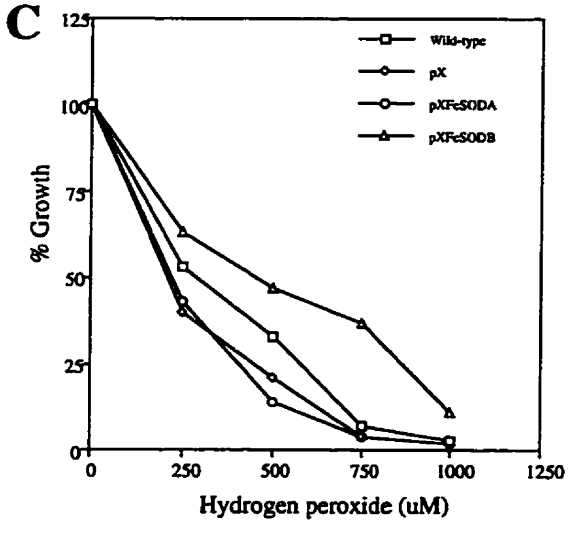
4. Growth curves of SOD overexpressing parasites treated with various oxygen free radical producing agents.

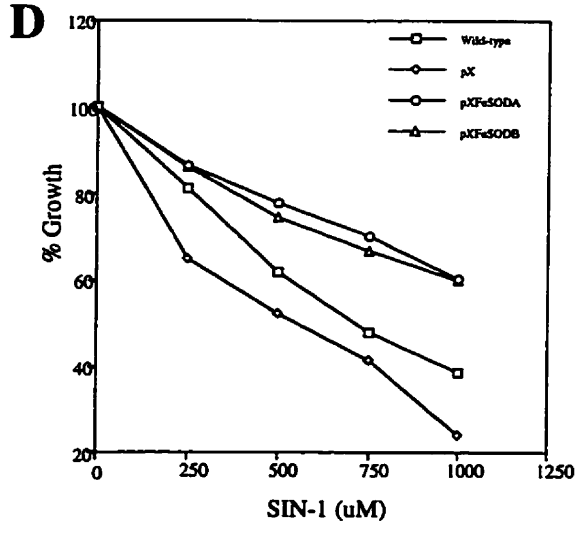
The effect of overexpressing SODs on the sensitivity of parasites to oxidative stress agents was measured by an assay of promastigote motility. Channon and Blackwell (1985) and Zarley *et al.,* (1991) established that the motility of *Leishamnia* sp. correlates with survival. *L. chagasi* parasites (wildtype and pX , pXFeSODA as well as pXFeSODB transfectants) were treated with increasing amounts of paraquat (Fig.4.11A), nitroprusside (B), hydrogen peroxide (C) or SIN-1 (D) and the presence of flagella was assayed on day 6 of growth. Transfectants overexpressing both L.c.FeSODA and L.c.FeSODB were more resistant to paraquat, nitroprusside and SIN-1 as compared to wildtype parasites or pX transfectants. This resistance was observed even at normally toxic levels of these reagents (1 mM concentration). These results suggest that overexpression of either L.c.FeSODA or L.c.FeSODB provides protection to *L. chagasi* parasites when exposed to ROI's. The overexpressing parasites showed the most protection against paraquat, with no less than 80% growth achieved.Since paraquat is known to produce O_2^-, and SODs function to eliminate O_2^-, then it would follow logically that they would provide a good defense against this agent. Nitroprusside produces both O_2^- and NO and the FeSOD overexpression provided protection to 70% growth. A similar pattern was seen with the agent SIN-1, which produces NO and O_2^-, with the overexpressing cells displaying 60% growth. This indicates that the overexpressing SOD

Figure 4.11. Effect of paraquat (A), nitroprusside (B), hydrogen peroxide (C) and SIN-1 (D) on *L. chagasi* parasites ovexpressing L.c.FeSODA and L.c.FeSODB. Wild-type, pX control, pXFeSODA and pXFeSODB parasites were seeded at 1 X 10^6 cells and treated with varying amounts of agents as indicated in the graph and their motility was monitored on day 6 as detailed in the materials and methods. Three trials were averaged in each case and presented as % growth.


A
% Growth
Paraquat (uM)
Wild-type
pX
pXFeSODA
pXFeSODB


B
% Growth
Nitroprusside (uM)
Wild-type
pX
pXFeSODA
pXFeSODB


C
% Growth
Hydrogen peroxide (uM)
Wild-type
pX
pXFeSODA
pXFeSODB


D
% Growth
SIN-1 (uM)
Wild-type
pX
pXFeSODA
pXFeSODB

enzymes do not provide as good of a defense to the parasites for agents which produce NO as well. This could be due to the fact that the O_2^- and NO are forming peroxynitrite at a faster rate than the SOD enzymes are able to eliminate the O_2^- and thus the peroxynitrite is adversely affecting the overexpressing parasites. Treatment of FeSOD overexpressing cells with H_2O_2 (Fig.4.11C) did not result in protection as compared to the controls. This reflects the cascade nature of anti-oxidant enzymes, since H_2O_2 is downstream of SOD and cannot be eliminated by SOD. Also, H_2O_2 is known to inactivate iron-containing SODs by oxidizing tyrptophan residues, resulting in probable modification of the active-site environment (Beyer and Fridovich, 1987).

D. L.c.FeSODA and L.c.FeSODB gene knock-outs

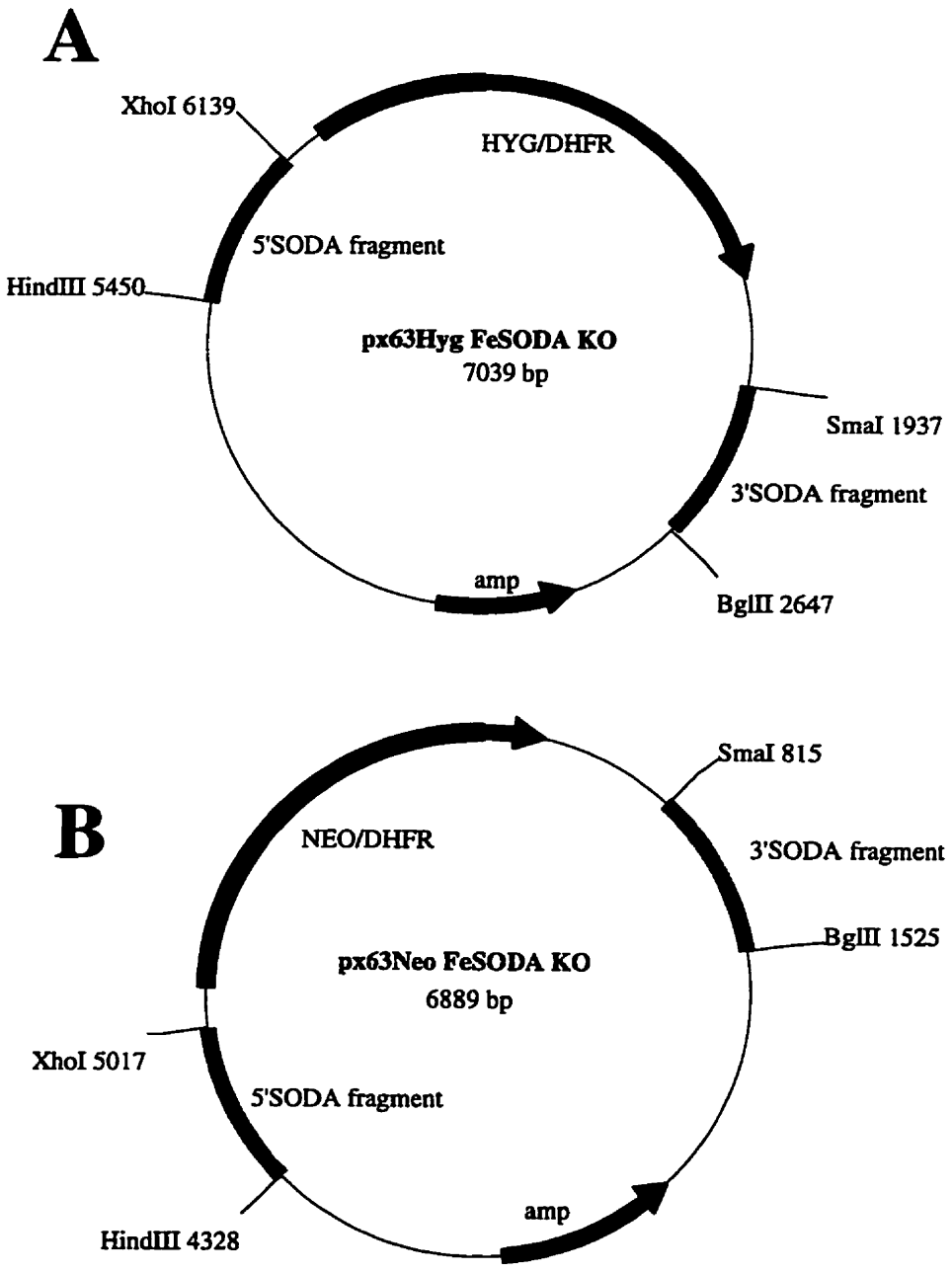
In an attempt to further study the functional role of the L.c.FeSODA and L.c.FeSODB genes in *Leishmania*, steps for creating *L. chagasi* SOD-null mutants were initiated using vectors containing the selectable markers neomycin and hygromycin phosphotransferases. Gene replacement for the purpose of creating null mutants has been widely used in the *Leishmania* species in the past few years. With some *Leishmania* with frequencies of homologous recombination close to 100% (Cruz *et al.*, 1991), this method allows the sequential targeting of one allele and then the second with constructs bearing two different selectable markers. This method has successfully worked to create null mutants in *L. major* for the dihyrofolate reductase-thymidylate synthase

gene (Cruz *et al.*, 1991), in *L. mexicana* for the cysteine proteinase gene (Souza *et al.*, 1994) and more recently the trypanothione reductase gene in *L. donavani* and *L. major* (Dumas *et al.*, 1997). Interestingly, this method has also been used to target multi-copy gene families and replace the entire family as was performed in *L. mexicana* for the cysteine protease family of genes consisting of 19 tandemly arranged genes (Mottram *et al.*, 1997) and in *L. enriettii* where the α-tubulin gene family was deleted (Curotto de Lafaille and Wirth, 1993). Gene replacement through homologous recombination has proven to provide alot of information pertaining to the functional roles various genes play within these *Leishmania* species.

1. Construction of pX63Hyg FeSODA, pX63Neo FeSODA, pX63Hyg FeSODB and pX63Neo FeSODB constructs.

Knock-out vectors were constructed to perform single allele knock-outs of the L.c.FeSODA and L.c.FeSODB genes. The vectors used were pX63Hyg and pX63Neo which are *Leishmania* specific homologous gene replacement vectors used for stable transfection (Cruz *et al.*, 1991). The vectors were constructed as shown in Fig.4.12. A 5' homologous region from each gene was subcloned into the *Hind*III/*Xho*I site and a 3' homologous region from each gene was subcloned into the *Sma*I/*Bgl*II site of each of the vectors. In each case, the 2837 bp HYG/DHFR region between the *Xho*I and *Sma*I site as well as the 2687 bp NEO/DHFR region between the *Xho*I and *Sma*I site acts as the fragment that replaces the sequence of the gene intended for knock-out. The completed

Figure 4.12. Circular maps of the L.c.FeSODA and L.c.FeSODB gene knock-out plasmids. (A) pX63Hyg FeSODA KO plasmid and **(B)** pX63Neo FeSODA KO plasmid. 5'SODA and 3'SODA homologous recombination fragments are shown and cloned into the *Hind*III/*Xho*I and *Sma*I/*Bgl*II plasmid sites respectively. **(C)** pX63Hyg FeSODB KO plasmid and **(D)** pX63Neo FeSODB KO plasmid. 5'SODB and 3'SODB homologous recombination fragments are shown and cloned into the *Hind*III/*Xho*I and *Sma*I/*Bgl*II plasmid sites respectively. In each plasmid, the 2837 bp HYG/DHFR region or the 2687 bp NEO/DHFR region act as the replacement fragment and it is these regions that replace the sequence of the gene intended for knock-out.


A
XhoI 6139
HYG/DHFR
5'SODA fragment
HindIII 5450
px63Hyg FeSODA KO
7039 bp
SmaI 1937
3'SODA fragment
amp
BglII 2647
B
SmaI 815
NEO/DHFR
3'SODA fragment
BglII 1525
px63Neo FeSODA KO
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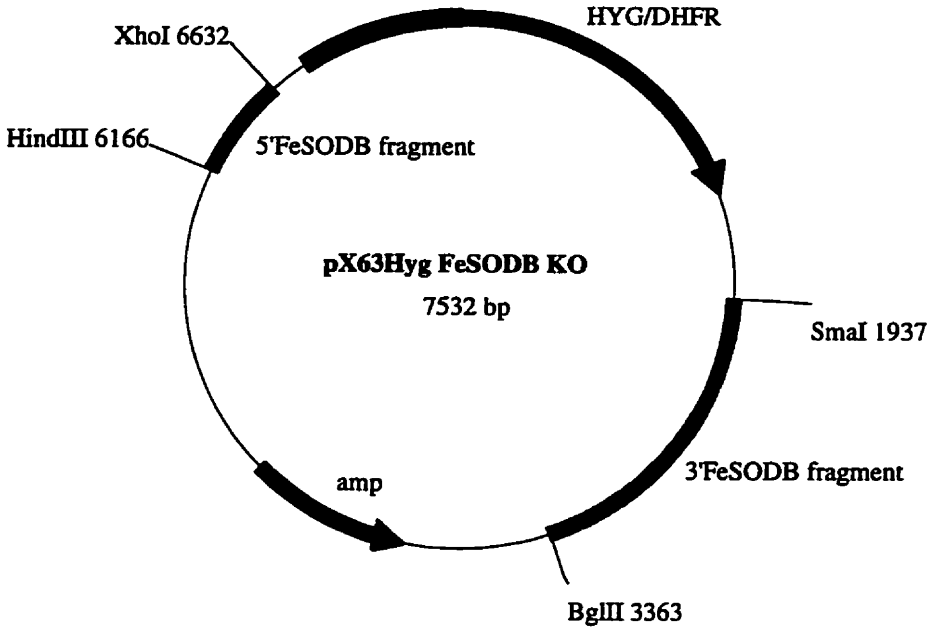
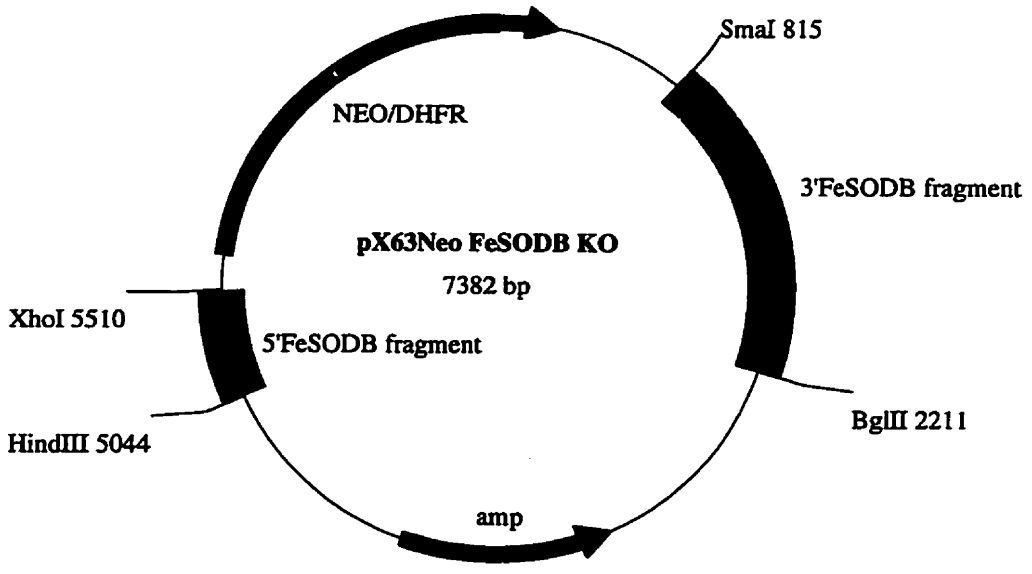
XhoI 5017
5'SODA fragment
amp
HindIII 4328

C


HYG/DHFR
XhoI 6632
HindIII 6166
5'FeSODB fragment
pX63Hyg FeSODB KO
7532 bp
SmaI 1937
amp
3'FeSODB fragment
BglII 3363

D


SmaI 815
NEO/DHFR
3'FeSODB fragment
pX63Neo FeSODB KO
7382 bp
XhoI 5510
5'FeSODB fragment
BglII 2211
HindIII 5044
amp

knock-out constructs were linearized with *Hind*III and *Bgl*II and used for transfection of *L. chagasi.*

The regions used for construction of the two knock-out vectors used for the single allele knock-out attempt of the L.c.FeSODA gene is outlined in Fig.4.13. The sense primer HINDSODAKO and the antisense primer XHOSODAKO (primers underlined in Fig.4.13) were used to amplify a 465 bp fragment for the 5' homologous region insert and cloned into the *Hind*III/*Xho*I sites of pX63Hyg and pX63Neo. As well, the sense primer SMASODAKO and the antisense primer BGLSODAKO (primers underlined in Fig.4.13) were used to amplify a 765 bp fragment for the 3' homologous region insert and cloned into the *Sma*I / *Bgl*II sites of pX63Hyg and pX63Neo. The region of L.c.FeSODA which would be replaced by the Hyg or Neo gene upon homologous recombination is highlighted in green in Fig.4.13. As well, the two amino acid residues, histidine and aspartate, involved in coordinating the metal in the active site of FeSOD enzymes near the carboxy terminus (highlighted in red in Fig.4.13) will be lost upon gene replacement. The two histidine residues near the amino terminus which are involved in the active site coordination (highlighted in red in Fig.4.13) will still be present upon a gene replacement event.

Similarily, the regions used to construct the knock-out vectors for the L.c.FeSODB gene are outlined in Fig.4.14. The sense primer HINDSODBKO and the antisense primer XHOSODBKO (primers underlined in Fig.4.14) were used to amplify a 466 bp fragment for the 5' homologous region insert and

Figure 4.13. Complete nucleotide sequence of the L.c.FeSODA cDNA showing the 450 bp knock out region. The 5' homologous recombination fragment between the two underlined primers HINDSODAKO and XHOSODAKO is the region cloned into the *Hind*III/*Xho*I sites of pX63Neo and pX63Hyg (see Fig. 4.13 A and B). The 3' homologous recombination fragment between the two underlined primers SMASODAKO and BGLSODAKO is the region cloned into the *Sma*I/*Bgl*II sites in pX63Hyg and pX63Neo. The sequence that is being replaced by the HYG/DHFR or NEO/DHRF regions is shown in green. The residues shown in red are the amino acids which are involved in coordinating the Fe metal at the active site of the enzyme. Two of these residues are present in the region that is to be replaced. Residues shown in purple are the four invariant residues thought to be completely conserved through all iron-containing SODs.

```
   1 gcacgaggtttctgtactttattgttgcactttatcgagttctttgcactgaaaagcatcgcacgactccacgcctccac
                                                                  HINDSODAKO ⇒
  81 tctctccctttcccccaagcactccagtcctcccgcattccga ATG TTC CGC CGC GTC TCG ATG AAA GCC
                                                    M   F   R   R   V   S   M   K   A

 151 GCC ACG GCC ACG GCT CCC GTC GGG TTT GCG TTC CTG TGC TAC CAT ACC CTT CCT CTA CTT
     A   T   A   T   A   P   V   G   F   A   F   L   C   Y   H   T   L   P   L   L

 211 CGG TAC CCG GCC GAG CTG CCG ACG CTC GGC TTC AAC TAC AAG GAC GGC ATC CAG CCC GTC
     R   Y   P   A   E   L   P   T   L   G   F   N   Y   K   D   G   I   Q   P   V

 271 ATG AGC TCC CGT CAG TTG GAG CTG CAC TAC AAG AAG CAC CAC AGC GCG TAC GTG GAC AAG
     M   S   S   R   Q   L   E   L   H   Y   K   K   H   H   S   A   Y   V   D   K

 331 TTG AAC ACG CTC GGC AAG GGC TGC GAG GGG AAG ACG ATT GAG GAG ATC ATC TTG GCG ACC
     L   N   T   L   G   K   G   C   E   G   K   T   I   E   E   I   I   L   A   T

 391 AGC GGC ACG ACT GAG AGC AAG GTC ATG AAC AAC CAG GCC GCT CAG CAC TTC AAC CAT TCC
     S   G   T   T   E   S   K   V   M   N   N   Q   A   A   Q   H   F   N   H   S

 451 TTC TTC TGG AAG TGC CTC TCG CCT GGT GGC AAG AAG ATC CCG AAG ACG CTC GAG AAT GCC
     F   F   W   K   C   L   S   P   G   G   K   K   I   P   K   T   L   E   N   A
                                                        ⇐ XHOSODAKO

 511 ATC GCG AAC GAG TTC GGA AGC GTC GAC GAC TTC ACG GTT TCC TTC CAG CAG GCC GGC GTG
     I   A   N   E   F   G   S   V   D   D   F   T   V   S   F   Q   Q   A   G   V

 571 AAC AAC TTT GGC TCT GGC TGG ACG TGG CTC TGC GTC GAT CCC CGG ACG AAG GAG CTT CGC
     N   N   F   G   S   G   W   T   W   L   C   V   D   P   R   T   K   E   L   R

 631 ATC GAC AAC ACG AGC AAC GCG GGC TGC CCG CTG ACC TCT GGC TTG CGC CCC ATC TTC ACC
     I   D   N   T   S   N   A   G   C   P   L   T   S   G   L   R   P   I   F   T

 691 GCT GAT GTG GGG GAG CAC GCC TAC TAC AAA GAC TTT GAG AAC CGC CCG CGC GAC TAC CTG
     A   D   V   G   E   H   A   Y   Y   K   D   F   E   N   R   P   R   D   Y   L

 751 AAA GAG CTC TGG CAG ATC GTC GAC TGG GAG TTT GTC TGC CAG ATG TAT GAG AAG GCC ACG
     K   E   L   W   Q   I   V   D   W   E   F   V   C   Q   M   Y   E   K   A   T

 811 AAG TAA gagagtcgtagatgcatctgtacccaatatgctgaggtgaggaagggagggaccgagcgtgacagcgcgtgc
     K   †

 889 ctacaccacgtagacgaggggctcgcgcggaacgtagaatgacggaggccgtgggcagatggcacacacgcgcacgcaat
                                                  SMASODAKO ⇒
 969 gagaaggagtgttggacgtgctggtggtggtgacagtggcaggcatgcggcgagcgctcatctaatggccgcccgccgcc

1049 atcacgactaccacagcctcgtgagtgtcgtacggttggtggaagcgcacccccccccggctcgctcgctcgcttccact

1129 ttcttccgtctctcatgctgcggcatgtgcgcgaagcagcggtagctctacccttgcttctccggcctcctcacacacac

1209 acaccacgccaacgctgcccctctaggttcctcgtctccgcccggtgccgtcacccaatccctgtcgcctttagccgtct

1289 gtttctttgccgttcgccctcccccacccttcctgagcaagtcagcggtgagagtgtgatgcgcacgtggaggagcag

1369 cctcgccgcccttctctctgtctccctgtgcgcgcaggtcgggcgggtggtgggcggctggcgtgcgcgtggaaggtgtg

1449 taggccggttggaaggagtcacgacttgcatccatgttgccctattctgtagacacacagacacgacgtaatgggcgcgc

1529 gcgcactcgtcacttgaggatcttgctgctgatcaccatcacatacatagagagagtttgcgcatccttctctccaacgt

1609 tgaccgcccgcgcccgtaccttatacatcctaccacttcccagcacaacatttgctcgttgtcgcgttgattcttcgattg
                    ⇐ BGLSODAKO

1689 tttttt
```

Figure 4.14. Complete nucleotide sequence of the L.c.FeSODB cDNA showing the 364 bp knock out region. The 5' homologous recombination fragment between the two underlined primers HINDSODBKO and XHOSODBKO is the region cloned into the *Hind*III / *Xho*I sites of pX63Neo and pX63Hyg (see Fig. 4.13 C and D). The 3' homologous recombination fragment between the two underlined primers SMASODBKO and BGLSODBKO is the region cloned into the *Sma*I / *Bgl*II sites in pX63Hyg and pX63Neo. The sequence that is being replaced by the HYG/DHFR or NEO/DHRF regions is shown in green. The residues shown in red are the amino acids which are involved in coordinating the Fe metal at the active site of the enzyme. Two of these residues are present in the region that is to be replaced. Residues shown in purple are the four invariant residues thought to be completely conserved through all iron-containing SODs.

```
   1 ggcacgagctttattgaatgaagcgctttctccctctcactcatcccttattattattatttccgttttctttcgctgcc

  81 gcgtgtagcctcaccatctttgctttcggttctgcaggaggaaaacagcatctctccacctcagccagctaaggaatcgg
                HINDSODBKO ⇒
 161 acgacgtacacaactcacgtgcctttctcacggttcttctacatccctgtttgctttctgctcgtgcgtc ATG CCG
                                                                            M   P

 237 TTC GCT GTT CAG CCG CTG CCG TAC CCC CAC GAT GCG CTC GCG TCG AAG GGC ATG TCG AAG
     F   A   V   Q   P   L   P   Y   P   H   D   A   L   A   S   K   G   M   S   K

 297 GAG CAG GTC ACC TTC CAC CAC GAG AAG CAC CAC AAG GGG TAC GCC GTG AAG CTG AAC GCT
     E   Q   V   T   F   H   H   E   K   H   H   K   G   Y   A   V   K   L   N   A

 357 GCC GCG GAG TCA AAC TCG GGT CTT GCG TCG AAG TCG CTG GTG GAC ATC ATC AAG TCT GAG
     A   A   E   S   N   S   G   L   A   S   K   S   L   V   D   I   I   K   S   E

 417 AAG GGC CCC GCC TTC AAC TGT GCG GCG CAG ATT TTC AAC CAC GAC TTC TTC TGG CGC TGC
  63 K   G   P   A   F   N   C   A   A   Q   I   F   N   H   D   F   F   W   R   C

 476 CTG TCC CGA GAG GCC GGT GGC GAG CCG TCG GGC CCT TTG GCG AGT GCG ATC GTC GAC AGT
  83 L   S   R   E   A   G   G   E   P   S   G   P   L   A   S   A   I   V   D   S
                                                        ⇐ XHOSODBKO
 537 TTT GGC ACG TTC GCG AGC TTC AAG AAG GAA TTC ACG GAT GCG CCC AAC GGC CAC TTT GGC
 103 F   G   T   F   A   S   F   K   K   E   F   T   D   A   P   N   G   H   F   G

 597 TCC GGC TGG GCG TGG CTC GTG AAG GAC AAG TCG AGT GGC AAG CTG AAG GTG CTC CAA ACG
 123 S   G   W   A   W   L   V   K   D   K   S   S   G   K   L   K   V   L   Q   T

 657 CAC GAC GCG GGC TGC CCG CTC ACG GAG CCC AAC CTC GTG CCG ATG TTG ACG TGC GAT ATA
 143 H   D   A   G   C   P   L   T   E   P   N   L   V   P   M   L   T   C   D   I

 717 TGG GAG CAC GCG TAC TAT ATC GAC TAC AGG AAC GAC CGC GCG TCC TAC GTG AAC GCA TTT
 163 W   E   H   A   Y   Y   I   D   Y   R   N   D   R   A   S   Y   V   N   A   F

 777 TGG AAC ATG GTG GAC TGG GAT TTC GCC TCT AGC CAG CTT TAA gctcttctgcgatgcgcagtttgg
 183 W   N   M   V   D   W   D   F   A   S   S   Q   L   †
 843 agttcacgttgagaaagaggccgagcgagtaacgggtggcgcgccgggggaggggggaagcggaactgtgctggcgcgctc
                                                    SMASODKO ⇒
 923 gtggcaacaacaaagatccaaagacaagagaaagaagcgaacagtgtagtataagtagcgcggtgcttttgtgtgtgtga

1003 cgtgtgaagagctctgccactatttcgttttctggtttcattgttttctttctctctcgctctctatcccagcattgtcg

1083 tcttgaccgaacgtctgtctcttcgacggtgctgtttcaacgagaagtgaaatctccctcaggggttatgcggcggcgt

1163 tcctgcttgtttcttctctttttttctgaaattgccgtttcatacacacacttacgccccgcacaatgccgtgtgctcat

1243 gcttgcgcgttcaagtgctgagtagggtgggaatatgatggaaggaagcactgcactgctctgtaaaccctataacacac

1323 cttctctcacttgtcactgtttgtctctaacatcttctagcactgctgctgccatggctttctataaagccgtaagcgaa

1403 acgaagatgcaagcactcgcagccacggcagcacctatcctccccctttcactcgctgcttctcgatctctttctctct

1483 ttcgttctcctgcaatctcacgaaacggctttattgacgcatcgacgtttattagcacgggatacgtcgacacctcccct

1563 ccccgctgtctcccttctgtttctggcgataggtttggctccgtttttttttcccgtcgccttttctgatgcgttagtt

1643 tgtattctctttccatttttttctttttttttggactattgggccctgagattcacacgcgttttctctttttctttttt

1723 ttgtcgtcgttactctcgtgcttttcatgttgcttcccctctctcgccattcccccttctgcccttctcttctcgccctct

1803 cccgtatcgatgtctgtgtcattgttgttgcataggtgcgtcgtttctttggcgcgaaagcagaaaagaggcaaatggga
```

1883 gtctcctcttcttccgtccaccaccacacttctcgggtgtgtcgtatggcatgcctgcctttcatctccctggcgcttcc

1963 tcattccttcctgtcttgcccccccccccacctcgtctcgcggctctctctctctctcggttgtgtctttgctgttgttg

2043 ttgttgtgtctgtgtgcgtgtgatagcacgagcaagtgaccgaaaccatagttggaacagtaacaacagagcgtgatgga

2123 catgatggacgggtggggcggggtgggggaaggagtgggccggacgtccaggaaatcatacagaaagagctctcttccgc

2203 acgtggtctcatttgggtgtaacccccgccctcttcccccccactttgtttttacttcccctttctttcctgtgcttttcat

⇐ BGLSODBKO

2283 ccccgccaacggtggtccatgacgagatggtattgacacgactatcgaaaaaaaaagcgggaaagaaggagcacgcacaac

2363 aactgcaaagagaacaacgtggaaaataggcgcgaaaaatgcgaactgacgtacagaggaatgttgaagaattcaagaat

2443 cgttccttttgcgcacatgtaacgatccttcaggatcgaggaaagatgagctggagaaaaagcgtctgcgcgtgcactca

2523 aattgttgaaggaatcggaaagttactcatgcgcttctcagcgtctgtgacttttcgttttccctcttcttgtgtctcca

2603 gcctgcgtgcgtctggctgctgtgacctttcctcacccctcctttgccgcccccgctgtgccgtccgtctccctctccta

2683 ttcagcctcacgttaatggcacgttgagcgcaggtatacctctgcctcactgttagccagtgtgaaaggcggtgagctgt

2763 aaagacaacggtagggtcgccagagagggtgaagggtacaccgttttgcaactctctatgctttaggtctacagagtagt

2843 agcaggtgcgccttttatttcttttgccgcactgtttctccgtttttttttttttcgtctgtgaatcgctatcttctgcg

2923 acccctccgtccccctcacttttacatctgccagtcgttgatgtgtggtacggttcttgtactcttcttctctccccagc

3003 gtttctttggtgctcaagaaccgcaaagatcgccacaagagaaggagcaaacgaaaacaaaaaaagaacaccataaagac

3083 gacgagaaggcggtaagaggagtgtggtttcagcgcacgtacatacatagacacacacatgtgtatgtattctgcagcaa

3163 gacgatgccaaacgagtggcggtacggatgaggggagaaatacagggaaaaaagacgtgcccgcgtacgacacacacgga

3243 cggtcgtttgaaaacaaggtgggagcagggaaagtaaagtgactgcaagagttttcgctttcccgcaagtgcacttcagt

3323 gaccctgctgcgcatctgctctcctcctcgtgcccagccagtctcgcttgtgtgccgggctagtgtaaagtgccacgtaa

3403 gcgcctgtatccgccggtgtactgtgctagttaccactctttctctatgcgtatctgccgctctgtcactcgttgtggct

3483 gctcagcggtctctctccttcaccgtgtcctctttgcgcgtgtacgtgtgccctatgtgcgtggaatgcaatatcctccc

3563 tccgtcaacgttccgcgatctcttcgtcttgtttttttttcctctgcatgtattgttgccgtttctcctccagagtgtga

3643 acgctacctctctcctctctcctgttcttctttgcttgcttgcttggctctgcgtgggggttttatgccttacacagtta

3723 tgactaagcactcaaaacatatatgcggtctatcaacggcagtgaagggaggccgaaaagtgtgctgccaaaaaaaaaaa

3803 aaaaaaaaaaaaaaaaaaaa

cloned into the *Hind*III/*Xho*I sites of pX63Hyg and pX63Neo. As well, the sense primer SMASODBKO and the antisense primer BGLSODBKO (primers underlined in Fig.4.14) were used to amplify a 1465 bp fragment for the 3' homologous region insert and cloned into the *Sma*I/*Bgl*II sites of pX63Hyg and pX63Neo. The region of L.c.FeSODB which would be replaced by the Hyg or Neo gene upon homologous recombination is highlighted in green in Fig.4.14. As mentioned above, the same two amino acid residues, histidine and aspartate, involved in coordinating the metal in the active site of FeSOD enzymes near the carboxy terminus (highlighted in red in Fig.4.14) will be lost upon gene replacement whereas, the two histidine residues near the amino terminus (highlighted in red in Fig.4.14) will still be present. The L.c.FeSODB constructs were designed before it was known that this gene is present in a multi-copy gene family, so the resulting knock-out will result in the replacement of a single gene of the family. In order to study the effect of the deletion of the entire gene family the 5' and 3' extreme intergenic regions of the cluster would need to be characterized and used for the homologous recombination regions.

2. Transfection and analysis of the L.c.FeSODA and L.c.FeSODB single allele knock-outs.

Wild type *L. chagasi* parasites were transfected with 5 μg of the linear knock-out constructs (refer to the Material and Methods for a detailed transfection protocol) for selection of hygromycin B and neomycin resistant transformants. Transfections were performed until viable cultures were obtained

at a final concentration of 50 μg/ml for each of the selectable markers. Genomic DNA was isolated from these cultures, digested with various enzymes and analyzed by Southern blot analysis.

Southern blot analysis of the L.c.FeSODB single allele knock-out replaced with the hygromycin gene is shown in Fig.4.15A and B. A summary of the results obtained from digests of the genomic DNA with either *Bam*HI or *Pst*I and hybridized with the L.c.FeSODB coding region probe or a hygromycin specific probe are shown in Fig.4.16. Digestion of wild type DNA with *Bam*HI and hybridized with the L.c.FeSODB coding region probe resulted in two bands (>12 kb and ~2.4 kb, Fig.4.15A, lane1). *Bam*HI digests of the pX63Hyg FeSODB KO DNA hybridized with the L.c.FeSODB coding region probe resulted in three bands (lane 3). One band >12 kb, is similar to the band seen in the wild type digest, although it is half the intensity. The second band is ~3.5 kb which is not observed in the wild type lane. This band resulted from an internal *Bam*HI site which exists at the 3' end of the HYG/DHFR region (Fig.4.16A) cutting the >12 kb band arising from the wild type DNA digest. The third band at ~2.4 kb (Fig.4.15A, lane 3) was similar to the 2.4 kb band seen in the wild type digest. This band was a result of cross-hybridization with a member of the FeSODB gene family. The intensity of this band remained constant in the wild type and knock-out lanes (lanes 1 and 3). A similar blot was hybridized with the hygromycin specific probe as shown in Fig.4.15B. No hybridizable bands were observed in the wild type lane digested with *Bam*HI (Fig.4.15B, lane 1). A band

Figure 4.15. Results of the Southern blot analysis of DNA from wild type and pX63Hyg FeSODB KO transformants. Lane 1: wild type DNA digested with *Bam*HI; lane 2: wild type DNA digested with *Pst*I; lane 3: px63Hyg FeSODB KO DNA digested with *Bam*HI; and lane 4: px63Hyg FeSODB KO DNA digested with *Pst*I. **(A)** Southern blot hybridized with the L.c.FeSODB coding specific probe (SODBATG/SODBTAA). **(B)** Southern blot hybridized with the Hygromycin specific probe. Positions of molecular weight markders are shown to the left of each panel.

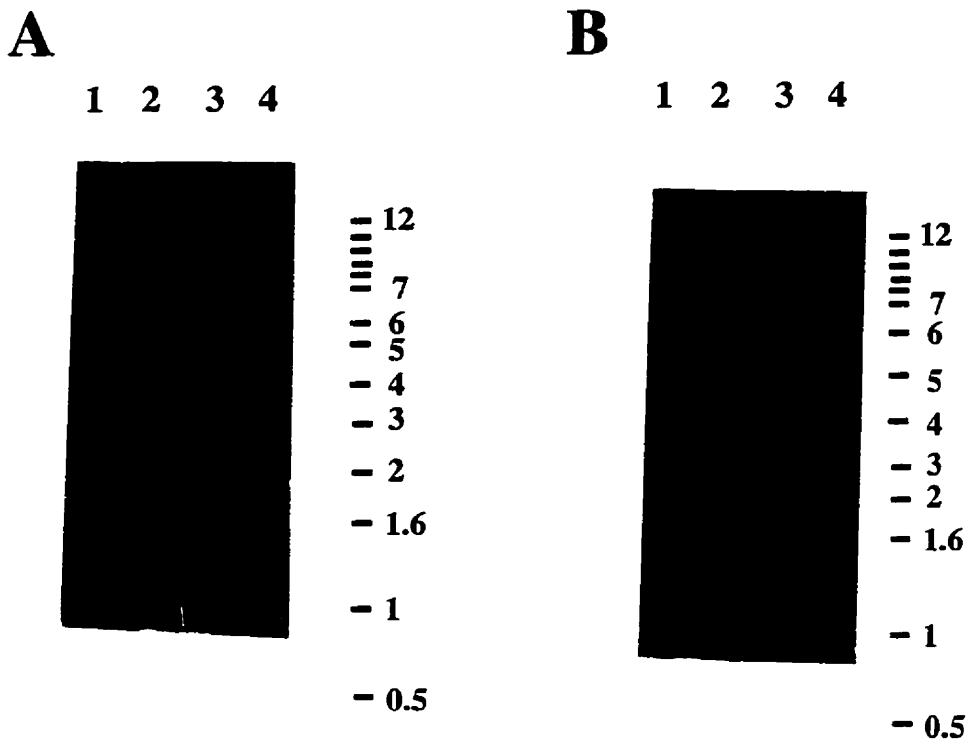
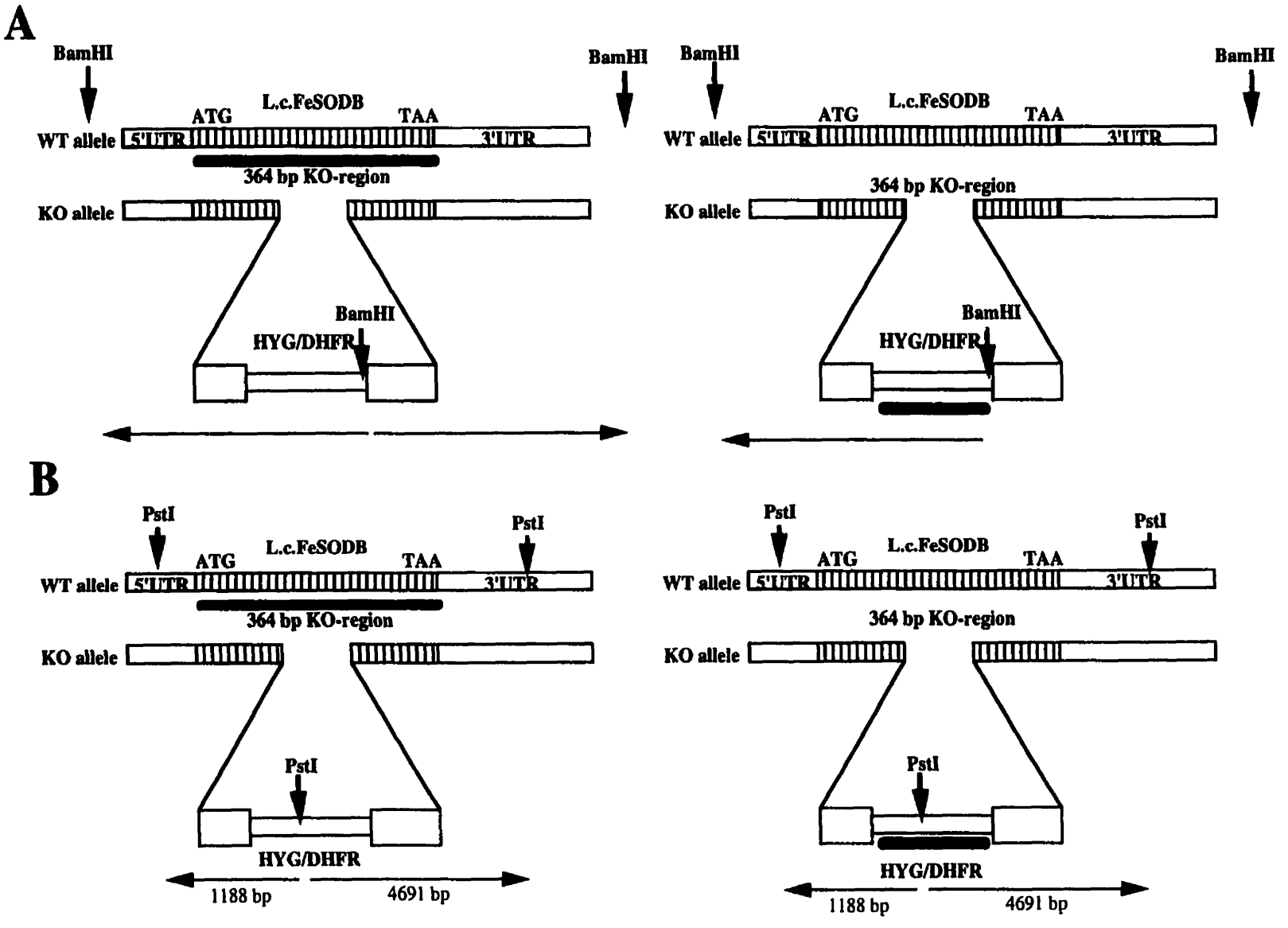

A
1 2 3 4
12
7
6
5
4
3
2
1.6
1
0.5
B
1 2 3 4
12
7
6
5
4
3
2
1.6
1
0.5

Figure 4.16. Summary of pX63Hyg FeSODB KO Southern blot analysis shown in Fig.4.15. Schematic drawing showing the fragments expected from Southern analysis of the pX63Hyg FeSODB KO genomic DNA digested with **(A)** *Bam*HI and **(B)** *Pst*I if one of the L.c.FeSODB alleles is disrupted at the expected position. WT allele represents the wild type allele and KO allele represents the knock-out allele. Positions of the probes used to hybridize to the Southern blot are shown with a solid black bar. Expected fragments hybridized to the various probes are shown as arrows below the schematics. Fragments shown are from obly the KO allele.


A
BamHI
BamHI
ATG
L.c.FeSODB
TAA
WT allele
5'UTR
3'UTR
364 bp KO-region
KO allele
BamHI
HYG/DHFR
BamHI
BamHI
ATG
L.c.FeSODB
TAA
WT allele
5'UTR
3'UTR
364 bp KO-region
KO allele
BamHI
HYG/DHFR
B
PstI
PstI
ATG
L.c.FeSODB
TAA
WT allele
5'UTR
3'UTR
364 bp KO-region
KO allele
PstI
HYG/DHFR
1188 bp
4691 bp
PstI
PstI
ATG
L.c.FeSODB
TAA
WT allele
5'UTR
3'UTR
364 bp KO-region
KO allele
PstI
HYG/DHFR
1188 bp
4691 bp

of ~3.5 kb hybridized in the *Bam*HI digested pX63Hyg FeSODB KO DNA lane (lane 3). This band is equivalent to the extra 3.5 kb band seen in Fig.4.15A, lane 3.

*Pst*I digested wild type DNA hybridized with the L.c.FeSODB coding region probe resulted in two bands (~2.4 and 2.8 kb, Fig.4.15A, lane 2). Digestion of the pX63Hyg FeSODB KO DNA with *Pst*I resulted in the appearance of three bands when hybridized with the coding region probe. The two bands at ~2.4 and 2.8 kb (Fig.4.15A, lane 4) were similar in size to the two bands seen with the wild type digest (lane 2). The 2.8 kb band is approximately half the intensity in the knock-out lane (lane 4) as compared to the wild type lane (lane 2), whereas the 2.4 kb band remained the same intensity in both lanes (lanes 2 and 4). The third band (~1.2 kb, lane 3), was not seen in the wild type digestion. This band hybridized due to the *Pst*I site within the 5' UTR of L.c.FeSODB and the internal *Pst*I site within the hygromycin gene (Fig. 4.16B). A fourth band was expected to hybridize at ~4.7 kb (Fig.4.15A, lane 4) which would be a result of the PstI site within the 3' UTR of L.c.FeSODB and the internal PstI site within the hygromycin gene. The absence of this band cannot be explained without further characterization. However, a similar blot hybridized with the hygromycin specific probe (Fig. 4.15B), showed the absence of any bands hybridizing to wild type DNA digested with *Pst*I (lane 2) and the presence of the two bands in the knocked-out lanes. Digestion of pX63Hyg FeSODB KO DNA with *Pst*I resulted in two bands, ~1.2 kb and ~4.6 kb (Fig.4.15B, lane 4). These

are the expected sizes of bands if the hygromycin gene inserted at the expected position within the L.c.FeSODB gene (Fig.4.16). Taken together, these data indicate that we have successfully replaced one allele of the L.c.FeSODB gene with the hygromcyin gene. The half intensity of the >12 and ~2.8 kb bands (Fig.4.15A, lanes 3 and 4 respectively) indicates that one allele was replaced from the same L.c.FeSODB gene which was characterized in Chapter 3. The constant intensity of the ~2.4 kb bands seen in lanes 3 and 4 (Fig.4.15A) as compared to the wild type indicate that these bands resulted from cross-hybridization with a member of the FeSODB gene family and was not affected by the replacement that took place.

An attempt to replace the L.c.FeSODB gene with the neomycin gene was unsuccessful. The results of the Southern used to analyze the L.c.FeSODB single allele knock-out using the neomycin gene resulted in the same pattern as was seen in the wild type genomic DNA when hybridized with the L.c.FeSODB coding region probe or the neomycin specific probe (data not shown).

Similar attempts to knock-out the L.c.FeSODA gene with the neomycin gene were unsuccessful. Southern blots hybridized with the L.c.FeSODA coding region probe or the neomycin specific probe resulted in the same pattern seen with the wild type DNA, indicating the neomycin gene was not incorporated into the *L. chagasi* genome (data not shown). However, knock-out attempts using the hygromycin gene revealed different results. The *Leishmania* culture containing the single allele knock-out attempt of L.c.FeSODA with hygromycin exhibited

very slow growth and an attempt to isolate genomic DNA from this culture was unsuccessful due to the lack of viable cells. Viable cells are currently growing from this knock-out attempt and have yet to be analyzed.

III. Summary

1. The L.c.FeSODA and L.c.FeSODB genes demonstrate differential expression 1.5 and 3 fold increase in the level of mRNA respectively in the amastigote stage compared to the levels in the stationary phase promastigote.

2. Overexpression of the L.c.FeSODA protein in SOD null *E. coli* cells complemented their SOD function when treated with the toxic oxygen producing agent, paraquat.

3. Cloning of the L.c.FeSODA and L.c.FeSODB cDNA coding regions in *L. chagasi* cells, using the pX expression vector, showed an increased transcript level which correlated to an increased level of protein activity. These overexpressing cells showed protection of the *Leishmania* parasite against ROI and RNI producing agents, suggesting a role for these SODs in the elimination of superoxide radicals.

4. Attempts at single allele knock-outs of the L.c.FeSODA and L.c.FeSODB genes resulted in the apparent successful replacement of one allele of the L.c.FeSODB gene using the Hygromycin gene.

CONCLUDING REMARKS AND FUTURE EXPERIMENTS

This study consisted of the characterization and functional analysis of two cDNA clones L.c.FeSODA and L.c.FeSODB, encoding the *L. chagasi* iron superoxide dismutase A and B enzymes. L.c.FeSODA and L.c.FeSODB have been shown to contain all of the conserved residues involved in metal coordination at the active site of iron-containing SOD enzymes. The L.c.FeSODA gene was shown to be present as a single copy within the *L. chagasi* genome, whereas the L.c.FeSODB gene was present as a multi-copy gene family. Both of the SOD genes are differentially regulated. Treatment of overexpressing L.c.FeSODA and L.c.FeSODB *Leishmania* cells with various ROI and RNI producing agents resulted in protection of parasites against superoxide radicals. An initial attempt at the single allele knock-out of the L.c.FeSODA and L.c.FeSODB genes was also undertaken. Characterization of the knock-out attempts resulted in the successful single allele knock-out of the L.c.FeSODB gene.

The inability of macrophage cells to control or eliminate the replication of intracellular microorganisms, such as *Leishmania*, leads to the manifestation of the disease leishmaniasis. Upon entry of *Leishmania* into the mammalian host, the parasite is phagocytosed by the host macrophage cells which initiates a cascade of defense machanisms, including an oxidative burst. In order for the parasite to survive an infection, it must be able to combat this oxidative burst. Many anti-oxidant enzymes are known to be involved in the elimination of

oxygen free radicals such as superoxide dismutase, catalase and glutathione peroxidase.

Superoxide dismutase enzymes have been implicated as virulence factors in a variety of intracellular pathogens. SOD has been shown to play an important role in the pathogenesis of the the intracellular bacteria, *Shigella flexneri* (Franzon *et al.* 1990). These researchers produced a strain of *S. flexneri* which was FeSOD deficient and showed that this strain was extremely sensitive to killing by phagocytes when compared to a wild-type infection. The importance of SOD enzymes was also shown by Beaman and Beaman (1990) in the intracellular bacteria *Nocardia asteroides*. Using a monoclonal antibody to deplete a SOD enzyme of *N. asteroides*, they showed that the SOD enzyme activity was inhibited and therefore unable to eliminate the superoxide radicals generated during infection. The data suggested that SOD protects *N. asteroides* from oxidative killing *in vivo* during all stages of infection. As well, a gene encoding a superoxide dismutase from the intracellular bacterium *Listeria monocytogenes* was cloned by functional complementation of an SOD-deficient *E. coli* mutant and is thought to play an important role in the development of successful infections of *L. monocytogenes* in phagocytic cells, although this has yet to be shown (Brehm *et al.*, 1992). Although the level of SOD expression from these intracellular pathogens has not been studied at the time of infection, the disruption and elimination of SOD function within these bacterium suggests a

possible role for the superoxide dismutase enzyme as a virulence factor in the pathogenesis of these intracellular pathogens.

The ability of macrophage cells to control the infection of intracellular pathogens has recently been shown to be influenced by a group of growth factors and cytokines (Abbas *et al.*, 1996; Lehn *et al.*, 1989; Scott, 1989). Tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) have been shown to act synergistically to induce the killing action of macrophage cells (Bogdan *et al.*, 1990; Liew *et al*, 1990). The mechanism by which TNF-α and IFN-γ mediate intramacrophage killing of *Leishmania* was shown by Wei *et al.* (1995) to be dependent on the production of nitric oxide (NO) by nitric oxide synthase (NOS). By disrupting the mouse NOS gene by knock-out techniques, they were able to show that the presence of NO is necessary to control the overproduction of IFN-γ and thus the overexpansion of TH1 cells. Without this control, the mice became susceptible to *L. major* infections. This shows that the presence of RNI species are very important for macrophage cells to fight off *Leishmania* infections. Therefore, if the action of IFN-γ and TNF-α is activated through these reactive species, then susceptibility of *Leishmania* elimination by macrophage cells may be influenced by the presence of RNI scavenging enzymes. Recently, studies have reported that both TNF-α and IFN-γ induce the expression of SODs in various cell types, including macrophages (Harris *et al.,* 1991; Wong *et al.,* 1989). This could indicate that these cytokines are also being induced through the action of ROI species, thus implicating the importance of ROI scavenging

enzymes in the infection process. Taken together, these studies indicate the importance of free radical species, and specifically $^{\cdot}O_2$, in the host's defense against invading microorganisms. It would thus be beneficial that enzymes which neutralize the effects of the macrophage-produced $^{\cdot}O_2$ be expressed within the pathogen.

This study has evaluated the possible role(s) SOD enzymes may play within the pathogeneis of the intracellular parasite, *Leishmania chagasi*. Differential gene expression of L.c.FeSODA and L.c.FeSODB within the various life cycle phases of the parasite indicates the need for the SOD genes during the infective phase of the parasite. As well, protection by overexpressing SOD cultures from various free radical producing agents indicates the oxygen free radical eliminating role played by the SOD enzymes of this parasite. The presence of the putative mitochondrial transit peptide at the amino terminus of the L.c.FeSODA enzyme and the absence of an extension on the L.c.FeSODB enzyme suggests a possible compartmentalization of these two enzymes within the *L. chagasi* parasite. Most eukaryotic manganese containing SOD enzymes analyzed to date have been found to reside within the mitochondria. The presence of two SOD enzymes containing the same metal at their active site in the *L. chagasi* parasite may be explained through this compartmentalization phenomenon. L.c.FeSODA, which may be trageted to the mitochondria of this parasite, may play the function of an "Mn"SOD as seen in higher eukaryotes,

whereas the L.c.FeSODB enzyme, which potentially remains within the cytosol of the parasite, may play a true "Fe"SOD function. The localization of these enzymes has not been studied to date and requires investigation to elucidate the *in vivo* function of these two enzymes. Taken together, the evidence presented in this study suggests a possible role for SODs in the protection of the parasite within the promastigote stage. Further studies need to be carried out to determine the importance of SODs as a virulence factor in the protection of this intracellular pathogen within its host.

Some future experiments which would further analyze the role L.c.FeSODA and L.c.FeSODB genes play in *Leishmania* pathogenesis and survival are as follows:

1. Raising an antibody to L.c.FeSODA and L.c.FeSODB in order to study the levels of protein within the cell comparing protein levels in wild type, overexpressing and knock-out strains and correlate them to the transcript levels and protein activity levels.

2. To study the localization of the L.c.FeSODA and L.c.FeSODB proteins within the *Leishmania* parasite using specific antibodies and the green fluorescent protein (GFP+)/SOD fusions which would allow the localization of proteins.

3. Establishing a quantitative SOD assay system to quantitate activity levels within *Leishmaina* whole cell extracts and comparing them to the various overexpressing and knock-out strains.

4. To establish and characterize single and double allele knock-outs of the L.c.FeSODA and L.c.FeSODB genes. As well, the depletion of these gene products could be carried out using specific antibodies.

5. To establish a quantitative method of determining parasite load after infecting macrophage cells with the various overexpressing and knock-out strains. As well, to quantitatively study the levels of superoxide radicals and cytokines being released upon infection and correlate survival of parasites to parasite load.

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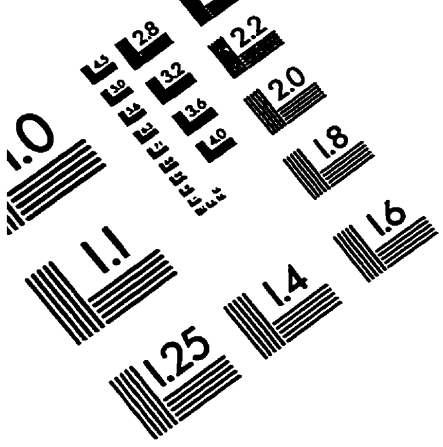

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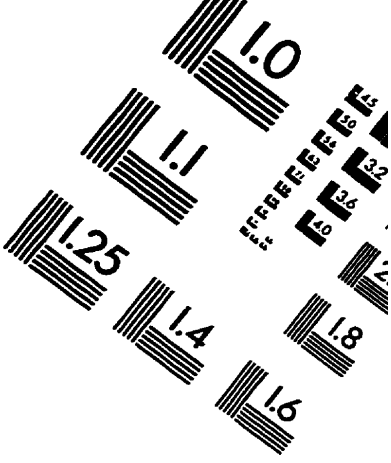

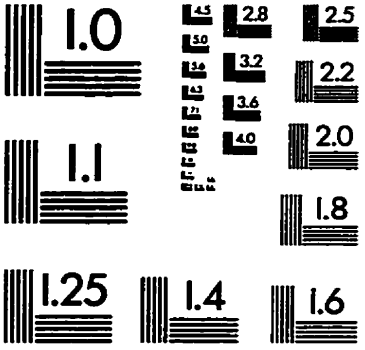

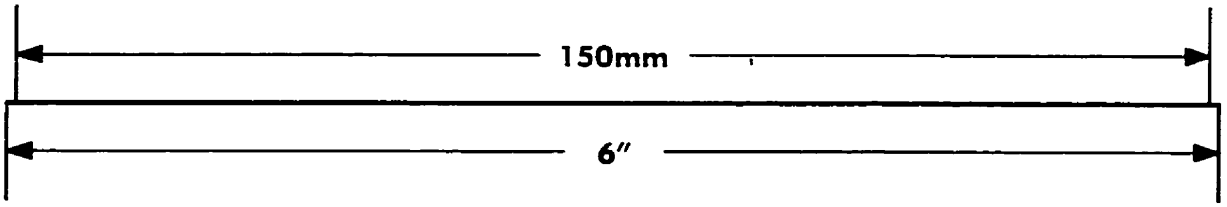

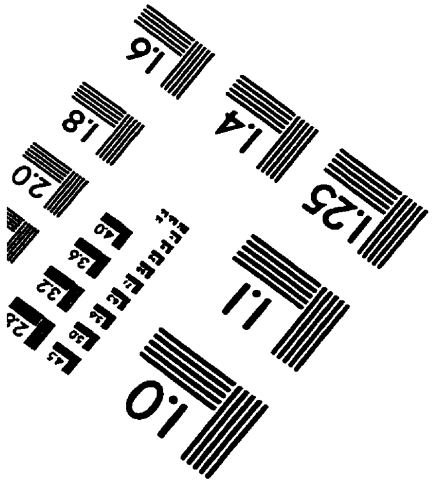

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