

**EFFECT OF OXIDATIVE STRESS ON *Escherichia coli*
sodA-sodB⁻ : PROTECTION BY THE MIMIC OF
SUPEROXIDE DISMUTASE , Mn(III)-SALOPHEN**

by

Vipavadee Kittiponkul

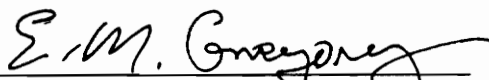
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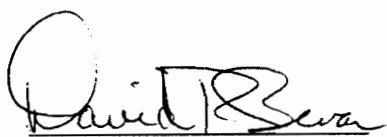
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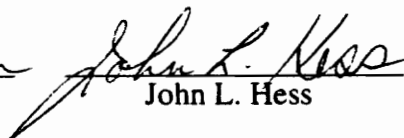
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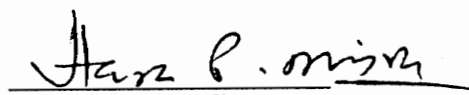
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EFFECT OF OXIDATIVE STRESS ON *Escherichia coli* *sodA*⁻*sodB*⁻ :
PROTECTION BY THE MIMIC OF SUPEROXIDE DISMUTASE,
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Department of Biochemistry

(ABSTRACT)

The effect of Mn(III)-salophen, a superoxide scavenger, against oxidative stress was evaluated in *Escherichia coli* *sodA*⁻*sodB*⁻. Oxidative stress was imposed by exposure of the cells to paraquat or hyperoxia. Cells were grown in LB medium overnight, washed and resuspended in the indicated glucose / salts medium supplemented with casamino acids. The effect of Mn(III)-salophen in the oxidative stress model *in vivo* was measured in terms of the cell growth. Mn(III)-salophen (60 nM) completely protected *E. coli* JI132*sodA*⁻*sodB*⁻ against 1.0 μ M paraquat. Equivalent amounts of Mn(III) acetate, a Mn(III)-salophen component, also protected against paraquat toxicity in aerobic *E. coli* JI132*sodA*⁻*sodB*⁻. Fe(III)-salophen which has no superoxide scavenging activity, did not protect the cells against paraquat toxicity. The protective effect of Mn(III)-salophen against the paraquat toxicity was proposed to come from the intracellular superoxide scavenging activity of either the complex itself, its component Mn(III), or both, but not by inhibiting the uptake of paraquat. The protective effect of Mn(III)-salophen and Mn(III) in the glucose / salts medium containing casamino acids was also observed in *E. coli* *sodA*⁻*sodB*⁻ in 100 % and 50 % oxygen.

Hyperoxia increases intracellular levels of superoxide radicals that are intercepted by Mn(III)-salophen and Mn(III).

Dedicateto the special one who always stays in my mind

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LIST OF ABBREVIATIONS

SOD	superoxide dismutase
MnSOD	manganese - containing superoxide dismutase
FeSOD	iron - containing superoxide dismutase
Cu,ZnSOD	copper,zinc - containing superoxide dismutase
<i>sodA</i>	gene of Mn-SOD
<i>sodB</i>	gene of Fe-SOD
CAT	catalase
GSH	glutathione (reduced form)
GSSG	glutathione (oxidized form)
GSH reductase	glutathione reductase
GSHPx	glutathione peroxidase
Mn(III)-salophen	Manganese (III)-salophen
CuDIPS	Cu(II) (3,5 - diisopropylsalicylate) ₂
Fe-TPEN	Fe(II)-tetrakis-N,N,N',N'[2 - pyridylmethylethylenediamine]
Fe-TPAA	Fe(III)-tris[N-(2-pyridylmethyl) - 2 - aminoethyl] amine
TEMPO	2,2,6,6 - tetramethylpiperdin-1 -yloxy
TEMPOL	4 - hydroxy - 2,2,6,6 - tetramethylpiperidin-1 -yloxy
NBT	nitro blue tetrazolium
BSA	bovine serum albumin
Salophen	N,N' - bis - (salicylidene) - 1,2 - phenylenediamine
Desferal	desferrioxamine mesylate
Mn(II)	manganese(II)chloride
Mn(III)	manganese(III)acetate dihydrate
Fe(III)	ferric(III)chloride hexahydrate
DF-Mn	Desferrioxamine - manganese
MnTMPyP	Manganic(III)-porphyrin
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
PQ	paraquat
CHO	Chinese Hamster Ovary
DHAD	α,β - dihydroxy - acid dehydratase
LB	Luria - Bertani medium
OD	optical density

I INTRODUCTION

Superoxide radicals can be produced in cells during normal metabolism and their production is augmented by exposure to oxidative stress. Superoxide mediates cellular toxicity in several ways and has been associated with several disease processes, including inflammation, carcinogenesis, aging and reperfusion injury [Nagano *et al.*, 1989 ; Darr *et al.*, 1988]. Additionally, superoxide inactivates certain enzymes including some dehydratases, catalase, and glutathione peroxidases [Gardner and Fridovich, 1992 ; Kuo and Fridovich, 1987 ; Darr *et al.*, 1989]. Superoxide dismutase (SOD), an enzyme, is a cellular defense against the deleterious effect of superoxide [Fridovich, 1995 ; Nagano *et al.*, 1989 ; Faulkner *et al.*, 1994]. Furthermore, SOD protects against the toxicity of dioxygen and dioxygen - dependent toxicities of viologen and quinones [Rabinowitch *et al.*, 1987]. However, there are many problems associated with the use of protein SODs as therapeutic agents such as a short circulating half life of protein SODs, non specific tissue targeting, and immunogenicity [Darr *et al.*, 1988 ; Iuliano *et al.*, 1992]. Various low molecular weight compounds or complexes with SOD - like activity have been synthesized in an effort to overcome the problems associated with therapeutic use of SOD. The effects of these complexes have been studied *in vitro* and *in vivo* . A number of manganese complexes have been developed and characterized. However, some of them have been shown to react stoichiometrically, but not as catalytic superoxide scavengers. Mn(III)-salophen, a SOD - mimic, has been synthesized and characterized by Liu *et al.* [1994]. Mn(III)-salophen has superoxide scavenging activity *in vitro* . This complex appears to promote aerobic growth of *E. coli* double mutant QC779 in glucose - salts

minimal medium, and protects against the inhibitory effect of paraquat on aerobically grown *E. coli* BB₁₂⁻ in the minimal medium [Liu *et al.*, 1994]. However, the effects of Mn(III)-salophen on paraquat exposure or hyperoxygenation in the SOD double mutants of *E. coli* has not been investigated. This report shows the effect of Mn(III)-salophen on oxidative stress imposed by paraquat exposure and hyperoxygenation in the *E. coli*.

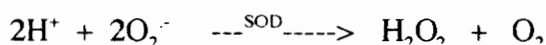
II LITERATURE REVIEW

2.1 Superoxide radicals and Superoxide dismutases

Aerobic organisms use oxygen as the terminal electron acceptor. In mitochondria, oxygen reduction by succinate and NADH releases energy that is subsequently used to synthesize useful metabolic end products. However, oxygen is known to be both toxic and mutagenic [Hassan and Schellhorn, 1988]. Diseases that involve toxic oxygen metabolites include inflammation, ischemia - reperfusion injury, and degenerative diseases such as cancer [McCord and Fridovich, 1988 ; Cerutti, 1985]. The toxicity of oxygen can occur from reduction of oxygen to reactive oxygen species , i.e., superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\cdot$). H_2O_2 is the most stable among the species listed, whereas $OH^\cdot$ is the most reactive. These reactive oxygen metabolites are produced during normal oxygen metabolism, and their levels can be increased in cells exposed to hyperoxygenation or to redox cycling compounds such as methyl viologen (paraquat), menadione, streptonigrin, plumbagin, juglone, and methylene blue [Hassan, 1988].

Cells have developed several mechanisms of defense against toxic oxygen metabolites. These defenses included antioxidant enzymes and low molecular weight substances such as vitamins C and E , carotenoids , glutathione (GSH), transferrin, and ceruloplasmin [Diplock, 1994 ; Goldstein *et al* ., 1982]. However, the primary defense against oxygen toxicity depends on enzymatic reactions requiring small amounts of Mn, Cu, Zn, and Se [Fridovich, 1995].

Superoxide dismutases (SODs), (EC 1.15.1.1.), are a family of metalloproteins that provide the first line of defense against the deleterious effects of $O_2^{\cdot -}$ in aerobic organisms, aerotolerant anaerobes, and some obligate anaerobes [Gregory and Fridovich, 1978 ; Gregory and Dapper, 1980 ; Hassan, 1988]. SODs dismutate $O_2^{\cdot -}$ by alternate reduction and oxidation of an active site transition metal by consecutive molecules of $O_2^{\cdot -}$ according to the reaction :



SODs are distinguished by the metal in their active site. These metals include Cu/Zn, Mn, or Fe.

The distribution of these three isozymes is different among eukaryotic and prokaryotic cells. Cu,ZnSODs are typically found in eukaryotic cytosol and in the peroxisome of plants, yeasts, and in a small number of gram - negative bacteria [McCord and Fridovich, 1969 ; Steinman, 1985]. Bacterial Cu,ZnSOD, called bacteriocuprein, is found in the periplasm but is absent from the cytosol of *Photobacter leiognathi*, some species of *Pseudomonas*, *Brucella abortus*, and some species of *Haemophilus* [Puget and Michelson, 1974 ; Steinman, 1985 ; Beck *et al.*, 1990 ; Paul *et al.*, 1992]. Recently, Benov and Fridovich found Cu,ZnSOD in the periplasmic space of *Escherichia coli* [Benov and Fridovich, 1994 ; Benov *et al.*, 1995]. The periplasmic location of the bacteriocuprein may intercept externally - generated reactive superoxide radicals. In *E. coli*, *sod A* and *sod B* genes encode its cytosolic Mn and FeSOD isozymes respectively, while *sod C* gene encodes its periplasmic Cu,ZnSOD [Imlay, 1996]. Additionally, there is evidence that *E. coli* has SOD located in specific regions within the cell ; MnSOD is associated with the nucleoid region ; FeSOD is peripheral to the nucleoid and is in the

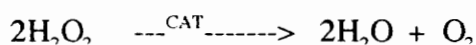
cytosol ; Cu,ZnSOD is in the periplasm [Benov *et al.* , 1995]. Therefore, *E. coli* Cu,ZnSOD may protect the cells against the extracellular O_2^- formed during autoxidation of medium components or as a result of respiratory burst of phagocytes from host organism [Benov *et al.* , 1995]. About 70 % of total O_2^- from aerobically grown *E. coli* is produced by the inner membrane fraction and 30 % by the cytosolic fraction [Benov *et al.* , 1995]. However, there is no report about the exact source of O_2^- in *E. coli* periplasm.

Iron - containing superoxide dismutases (FeSODs), are typically found in cytoplasm of aerotolerant and aerobic bacteria. However, some obligately anaerobic bacteria such as *Bacteroides fragilis* , *B. thetaiotaomicron* and *B. distasonis* also have FeSOD and as a consequence they can tolerant some exposure to oxygen [Gregory *et al.* , 1977 ; Gregory and Dapper, 1980 ; Pennington and Gregory, 1986 ; Privalle and Gregory, 1979].

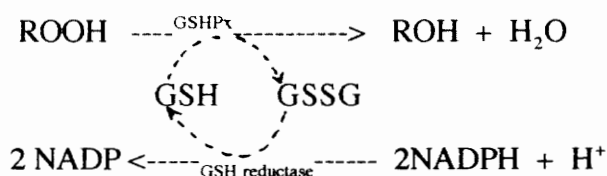
Manganese - containing superoxide dismutases (MnSODs) are found in cytosol of some prokaryotes and in eukaryotic mitochondria [Halliwell *et al.* , 1989]. FeSODs and MnSODs are known to defend bacterial cells against superoxide generated from bacterial metabolism, to protect enzymes that use 4Fe-4S centers at the active site, and to prevent oxidative damage to DNA [Flint *et al.* , 1993]. MnSOD is induced by oxygen exposure in aerobically grown *E. coli* and the SOD - induced cells gained resistance to hyperbaric oxygen lethality [Gregory *et al.* , 1973 a, b]. Some obligately anaerobic bacteria such as *Actinomyces* spp. also have MnSOD [Barkley and Gregory, 1990]. MnSODs and FeSODs are structurally similar to each other, but differ from the structure of Cu,ZnSODs [Hassan, 1988].

MnSOD, FeSOD and Cu,ZnSOD activities can be differentiated by their inhibition patterns. Cyanide selectively inhibits Cu,ZnSOD, but not FeSOD and MnSOD. Conversely, azide inhibits all three kind of the isozymes, but to different degrees. The order of the inhibition at a given azide concentration is $0 < \text{Cu,ZnSOD} < \text{MnSOD} < \text{FeSOD}$ [Misra and Fridovich, 1978].

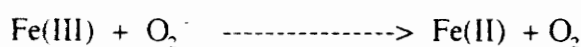
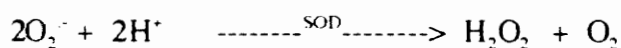
Cells have developed SODs and other antioxidant enzymes such as catalase and glutathione peroxidase (GSHPx) to alleviate oxygen toxicity. SODs directly decrease cellular $\text{O}_2^{\cdot -}$ level, whereas catalase and GSHPx remove H_2O_2 or alkyl peroxides, respectively. Both catalase and GSHPx can be inhibited by $\text{O}_2^{\cdot -}$. The reaction catalysed by catalase is :



Catalase is composed of four protein subunits, each containing a heme (Fe(III)-protoporphyrin) group in the active site that is stabilized by one molecule of tightly bound NADPH [Diplock, 1994]. Glutathione - peroxidase (GSHPx), a selenoenzyme, contains selenocysteine in each of the four identical subunits. The role of GSHPx is not only to catalyze the reduction of H_2O_2 , but perhaps more importantly, to reduce lipid hydroperoxides according to the reaction [Diplock, 1994] :



There is no known mechanism to remove hydroxyl radical catalytically; nevertheless, the efficient elimination of both superoxide radical and hydrogen-peroxide can modulate the formation of hydroxyl radical via the iron-catalysed Haber-Weiss reaction :



SOD decreases the rate of reduction of Fe(III) by $\text{O}_2^{\cdot -}$ and catalase decreases amount of H_2O_2 , effectively decreasing $\text{OH}^{\cdot}$ formation. Certain iron chelators prevent iron from participating in the above reaction.

Increased level of SODs enhance cellular antioxidant defenses [Omar *et al.*; Cutler, 1991; Mc Cord, 1992; Halliwell and Gutteridge, 1989; Gregory and Fridovich, 1973; Freeman *et al.*, 1983; Werns *et al.*, 1985]. However, increasing the level of cellular SODs by using the protein SODs is limited because the high molecular weight SODs can not cross the plasma membrane [Huber *et al.*, 1980]. However, complexes that have SOD-like activity (SOD mimics) have been studied [Gardner and Weser, 1984; Sorenson, 1984; Beyer and Fridovich, 1990; Emerit *et al.*, 1990; Liu *et al.*, 1994]. The complexes are based on the transitional elements found in the active sites of SODs. Although many studies have examined the effect of SOD mimics *in vitro* and *in vivo*, there is no clear indication of the relationship between SOD mimics and levels of other cellular antioxidant enzymes, especially SODs [Darr *et al.*, 1989; Elray-Stein *et al.* 1986; Steinkeehler *et al.*, 1990; Benov and Fridovich, 1995].

2.2 Types and Development of SOD Mimics

Superoxide dismutases can alleviate the deleterious effect from direct and indirect reactions of superoxide radical [Nagano *et al.*, 1989; Darr *et al.*, 1988; Mashino and Fridovich, 1987; Gardner and Fridovich, 1991; Faulkner *et al.*, 1989]. It is therefore

important to test native protein SODs or modified SODs as pharmaceuticals. Advantageous use of SODs in pharmacotherapy has been reported [Nagano *et al.*, 1989]. However, it is difficult to use native SODs because the protein will not enter cells unless special techniques such as liposome - fusion or scrape loading are applied [Rabinowitch *et al.*, 1989]. Additionally, native SODs are readily cleared from circulation by the kidneys [Nagano *et al.*, 1989]. There are also other problems associated with the use of native SODs. These problems are systemic injection of foreign protein, small circulation life - time, imprecise tissue targeting of injected SOD, and high costs of the pure protein [Iuliano *et al.*, 1992 ; Darr *et al.*, 1988]. Various low molecular compounds or complexes with SOD - like activity have been synthesized. To be an effective agent, the SOD mimic should be capable of membrane permeability, be biologically stable, be nonimmunogenic, nontoxic and specific for its substrate [Hahn *et al.*, 1991 ; Nagano *et al.*, 1989 ; Faulkner *et al.*, 1994].

The use of copper, manganese, and iron as the redox active components of native SODs led many investigators to search for low molecular weight complexes of these metals that have SOD activity. Conversely, these complexes might be ineffective in cells because of the free metal ions from their metal - ligand dissociation, subsequently bind to critical cellular components, causing deleterious effect [Darr *et al.*, 1987]. Therefore, metal - independent SOD mimics might be biologically useful [Mitchell *et al.*, 1990 ; An and Hsie, 1992 ; Krishna *et al.*, 1994 ; Hahn *et al.*, 1994]. However, the metal - dependent compounds or complexes have been vigorously sought and studied, compared with the metal - independent antioxidant compounds.

Certain copper chelates were found to dismute superoxide radical and provided

protection against *in vivo* oxygen lethality [Rabinowitch *et al.*, 1987]. Unfortunately, copper binds intensely to proteins and most copper complexes seem unlikely to retain activity *in vivo* as shown by Nagano and co-workers [Nagano *et al.*, 1989]. Addition of serum albumin depressed the activities of [Cu (Gly-His-Lys)], [Cu (salicylate)₂] and [Cu (DIPS)₂] in the cytochrome c reduction assay. Therefore, CuSOD mimics proved to be biologically ineffective because of their high rate of dissociation and chelation with groups ordinarily found in living cells [Nagano *et al.*, 1989 ; Hahn *et al.*, 1991]. The limited stability of CuSOD mimics in the presence of biological chelates and of the potential participation of copper in the Fenton reaction to generate hydroxyl radical promoted the search for MnSOD and FeSOD mimic complexes [Faulkner *et al.*, 1994].

Relatively few reports deal with FeSOD mimics [Nagano *et al.*, 1989]. Nagano and co-workers reported that Fe (II)-tetrakis-N,N,N',N' [2-pyridylmethyl] ethylene - diamine (Fe-TPEN) and Fe (III) - tris [N- (2-pyridylmethyl) - 2 - aminoethyl] amine (FeTPAA) have high SOD activity and can protect *E. coli* cells from paraquat toxicity [Nagano *et al.*, 1989]. However, Iuliano and co-workers demonstrated that Fe-TPEN acts as a Fenton catalyst, generating OH from H₂O₂ [Iuliano *et al.*, 1992]. Additionally, Weiss and co-workers reported that the compounds which have no SOD activity can give false positives in the cytochrome c reduction assay, if the compounds either inhibit the production of superoxide radicals or react with superoxide radicals stoichiometrically. FeTPEN, FeTMPP, TEMPO (2,2,6,6 - tetramethylpiperdin-1-yloxy), and TEMPOL (4 - hydroxy - 2,2,6,6 - tetramethylpiperidin-1-yloxy) can also give false positive for SOD activity in cytochrome c reduction assay by oxidizing reduced cytochrome c [Weiss *et al.*, 1993]. However, some of these compounds protect biological tissues against oxidative

damage [An and Hsie, 1992 ; Krishna *et al.*, 1994 ; Hahn *et al.*, 1994].

The limitation of the CuSOD and FeSOD mimics enhanced the search for a new kind of SOD mimic, based on manganese. MnSOD mimics are affected by bovine serum albumin in the assay but effectively removed superoxide radical [Hahn *et al.*, 1991]. However, the activity of most complexes was inhibited by EDTA. Most of the manganese complexes that have SOD-like activity have been studied for their ability to protect organisms from oxidative stress. The oxidative stress was provided by exposure to aerobic conditions greater than normally found in the cells environment or by redox cycling compounds. Superoxide scavenging activity of manganese complexes was studied in bacteria, green alga, and mammalian cells [Rabinowitch *et al.*, 1987 ; Darr *et al.*, 1988 ; Rabinowitch *et al.*, 1989 ; An and Hsie, 1992 ; Liu *et al.*, 1994 ; Faulkner *et al.*, 1994 ; Liochev and Fridovich, 1995].

Archibald and Fridovich (1981) demonstrated that *Lactobacillus plantarum*, an aerotolerant organism that is devoid of protein SOD, actively accumulated free Mn(II) and used a Mn - protein complex that protected against hyperbaric oxygen toxicity and the oxygen-dependent lethality of plumbagin [Archibald and Fridovich, 1981]. They also reported that *in vitro*, Mn(II) - protein complex scavenged superoxide radicals only in the absence of EDTA, and the activity could be mimicked by MnCl₂. Additionally, they had other evidence to support the superoxide scavenging activity of Mn(II). For example, Mn(II) can inhibit lipid - peroxidation in biological membrane, and Mn(II) within chloroplasts could act as a superoxide scavenger [Kono *et al.*, 1976 ; Archibald and Fridovich, 1981]. There is evidence that Mn(II) acted *in vitro* as a superoxide scavenger based on its ability to inhibit the reduction of NBT or cytochrome c [Lumsden and Hall,

1975 ; Halliwell, 1975 ; Halliwell and Ahluwalia, 1976]. However, Mn(II) has been reported to act as a stoichiometric rather than catalytic superoxide scavenger [Archibald and Fridovich, 1981; Weiss *et al.*, 1993].

A number of manganese complexes have been developed and characterized. Some complexes were effective superoxide scavengers *in vitro* and or *in vivo* under certain conditions. Among the manganese complexes that have SOD - like activity, manganese - desferal , manganic (III) - porphyrin , and manganese (III) - salophen have been well characterized. Although some of the manganese complexes such as manganese - desferal complexes, and manganic (III) - porphyrin act as stoichiometric, not catalytic, superoxide scavengers, those complexes still are effective superoxide scavengers *in vivo* [Weiss *et al.*, 1993 ; Faulkner *et al.*, 1994]. Mn(III)-salophen has been shown to be an effective superoxide scavenger *in vitro* , based on cytochrome c reduction assay and NBT assay and *in vivo* [Liu *et al.*, 1994].

Desferrioxamine - manganese (DF-Mn) complex exhibited superoxide scavenging activity and protected *Dunaliella salina* , a unicellular green alga, from paraquat toxicity [Darr *et al.*, 1987 ; Beyer and Fridovich, 1989 ; Rabinowitch *et al.*, 1987]. Rabinowitch *et al* also reported that the paraquat toxicity to *D. salina* was light dependent and alleviated by either prior induction of SOD or addition the complex, but not its components. The complex readily diffused out of the cells and could be washed away. Rabinowitch and co-workers showed that DF-Mn protected *Chlorella sorokiniana* against sulfite toxicity [Rabinowitch *et al.*, 1989]. Darr *et al* used DF-Mn to protect Chinese hamster ovary (CHO) cells against paraquat cytotoxicity. The protection was from the complex but not its components. However, they also reported that MnCl₂ provided little protection against

paraquat toxicity. Additionally, DF-Mn exhibits a dose - dependent toxicity to the CHO cells [Darr *et al.*, 1987]. Huang *et al* reported that DF-Mn enhanced the healing of surgically induced skin lesions in rats [Huang *et al.*, 1987]. Bhuyan *et al* showed that DF-Mn protected rabbits against toxicity of diquat - induced cataract [Bhuyan *et al.*, 1991]. DF-Mn protected against the over - production of superoxide radicals in many studies, and the SOD - like activity was from a reversible oxidation / reduction cycle of the complex [Darr *et al.*, 1987 ; Beyer and Fridovich, 1987]. Gray and Carmichael, however, reported that the SOD - like activity of DF-Mn originated from the Mn(II), not from the complex [Gray and Carmichael, 1992]. Weiss *et al* reported that Mn(II) and DF-Mn acted as stoichiometric superoxide scavengers [Weiss *et al.*, 1993].

Mn(III)-porphyrin (MnTMPyP) has SOD - like activity in the cytochrome c reduction method, and protects *sod A* ⁻ *sod B* ⁻ *E. coli* against dissolved oxygen and the parental strain against paraquat toxicity [Faulkner *et al.*, 1994 ; Liochev and Fridovich, 1995]. Additionally, the protective effect of MnTMPyP against paraquat toxicity was from the inhibition of paraquat uptake [Liochev and Fridovich, 1995]. MnTMPyP protected *E. coli* SOD double mutant in stationary phase from superoxide radical production, prevented the induction of the antibiotic resistance, and protected against heat kill [Benov and Fridovich, 1995]. However, this complex acted as an NADPH / GSH : O₂⁻ oxidoreductase rather than as an SOD mimic, *in vivo* [Faulkner *et al.*, 1994].

Mn(III)-salophen reacts with superoxide radical as measured by spectral changes and polarographic measurements [Matsushita and Shono, 1981]. Additionally, Mn(III)-complexed with salen or salophen (the diSchiff base) reacts with superoxide radical in DMSO [Coleman and Taylor, 1982].

Liu *et al* (1994) reported the preparation and catalytic characterization of Mn(III)-salophen with superoxide scavenging activity. *In vitro* , Mn(III)-salophen has superoxide scavenging activity based on the inhibition of cytochrome c reduction and inhibition of formazan in the absence of EDTA. The superoxide scavenging activity of this complex has also shown not to be a false positive because the complex did not reoxidize reduced cytochrome c in the cytochrome c reduction assay. The activity of the complex was retained in the presence of 1 mg / ml bovine serum albumin and *E. coli* crude extract , but was 50 % inhibited in the presence of 1 mM EDTA. Additionally, the activity of the complex was still high in minimal medium after 24 h at 37 C. The superoxide scavenging activity of 1:1 molar complex of Mn(III) and salophen in SOD units was equivalent to 4.6×10^8 / units / mol of the complex. *In vivo*, Mn(III)-salophen promoted aerobic growth of *E. coli* double mutant QC 779 in glucose minimal medium. The complex also exhibited a dose response protection pattern in *E. coli* . The complex's components have a slight effect on promoting the growth of QC779. However, Mn(III)-salophen does not accumulate in the cells and the complex could be washed away.

2.3 Increasing the level of the intracellular superoxide radicals

Since superoxide radicals can not cross the *E. coli* cell envelope, redox - cycling compounds and hyperbaric oxygen or hyperoxygenation have been used to increase the level of the intracellular superoxide radicals [Hassan and Fridovich, 1977 , 1979 ; Cerutti, 1985 ; Purucker and Lutz, 1992 ; Kim *et al.* , 1996]. Takabatake reported that intracellular

superoxide radical production was more important for toxicity than extracellular superoxide radical production [Takabatake *et al.*, 1992]. Intracellular superoxide radicals can have either a bacteriostatic or a bactericidal effect, depending on severity of the stress [Kuo *et al.*, 1987]. Additionally, Cerutti reported that radiation, xenobiotic metabolites Fenton - type reagents, and modulators of the cytochrome P-450 electron - transport chain increases concentrations of active oxygen, organic peroxides, and radicals which can be suppressed by cellular antioxidant defense enzymes and low molecular scavenger molecules [Cerutti, 1985]. Redox - cycling compounds such as paraquat (methyl - viologen), plumbagin, menadione, and phenazine methosulfate are used to produce superoxide radicals via redox cycling *in vivo* [Chan and Weiss, 1987 ; Clair *et al.*, 1991]. Paraquat can be actively taken up by *E.coli*, reduced by NAD(P)H - dependent diaphorase, and then reoxidized by oxygen to produce superoxide radicals [Liochev and Fridovich, 1995 ; Hassan and Fridovich, 1978]. The generation of superoxide radicals depends on entry of paraquat to the cells, which depends on pH of media and absence of certain inhibitory salts and polyamines [Kitzler and Fridovich, 1986 ; Liochev and Fridovich, 1995]. Therefore, paraquat toxicity depends on paraquat concentration, the availability of an electron supply, the concentration of available molecular oxygen, and the concentration of SOD within the cell [Hassan, 1983]. Additionally, the transition metals, Fe and Cu, play an important role in the paraquat toxicity, probably by redox cycling and producing hydroxyl radical via site - specific Fenton reaction [Korbashi *et al.*, 1986].

2.4 Parental strain and *sod A*⁻ *sod B*⁻ strains of *Escherichia coli*

E. coli, a facultative anaerobic bacteria, contains two cytoplasmic SODs (FeSOD

and MnSOD) and a periplasmic SOD (Cu,ZnSOD) [Hassett *et al.*, 1995]. *E. coli* double mutant strains (JI132, QC779), lacking both Mn- and Fe-SOD, were produced by consecutive transduction of *sod A*⁺ linked chloramphenicol resistance and *sod B*⁺ linked kanamycin resistance into AB1157, the superoxide dismutase - proficient parent [Imlay and Linn, 1987]. However , double mutant strains of *E. coli* do not completely lack SOD because they still have a Cu,ZnSOD [Benov and Fridovich, 1994]. The Cu,ZnSOD, an oxygen inducible enzyme, is present in both a parental strain and a double mutant strain [Benov and Fridovich, 1994]. Benov *et al* reported that *E. coli* Cu,ZnSOD is similar to human Cu,ZnSOD, but contains methionine and tyrosine [Benov *et al.*, 1995].

E. coli double mutant was hypersensitive to oxidizing agents and showed an oxygen - dependent and UV light - dependent mutagenesis [Benov Fridovich, 1995 ; Farr *et al.*, 1986 ; Hoerter *et al.*, 1989]. The double mutant was also auxotrophic for branched - chain amino acids in aerobic growth and sensitive to oxygen toxicity imposed by redox-cycling compounds [Carlioz and Touati, 1986 ; Steinman *et al.*, 1994].

Enzymes in branched - chain amino acid biosynthesis, oxidative defense, and the Krebs cycle are inactivated by superoxide radicals [Kuo *et al.*, 1987]. These include α,β - dihydroxy - acid dehydratase (DHAD), 6 - phosphogluconate - dehydratase, aconitase, catalase - peroxidase, and fumarase A and B [Kuo and Fridovich, 1987 ; Gardner and Fridovich, 1991 ; Gardner and Fridovich, 1992 ; Hassett *et al.*, 1995]. An Fe-S cluster in dehydratase enzymes is proposed to be a superoxide radical sensitive target [Gardner and Fridovich, 1991]. Recently, asparagine synthetase was found to be an oxidant - sensitive enzyme [Draczynska - Lusiak and Brown, 1994]. Additionally,

DNA and the cell envelope are the targets of the damage from superoxide radicals in a double mutant strain [Steinman *et al.*, 1994].

Because superoxide radicals can not cross membranes, superoxide radicals that cause any damage to the enzymes in a double mutant strain should be formed inside the margin of the cytoplasmic membranes [Imlay, 1995].

In this report, *E. coli* *sodA*⁻ *sodB*⁻ was used to evaluate the effective function of Mn(III)-salophen against the deleterious effects from an oxidative stress.

III MATERIALS and METHODS

3.1 Chemicals

Bacto tryptone, bacto yeast extract, bacto agar, and casamino acids were purchased from Difco. Ferricytochrome c, xanthine, bovine serum albumin, EDTA, thiamine-hydrochloride, methyl viologen (paraquat), and sucrose were purchased from Sigma Chemical Co. Monobasic and dibasic potassium phosphate, $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KOH , H_2O_2 , NaCl , NaN_3 , NaCN , and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ were purchased from Fisher Scientific Co. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were purchased from J. T. Baker Chemical Co. Tris was from Research Organics, Inc. Glucose was from ICN Biochemicals. Ethanol (200 proof) was from Aper Alcohol and Chemical Co. Chelex 100 was from Bio - Rad Labs. Manganese(III) acetate dihydrate, N, N'- bis - (salicylidene) - 1, 2, - phenylenediamine (salophen) was purchased from Aldrich Chemical Co. Mn(III)-salophen was prepared as described by Liu [Liu *et al.*, 1994]. Fe(III)-salophen was also prepared by a modification of the method described by Liu for Mn(III)-salophen preparation [Liu *et al.*, 1994]. Xanthine oxidase, from bovine milk , was prepared and purified in this lab [Rajagopalan, 1985]. Distilled, deionized water was used unless otherwise noted.

3.2 Bacterial strains

E. coli strains - *E. coli* K12 *sod A sod B* mutant was described previously [Carlizoz and Touati, 1986; Farr *et al.*, 1986]. The strains used in this study, QC779 and JI132, carry the mutations (*sodA* : MPR_{13})₂₀ ϕ (*sodB* -kan) 1- Δ 2, and (*sod A* :: Mud PR

13)₂₅ ϕ (*sodB* -kan) 1- Δ 2, respectively. The parental wild - type strain was AB1157 [Imlay and Linn, 1987]. The mutant of parental strains were a gift from Irwin Fridovich (Duke University). Bacteria used in this report were grown from one colony on LB plates.

3.3 Paraquat toxicity studies.

E. coli JI132 was grown in LB media [Miller *et al.* , 1972], adjusted pH to 7.0, at 37 C, 100 rpm (rotary platform, New Brunswick Model R26) overnight to an OD₆₀₀ of ≈ 3.5 . The overnight culture was diluted 1:14 (vol / vol) in fresh prewarmed LB media and incubated at 37 C, 100 rpm to OD₆₀₀ ≈ 1.3 . Cell suspensions were centrifuged at 5000 rpm for 5 minutes at 10 C, washed once and resuspended with modified M9 salts [Maniatis *et al.* , 1989] in tap water supplemented with 0.2 % glucose and 2.0 % casamino acids, adjusted pH to 7.0 and used to inoculate the same media. The inoculated medium typically had an initial OD₆₀₀ ≈ 0.06 . Graded amounts of filtered - sterilized Mn(III) - salophen, Mn(III), salophen , or Fe(III)-salophen , were then added along with paraquat to the final concentration indicated. Cultures to flask volumes were 1:10 to ensure proper aeration. Cultures were aerated by shaking at 100 rpm, 37 C in foil - covered 500 ml Erlenmeyer flasks. Cell growth was measured every 2 h at 600 nm using a Shimadzu UV-265 spectrophotometer [Koch, 1970]. Cells were Gram - stained and examined microscopically at the time of inoculation and at 8 h of incubation.

3.4 Sensitivity to aerobic growth

E. coli QC779 was grown in LB medium [Miller *et al.*, 1972], adjusted to pH 7.0, at 37 C, 100 rpm (rotary platform, New Brunswick Model R26) overnight to an OD₆₀₀ of ≈ 3.5 . The overnight culture was diluted 1:14 (vol / vol) in fresh prewarmed LB media and incubated at 37 C, 100 rpm to an OD₆₀₀ of ≈ 1.3 . Then, cell suspensions were centrifuged at 5000 rpm for 5 minute 10 C ,washed once and resuspended with M63 [Miller *et al.*, 1972], adjusted pH to 7.0, supplemented with 0.4 % glucose and 0.2% casamino acids, and used to inoculate the same media to an OD₆₀₀ of ≈ 0.04 . Then, graded amounts of filter - sterilized Mn(III)-salophen , Mn(III) , salophen , or MnCl₂ were added at the time of inoculation. Culture to flask volume was 1:10 to ensure proper aeration. Cultures were aerated by shaking at 100 rpm , at 37 C in foil - covered 500 ml Erlenmeyer flasks. Cell growth was measured every 2 h at 600 nm (OD_{600 nm}) using a Shimadzu UV-265 Spectro-photometer [Koch, 1970]. Cells were Gram - stained and examined microscopically at the time of inoculation and after 8 h of incubation.

3.5 Sensitivity to hyperoxygenation

E. coli JI132, and AB1157 were grown in LB media [Miller *et al.*, 1972], adjusted to 7.0, at 37 C, 200 rpm (oscillator shaker, New Brunswick Model G25) overnight to an OD₆₀₀ ≈ 3.5 . Inocula were prepared as described above but the casamino acids was 0.2 % and the inoculated medium typically had an initial OD₆₀₀ ≈ 0.04 . Graded amounts of filtered-sterilized Mn(III)-salophen, Mn(III), or salophen were then added. Cultures were bubbled with humidified oxygen gas mixed with nitrogen gas at the same partial pressure (20 Psi) to yield 50 % oxygen. The rate of application was 1L / min /

flask with shaking at 200 rpm at 37°C. A volume / flask ratio of 1:10 in foil - covered 500 ml Erlenmeyer flasks was used. Cell growth was monitored as described above.

3.6 Biochemical assays

The ability of Mn(III)-salophen to inhibit the reduction of cytochrome c by superoxide radicals was monitored at 25 °C as described by McCord and Fridovich [1969]. Assays were performed using a Shimadzu UV-265 UV-Visible Recording Spectrophotometer. Superoxide radicals were generated by a xanthine - xanthine oxidase system, in 50 mM phosphate buffer pH 7.8 without EDTA. An assay contained 10 µM ferricytochrome c and 50 µM xanthine [McCord and Fridovich, 1969].

IV RESULTS

4.1 Superoxide Scavenging activity

Mn(III)-salophen was prepared and used as described by Liu [Liu *et al.*, 1994]. This complex showed significant superoxide scavenging activity by the xanthine / xanthine oxidase cytochrome c reduction method in the absence of EDTA, without inhibiting the xanthine oxidase reaction. BSA (1mg / ml) did not interfere with superoxide scavenging by Mn(III)-salophen (data not shown). These results are consistent with those reported by Liu *et al* [Liu *et al.*, 1994]. Additionally, the activity of the complex was stable in the media bubbled with 100% oxygen (Table 4.3.3). The biological antioxidant effect of Mn(III)-salophen was studied in the growth of *E. coli* *sod A⁻ sod B⁻* in glucose / salts minimal medium supplemented with casamino acids exposed to oxidative stress conditions.

4.2 Effect of Mn(III)-salophen on paraquat exposure

The aerobic growth of *E. coli* J1132 *sod A⁻ sod B⁻* in M9 salts glucose medium supplemented with 2% casamino acids was apparently inhibited by a sub - lethal dose of paraquat (1.0 μ M) under these condition (Fig.4.2.1). The sub - lethal dose of paraquat effected the growth rate (Fig. 4.2.2, 4.2.3) or the doubling time (Table 4.2.2, 4.2.3). Mn(III)-salophen (0.0625 μ M) completely protected the cells from 1.0 μ M paraquat toxicity. Furthermore, Mn(III)-salophen at 0.0625, 0.125, 0.25, 1.0, and 2.5 μ M enhanced the growth rate, decreased the lag phase (Fig.4.2.2, 4.2.3), and decreased the

doubling time as compared to the control with paraquat alone (Table 4.2.2, 4.2.3).

Mn(III) acetate alone at the same concentration as Mn(III)-salophen also protected against the paraquat toxicity in the media containing casamino acids (Fig. 4.2.2, 4.2.3). Salophen alone did not protect the cells from paraquat toxicity in the media (data not shown).

Fe(III)-salophen, complex of Fe(III) and salophen (personal communication, Kristi Johnson) 0.125 μ M, which has no superoxide scavenging activity by based on cytochrome c reduction method, did not inhibit significantly the aerobic growth of *E. coli* J1132 *sod A⁻ sod B⁻* in glucose / salts medium supplemented with 2% casamino acids as compared with the positive control (Fig. 4.2.4). Fe(III)-salophen (0.125 μ M) also did not protect the cells from 1.0 μ M paraquat toxicity. Furthermore, Fe(III)-salophen enhanced the toxicity of paraquat as evidenced by the increased lag phase, decreased growth rate, and increased the doubling time of the bacteria (Fig. 4.2.4 ; Table 4.2.4).

Most of data showed in tabular form indicate the mean and standard error, while most of data points presented graphically represent mean values from at least duplicate experiments.

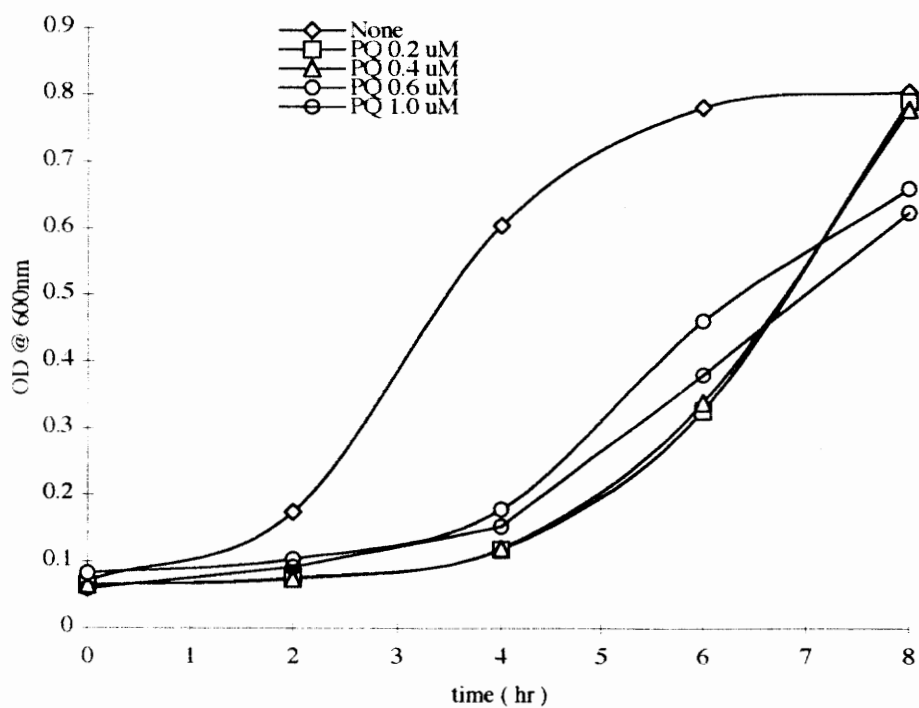


Fig. 4.2.1 The concentration dependent effect of paraquat on 8h. aerobic growth of *E. coli* JI132 *sodA* - *sodB* - . Cells were grown in modified M9 salts as described in Materials and Methods with the indicated concentration of paraquat.

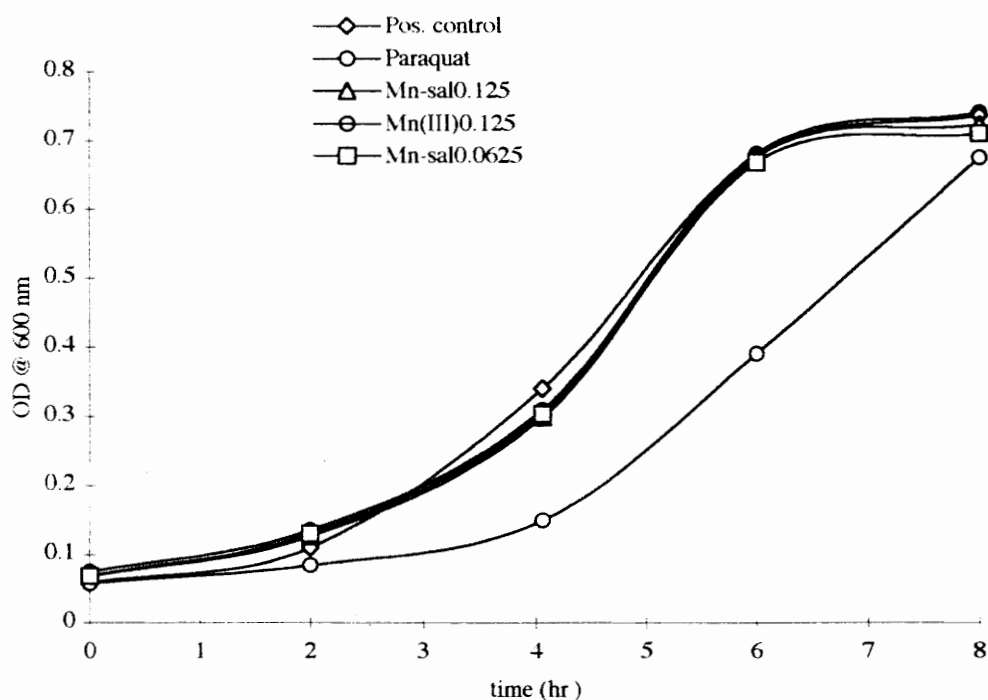


Fig. 4.2.2 The effect of Mn(III)-salophen and manganese salt @ 0.0625, 0.125 uM on protecting aerobic growth of *E. coli* JI132 from 1.0 uM paraquat. Cells were grown in the indicated media with 1.0uM paraquat as described in Materials & Methods.

Table 4.2.2 Doubling time of the aerobic growth of *E. coli* JI132 in the presence of Mn(III)-salophen, or manganese salt and 1.0 μ M paraquat.

Cells were grown as described in Materials and Methods. Absorbance at 8 h was determined at 600 nm [Koch,1970].

Doubling time (hr)

<i>E. coli</i> strain	Positive control	PQ 1.0 μ M	Mn(III)-sal 0.125 μ M + 1.0 μ M PQ	Mn(III) 0.125 μ M + 1.0 μ M PQ	Mn(III)-sal 0.0625 μ M + 1.0 μ M PQ
JI132	1.17 $\pm$ 0.06	2.08 $\pm$ 0.32	1.60 $\pm$ 0.02	1.64 $\pm$ 0.02	1.62

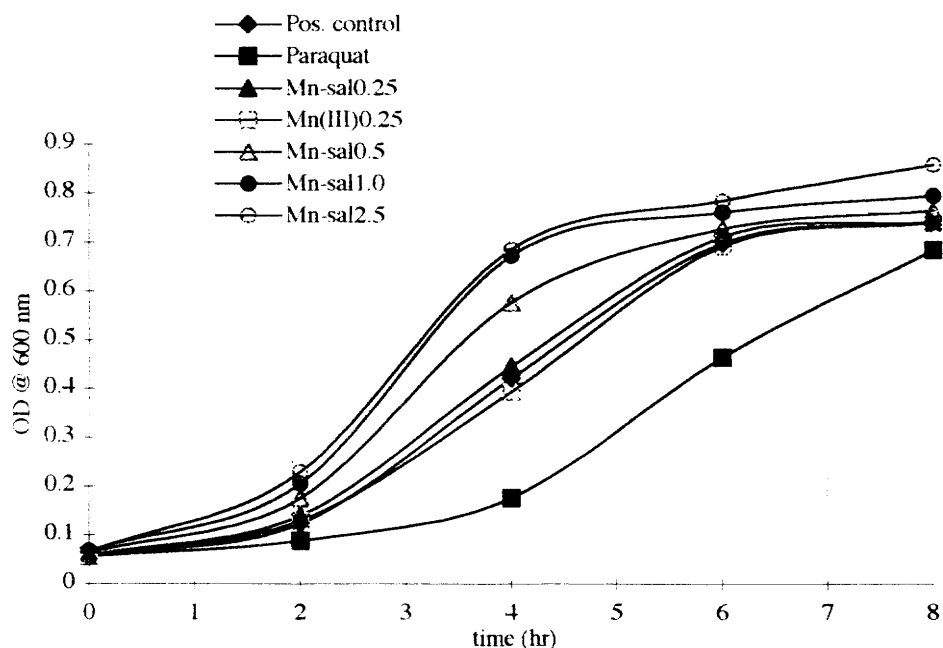


Fig. 4.2.3 The effect of Mn(III)-salophen and manganese salt @ 0.25, 0.5, 1.0, and 2.5 uM on protecting aerobic growth of *E. coli* JI32 from 1.0uM paraquat. Cells were grown in the indicated media with 1.0 uM paraquat as described.

Table 4.2.3 Doubling time of the aerobic growth of *E. coli* JI132 in the presence of Mn(III)-salophen or manganese salt and 1.0 μ M paraquat.

Cells were grown as described in Materials and Methods. Absorbance at 8 h was determined at 600 nm [Koch, 1970].

Doubling time (hr)

<i>E. coli</i> strain	Positive control	PQ 1.0 μ M	Mn(III)-sal 0.25 μ M + 1.0 μ M PQ	Mn(III) 0.25 μ M + 1.0 μ M PQ	Mn(III)-sal 0.5 μ M + 1.0 μ M PQ	Mn(III)-sal 1.0 μ M + 1.0 μ M PQ	Mn(III)-sal 2.5 μ M + 1.0 μ M PQ
JI132	1.14 $\pm$ 0.01	2.32 $\pm$ 0.38	1.3 $\pm$ 0.1	1.3	1.18 $\pm$ 0.003	1.23 $\pm$ 0.08	1.25

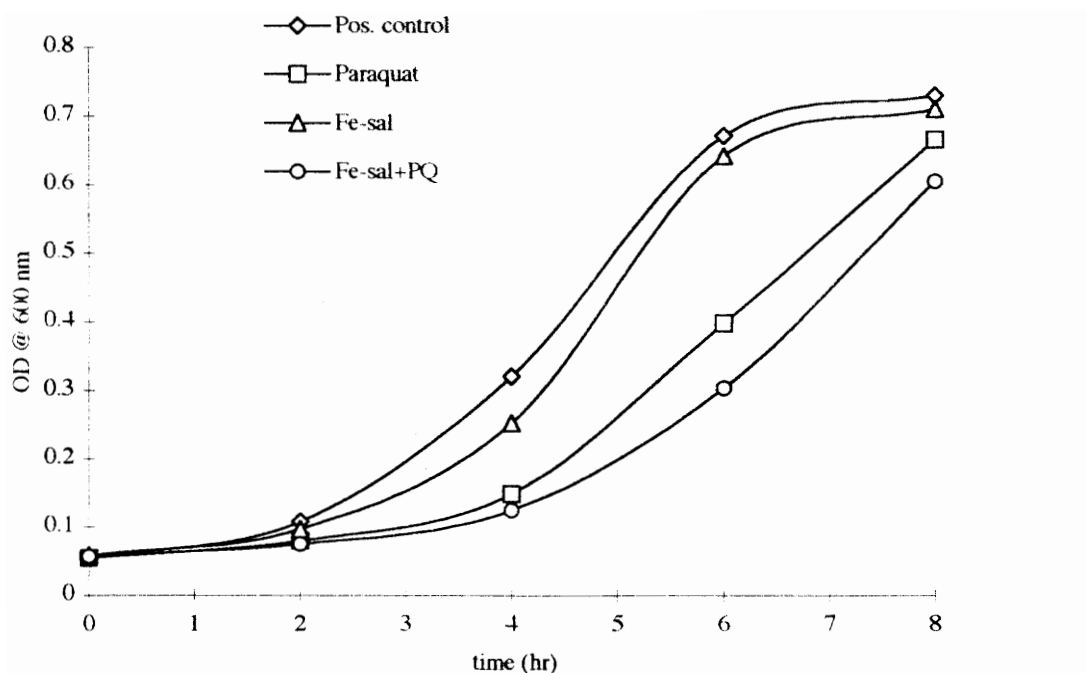


Fig. 4.2.4 The effect of 0.125 uM Fe(III)-salophen on aerobic growth of *E. coli* JI132 *sodA* - *sodB* - with 1.0 uM paraquat. Cells were grown as described in Materials and Methods with the indicated media.

Table 4.2.4 Doubling time of *E. coli* JI132 *sodA-sodB*- grown aerobically in the presence of Fe(III)-salophen and 1.0 μ M paraquat. Cells were grown as described in Materials and Methods with the indicated media. These results are the average of values from duplicate experiments. Absorbance at 8 h was determined at 600 nm [Koch, 1970]

Doubling time (hr)

<u>E. coli</u> strain	Positive control	PQ 1.0 μ M	Fe(III)-sal 0.125 μ M	Fe(III)-sal 0.125 μ M + 1.0 μ M PQ
JI132	1.25	2.2	1.45 $\pm$ 0.05	2.73

4.3 Effects of Mn(III)-salophen on aerobic growth and hyperoxia of *E. coli* *sodA*⁻ *sodB*⁻

E. coli QC779 *sodA*⁻ *sodB*⁻ show dioxygen - dependent branched - chain amino acids auxotrophy. They grow scantily in glucose / salts minimal medium (M63) in 8 h aerobically (data not shown). However, they can grow slowly in aerobic M63 glucose medium supplemented with 0.2 % casamino acids. Cell growth was enhanced by addition of Mn(III)-salophen, Mn(III) acetate, or MnCl₂ , each at 2.4 μM, to the medium. After a 4 h lag phase, cell grew to an OD₆₀₀ of 4.2, 4.3, and 4.5 absorbance units at 8 h in the presence of Mn(III)-salophen, Mn(III) acetate, or MnCl₂ , respectively (Fig. 4.3.1). These values are about 3 times higher than that of cells incubated in the medium but without the Mn(III)-salophen complex or manganese salts (Fig. 4.3.1). Additionally, the complex and manganese salts decreased the doubling time of aerobically grown *E. coli* QC779 as compared with the control (Table 4.3.1) ; cells grown in the medium but in the absence of Mn(III)-salophen and manganese salts served as control in this experiment. Salophen did not enhance the growth rate of *E. coli* QC779 in aerobic conditions in the concentration range of Mn(III)-salophen used in these experiments.

E. coli JI132 *sodA*⁻ *sodB*⁻ and *E. coli* AB1157 (parental strain) were grown in the M9 salts glucose medium supplemented with 0.2 % casamino acids (modified M9 salts glucose medium) in 50 % oxygen. As shown in Fig. 4.3.2, *E. coli* JI132 *sodA*⁻ *sodB*⁻ grew scantily in the modified medium as compared with its parental strain in 50 % oxygen. However, cell growth was enhanced by addition of Mn(III)-salophen or Mn(III) acetate (4.9 or 9.8 μM) to the medium. After an 2 h lag phase, cells grew to an OD₆₀₀ of 0.50 and 0.59 after 8 h exposure to 50 % oxygen in the presence of 4.9 and 9.8 uM Mn(III) -

salophen, respectively. The cells grew to an OD_{600} of 0.48 and 0.69 after 8 h exposure to 50 % oxygen in the presence of 4.9 and 9.8 μ M Mn(III) acetate, respectively (Table 4.3.2). Salophen alone at concentration equimicromolar to Mn(III)-salophen or Mn(III) acetate used in this study did not protect as compared with Mn(III)-salophen or Mn(III) acetate (data not shown).

4.3.1 The activity of Mn(III)-salophen and Mn(III) acetate in casamino acids medium and in *E. coli* JI132 *sodA*⁻ *sodB*⁻ in 100 % oxygen.

As shown in Table 4.3.3, Mn(III)-salophen exhibited superoxide scavenging activity that was stable in the M63 glucose medium supplemented with casamino acids in 100 % oxygen. The activity of the complex was quantitated using the xanthine - xanthine oxidase system. Additionally, casamino acids in the medium did not effect the superoxide scavenging activity of Mn(III)-salophen. Mn(III) acetate also exhibited the low level of antioxidant activity in the M63 glucose medium, but higher level in the medium supplemented with casamino acids.

However, *E. coli* JI132 *sodA*⁻ *sodB*⁻ growth was not significantly enhanced by addition of 2.4 or 4.9 μ M Mn(III)-salophen or Mn(III) acetate to the medium in 100 % oxygen (data not shown).

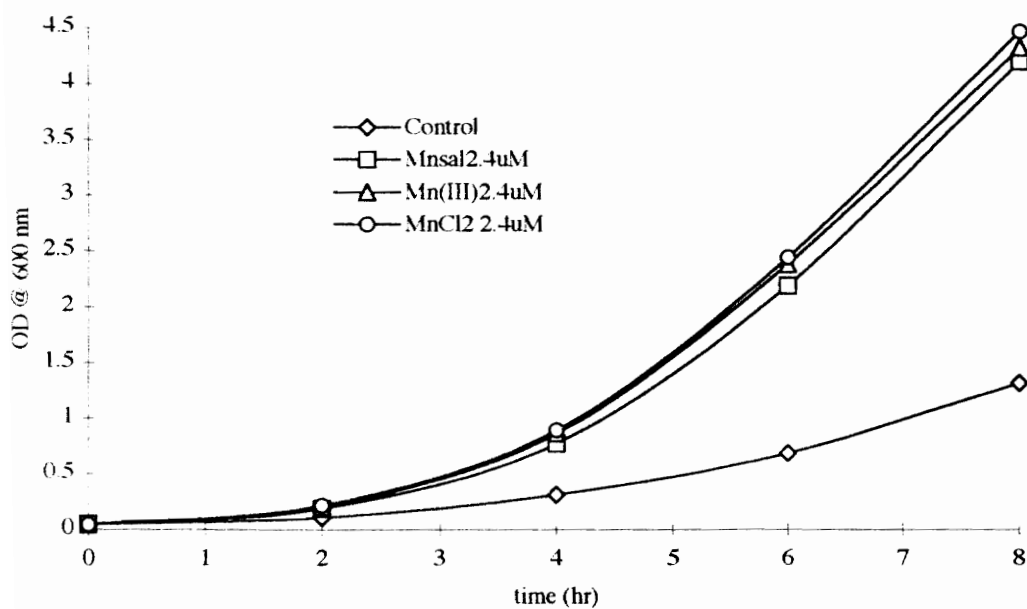


Fig. 4.3.1 The effect of Mn(III)-salophen and manganese salts on aerobic growth of *E. coli* QC779 *sodA* - *sodB* -. Cells were grown as described in Materials and Methods with the indicated media.

Table 4.3.1 Doubling time of *E. coli* QC779 *sodA-sodB*- in the presence of Mn(III)-salophen and manganese salts in aerobic condition. Cells were grown as described in Materials and Methods with the indicated media. Absorbance at 8 h was determined at 600 nm [Koch, 1970].

Doubling time (hr)

<u><i>E. coli</i></u> strain	Control	Mn-sal 2.1µM	Mn-sal 2.4µM	Mn(III) 2.4µM	MnCl ₂ 2.4µM
QC779	1.28	0.98	0.97	0.94	0.97

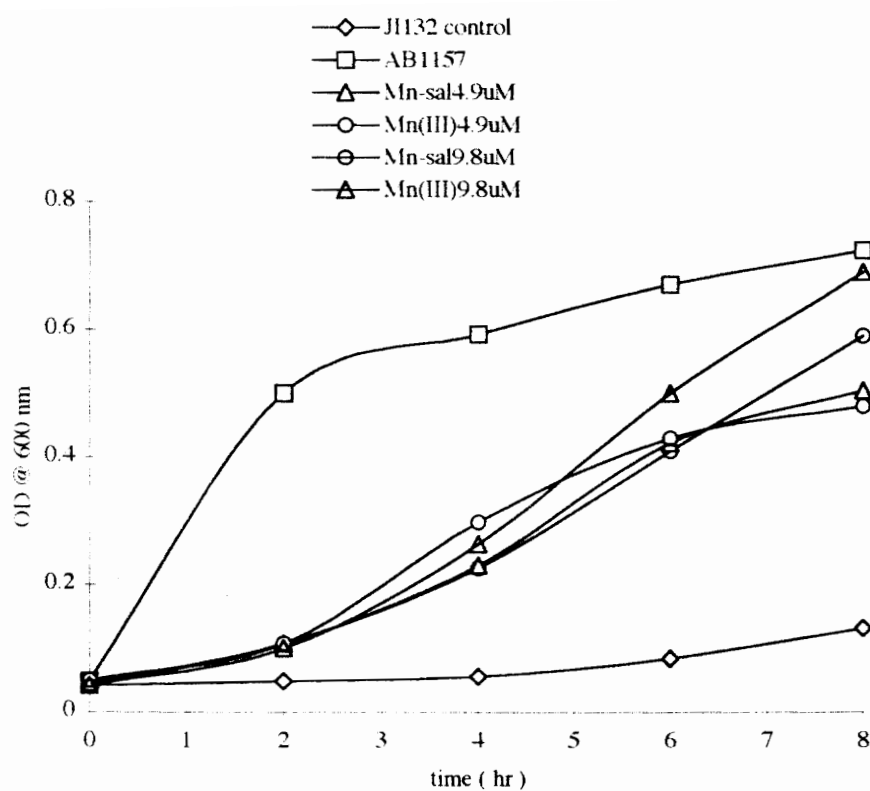


Fig. 4.3.2 The effect of Mn(III)-salophen and manganese (III) acetate on 50% oxygen growth of *E. coli* JI132 *sodA*⁻ *sodB*⁻ compare with the parental strain (AB1157). *E. coli* both strains were grown as described in Materials and Methods.

Table 4.3.2 Effects of Mn(III)-salophen and manganese(III) acetate on 50% oxygen growth of *E. coli* JI132 *sodA'sodB*. Cells were grown in M9 glucose medium + 0.2% casamino acids as described in Materials and Methods.

Addition	OD @ 600 nm*
Control (JI132)	0.13
Positive control (AB1157)	0.72
Mn(III)-salophen 4.9 μ M	0.50
Mn(III) 4.9 μ M	0.48
Mn(III)-salophen 9.8 μ M	0.59
Mn(III) 9.8 μ M	0.69

* OD₆₀₀ was measured after 8 h incubation at 37 C [Koch, 1970]

Table 4.3.3 The superoxide scavenging activity of Mn(III)-salophen and manganese(III) acetate 2.4 μ M in 100 % oxygen condition in the indicated media. The solution was assayed for superoxide scavenging activity at 0, 1, 2, 3, 5 h incubation at 37 C with shaking. Medium (M63 or M63 + casamino acids) exhibited no measurable superoxide scavenging activity in the absence of manganese. The activity was measured using the xanthine - xanthine oxidase cytochrome c reduction method [McCord and Fridovich, 1969].

Media	Mn(III)-salophen				Mn(III)			
	M63		M63 + 0.2% casamino acids		M63		M63 + 0.2% casamino acids	
time (hr)	% inhibition	U/ml	% inhibition	U/ml	% inhibition	U/ml	% inhibition	U/ml
0	52.0	5.5	52.0	5.5	42.8	3.8	52.0	5.5
1	52.0	5.5	54.0	5.9	44.0	4.0	52.0	5.5
2	52.0	5.5	52.0	5.5	42.8	3.8	52.0	5.5
3	50.0	5.0	54.0	5.9	44.0	4.0	52.0	5.5
5	50.0	5.0	52.0	5.5	44.0	4.0	52.0	5.5

V DISCUSSION

5.1 Superoxide Scavenging activity

The superoxide scavenging activity of Mn(III)-salophen was stable in the glucose / salts medium supplemented with casamino acids and the activity was not suppressed by amino acids in the medium (Table 4.3.3). The activity of the complex was not suppressed by 1 mg / ml of BSA but the activity was inhibited 50 % by EDTA. Mn(III)-salophen did not oxidize reduced cytochrome c in the xanthine - xanthine oxidase cytochrome reduction method (data not shown). Therefore, the superoxide scavenging activity of Mn(III)-salophen was not a false positive which was consistent with the observation by Liu *et al* [Liu *et al.*, 1994]. However, Mn(III) acetate, a component of Mn(III)-salophen, and MnCl₂ also exhibited the superoxide scavenging activity in the glucose / salts medium supplemented with casamino acids *in vitro*, using xanthine - oxidase system (Table 4.3.3) and presumably does so *in vivo* (Fig. 4.2.2, 4.2.3, 4.3.1, 4.3.2). The superoxide scavenging activity of Mn(III)-salophen in the glucose / salts medium supplemented with casamino acids might be from the complex itself or Mn(III) acetate (its component) or both. Therefore, it is apparent that these results are consistent with Liu 's observation [Liu *et al.*, 1994]. Mn(III) acetate and MnCl₂ probably reacted with amino acids in the media, and the compounds exhibited the superoxide scavenging activity.

5.2 Effects of Mn(III)-salophen on inhibitory effect of paraquat in *E. coli* JI132

sod A⁻ sodB⁻

E. coli JI132 *sod A⁻ sod B⁻* that lacks both MnSOD and FeSOD grew scantily in 8 h in glucose / salts medium unless the branched chain amino acids were supplied. Casamino acids, completely hydrolysed proteins, were used to supply the deficient amino acids in the paraquat studies. Paraquat was used to increase the intracellular level of superoxide radicals. Therefore, the inhibitory effect of paraquat to the *E. coli* depends on the production of superoxide radical due to the autoxidation of the univalently reduced paraquat. These reactions were documented by Hassan and Fridovich [1979]. For these reasons, the antioxidant activity of Mn(III)-salophen was tested against the inhibitory effect of paraquat in the *E. coli*. The present study reveals that Mn(III)-salophen protects against 1.0 μ M paraquat (Fig. 4.2.2, 4.2.3). The protective action of the complex was due to the superoxide scavenging activity of the complex itself, and or its component, Mn(III), but not from the salophen (the chelating agent). Mn(III) are equally effective in protecting the cells under this condition.

As shown in Fig. 4.2.4, we can further infer that the Mn(III)-salophen action was intracellular rather than extracellular. The reasons for the former are 1) paraquat produced superoxide radicals inside the cells 2) Fe(III)-salophen did not exert a protective effect. Fe(III)-salophen itself slightly enhanced the inhibitory effect of paraquat (Fig. 4.2.4) because the iron might play a central role in the deleterious effects of paraquat, probably by redox cycling and producing hydroxyl radical via the site - specific Fenton reaction. Mn(III) acetate in the media supplemented with casamino acids protected against paraquat toxicity in the *E. coli*. The protective effect likely arose from the reaction between Mn(III) acetate and amino acids in the media, forming mangano - complexes that have antioxidant

activity. Salophen alone did not protect against paraquat toxicity.

5.3 Effects of Mn(III)-salophen on aerobic growth and hyperoxia of *E. coli* *sodA*⁻ *sodB*⁻

Aerobic conditions were used to impose oxidative stress in *E. coli* by superoxide radical generation. Therefore, Mn(III)-salophen (2.4 μM) was used to provide the superoxide scavenging activity required for enhanced cell growth. Equimolar Mn(III) acetate and MnCl_2 also promoted cell growth in media supplemented with casamino acids (Fig. 4.3.1). The antioxidant effect of Mn(III) acetate and MnCl_2 can be explained as above. Salophen alone did not promote cell growth (data not shown).

Hyperoxia (50 % oxygen) was used to increase the oxidative stress in *E. coli* JI132 *sodA*⁻ *sodB*⁻ and the stress was opposed by Mn(III)-salophen at 4.9 and 9.8 μM . Additionally, Mn(III)-salophen exhibited a dose - dependent protection of the *E. coli* cells against the growth - inhibitory effect of hyperoxia (Fig. 4.3.2). Mn(III) acetate alone also protected against the oxidative stress in the media supplemented with casamino acids that can be explained as above.

VI SUMMARY

Compounds or complexes with superoxide scavenging activity have been studied *in vitro* and *in vivo*. The approach is to provide antioxidant activity of compounds or complexes *in vivo* for cells that lack significant amounts of SOD. Additionally, antioxidant activity of compounds or complexes were shown to be either catalytic or stoichiometric with respect to superoxide scavenging activity, and were shown to come from the compounds or complexes themselves and, or their components. SOD mutants were used to diminish the ambiguous explanation for studying the superoxide scavenging activity of compounds or complexes *in vivo* in cells containing protein SOD. The superoxide scavenging activity of compounds or complexes was challenged by an exposure of SOD mutant organisms to a variation of oxidative stress.

Mn(III)-salophen exhibited superoxide scavenging activity *in vitro* and *in vivo* [Liu *et al.*, 1994]. The superoxide scavenging activity of the complex was shown not to be a false positive *in vitro*. *In vivo*, this complex can enter *E. coli* cells and protect the cells from oxidative stress. The major goal of this report is to determine the superoxide scavenging activity of the complex against paraquat toxicity in *E. coli* *sodA*⁻ *sodB*⁻.

Paraquat toxicity depends on many factors such as an availability of oxygen, an abundance of intracellular reductant, a paraquat uptake system in the cell envelope, and composition of the medium [Liochev and Fridovich, 1995]. Paraquat toxicity comes from an increase in intracellular superoxide radicals [Hassan, 1977, 1979]. Therefore, Mn(III)-salophen was implied to protect against the paraquat toxicity by its superoxide scavenging activity inside the cells, but not from inhibition of paraquat uptake by the cells.

As shown in Fig. 4.2.2, and 4.2.3 , Mn(III)-salophen protect *E. coli* *sodA*⁻ *sodB*⁻ JI132 against 1.0 μ M paraquat toxicity in modified M9 salts / glucose medium. The minimum concentration shown to protect the cells was 60 nM. The protective effect of Mn(III)-salophen on aerobic growth of *E. coli* JI132 *sodA*⁻ *sodB*⁻ against paraquat toxicity was from the intracellular superoxide scavenging activity of the complex, but not from inhibiting uptake of paraquat. As shown in Fig. 4.2.4, Fe(III)-salophen slightly enhanced of paraquat toxicity that might be associated with the Fenton reaction [Keyer *et al.*, 1995]. In this study, we also showed that *E. coli* JI132 *sodA*⁻ *sodB*⁻ did not have impaired paraquat uptake, using aerobically grown cells with 1.0 μ M paraquat in modified M9 salts / glucose medium as a control (Fig. 4.2.2 , 4.2.3 , 4.2.4). However, control cells can qualitatively grow as well as the test cells, or as well as positive control at 8 h. The possible explanation for the growth of the control cells might be : 1) small amount of Cu,ZnSOD was produced (results not shown), 2) paraquat dose was bacteriocidal, 3) the medium contained small amount of required amino acids.

We can not exclude the protective effect of Mn(III) acetate in M9 salts / glucose medium supplemented with casamino acids on *E. coli* JI132 *sodA*⁻ *sodB*⁻ against paraquat toxicity. An amount of Mn(III) acetate equivalent to Mn(III)-salophen in the medium showed the same protective effect against paraquat toxicity on *E. coli* JI132 *sodA*⁻ *sodB*⁻. The possible explanation for the antioxidant effect of Mn(III) acetate in the condition might be the property of strong oxidizing Mn(III). In solution, Mn(III) is unstable, and is reduced to Mn(II) by a reductant in a solution [Archibald and Fridovich, 1982]. Therefore, 1) Mn(III) acetate, reduced by an available reductant in the medium to Mn(II), which reacts

with available amino acids (peptides) in the medium, secondarily acting as an SOD [Archibald and Fridovich, 1981], 2) Mn(III) acetate reduced by an available reductant in the medium to Mn(II) which acted as a stoichiometric superoxide scavenger [Archibald and Fridovich, 1981; Weiss *et al.*, 1993], according to the reactions:



X⁻ might represent superoxide radical or another cellular reductant [Archibald and Fridovich, 1982]. Additionally, the protective effect of Mn(III) acetate should occur inside the cells because paraquat produces intracellular superoxide radicals.

Mn(III)-salophen also protected *E. coli* JI132 *sodA*⁻*sodB*⁻ against hyperoxia in M9salts glucose / salts medium supplemented with casamino acids (Fig. 4.3.2) and *E. coli* QC779 *sodA*⁻*sodB*⁻ against aerobic condition (Fig. 4.3.1). Mn(III)-salophen showed dose - dependent protection against hyperoxia (Fig. 4.3.2). However, Mn(III) acetate and MnCl₂ also showed an antioxidant protective effect on hyperoxia and aerobic growth of *E. coli* *sodA*⁻*sodB*⁻ (Fig.4.3.1, 4.3.2). An equivalent amount of each manganese salt to Mn(III)-salophen showed the same protective effect against the oxidative stress. The possible explanation for the antioxidant effect of manganese salts might be explained as above.

In conclusion, the results reported here demonstrate that the protection afforded by Mn(III)-salophen against paraquat toxicity on SOD double mutant *E. coli* was the superoxide scavenging activity of the complex, but not by exclusion of paraquat. Mn(III)-salophen also protected against oxygen toxicity on SOD double mutant *E. coli*. However, the protective effect from the oxidative stress might also be from the Mn(III) in the media

containing amino acids. We do not know what is the major part of protective effect between the complex itself and the manganese component against the oxidative stress in the media containing amino acids.

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VITA

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