Cloning and Characterization of *katG*, encoding Catalase HPI, from Catalase Deficient Mutants of *Escherichia coli*

by

Tzay-Gao Chen

A Thesis
Submitted to the University of Manitoba
in Partial Fulfillment of the
Requirements for the degree of
Master of Science
in Microbiology

Department of Microbiology
University of Manitoba
Winnipeg, Manitoba, 1991

ISBN 0-315-76614-X

CLONING AND CHARACTERIZATION OF *katG*, ENCODING CATALASE HPI, FROM CATALASE DEFICIENT MUTANTS OF *ESCHERICHIA COLI*

BY

TZAY-GAO CHEN

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

DEDICATION

To my Parents

ACKNOWLEDGEMENTS

I am grateful to a number of people who helped me to complete this work:

Dr. Peter C. Loewen, for his guidance and advice during my studies; Jack Switala, for his technical assistance and helpful advice; Georg Hausner, for his sequencing of my PCR product and offering of **Winnipeg Free Press.** Ingemar von Ossowski, for his friendship and technical help; Michael Mulvey, for bringing me to bar and opening my eyes; Abbes, for his affairs and jokes.

Now I am ready to leave but will never forget Winnipeg, nice summer and "cool" winter.

ABSTRACT

ABSTRACT

Several mutant *katG* genes encoding inactive HPI catalase in *Escherichia coli* were amplified using the polymerase chain reaction and subsequently cloned into the Bluescript phagemid vector for sequencing and expression. The mutant HPI proteins were purified from strains harboring the cloned *katG* genes and were shown to have low catalase specific activity. According to absorption spectra of the proteins, all mutant HPIs bound much less protoheme IX than the wild-type HPI. The mutant genes were sequenced and the changes were identified, including a T to C change at 539 predicted to change Ser132 to Pro; a G to A change at 954 predicted to change Gly270 to Asp; an A to G change at 2268 predicted to change Lys708 to Arg, and a silent A to G change at 2107 which would not change Lys654. It is possible to predict that the change from a hydrophilic serine to a hydrophobic proline, which would also disrupt an α-helix section, and the change from a hydrophobic glycine to a hydrophilic aspartate would have the greatest effect on structure and activity. The change from lysine to arginine would not affect the charge of the protein, nor would it significantly change the spatial relationship with other residues.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	adenosine
A_{260}	absorbance at 260 nm
amp	ampicillin
Ap^R	ampicillin resistant
Arg	arginine
Asp	aspartic acid
bp	base pairs
BRL	Bethesda Research Laboratory
C	cytidine
Da	dalton(s)
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2'3'-dideoxyadenosine 5'-triphosphate
ddCTP	2'3'-dideoxycytidine 5'-triphosphate
ddGTP	2'3'-dideoxyguanosine 5'-triphosphate
ddTTP	2'3'-dideoxythymidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
G	guanosine
Gly	glycine

HPI	hydroperoxidase I
HPII	hydroperoxidase II
IPTG	isopropyl ß-D-thiogalactoside
kb	kilobase(s)
Klenow	DNA Polymerase I (Klenow fragment)
Leu	Leucine
NTP	ribonucleoside triphosphate
PCR	polymerase chain reaction
PFU	plaque forming units
Phe	phenylalanine
Pro	proline
RNase	ribonuclease
SDS	sodium dodecyl sulfate
Ser	Serine
T	thymidine
Taq	*Thermus aquaticus*
TEMED	N,N,N1,N1-tetramethylethylenediamine
Tn	transposon
TRIS	tris (hydroxymethyl) aminomethane
U	units
V	volts
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

HISTORICAL

Historical

Oxygen Stress

Oxygen-derived reactive molecular species have been implicated as causative agents of oxidative damage to a wide range of cellular components including DNA, lipids, proteins and amino acids. Oxidative damage can be caused by the superoxide anion (O_2^-), hydrogen peroxide and the highly reactive hydroxyl radical ($OH\cdot$). These species are formed as by-products of normal aerobic metabolism including redox reactions involving hydroquinone, haemoglobins, or glutathione. The highly reactive hydroxyl radical is produced from hydrogen peroxide in the presence of suitable transition metals, particularly iron(II) (Halliwell, 1986).

The nature of intermediates and the mechanism involved in reducing O_2 remain unsettled but the step-wise reduction may be written:

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-+2H^+} H_2O_2 \longrightarrow 2OH\cdot \xrightarrow{2e^-+2H^+} 2H_2O$$

Superoxide anion — Hydrogen peroxide — Hydroxyl radical

All three postulated intermediates are highly chemically reactive and it is interesting to note that living forms contain enzymes to protect the cell against these intermediates.

How cells cope with oxidative stress from normal or abnormal production of reactive oxygen species has been extensively studied in recent years. Although the reactive cytotoxicity of the various oxygen species is not fully understood, the major intracellular defence enzymes are thought to be superoxide dismutase, catalase and glutathione peroxidases. In addition, various molecular antioxidants including ascorbate, ß-carotene and α-tocopherol, have been identified in biological systems (Watson, 1990). α-Tocopherol is a lipid-soluble antioxidant; its unique function may be aided by the specific interaction between the phytyl residue and the fatty acid residues of the polyunsaturated phospholipids in the membrane (Sies, 1986). Ascorbate, together with glutathione, can react with radical chromanoxyl and can regenerate tocopherol in the membrane (Sies, 1986). ß-Carotene, called a provitamin, is a singlet oxygen quencher (Sies, 1986).

The superoxide radical is removed by superoxide dismutase (SOD) (Fridovich, 1975), which is widespread in aerobic organisms. Catalase which removes H_2O_2 is also present in almost all organisms. Peroxidases are relatively rare in animal cells, excepting leucocytes, erythrocytes, liver, and kidney but are common in all higher plants (Conn, 1976). The reactions catalysed by these enzymes are shown below:

SOD $O_2^- + O_2^- + 2H^+ \text{-------->} H_2O_2 + O_2$

Catalase $H_2O_2 + H_2O_2 \text{-------->} 2H_2O + O_2$

Peroxidase $H_2O_2 + RH_2 \text{-------->} 2H_2O + R$

Many organisms utilize molecular oxygen as a terminal electron acceptor. In *E. coli*, oxygen induces metabolic enzymes such as respiratory dehydrogenases and tricarboxylic acid cycle enzymes necessary for the oxidation of substrates, but represses other electron-accepting enzymes such as fumarate and nitrate reductases (Schiavone, 1988).

E. coli contains two SODs: an O_2^--inducible MnSOD encoded by *sodA* (Touati, 1983) and a constitutively produced FeSOD encoded by *sodB* (Sakamoto, 1984). SODs catalyze the dismutation of superoxide radicals to hydrogen peroxide, which in turn may be converted into molecular oxygen and water by the action of two catalases: the H_2O_2-inducible HPI encoded by *katG* (Triggs-Raine, 1987) positively regulated by *oxyR* (Christman, 1985) and HPII encoded by *katE* (Mulvey, 1988) and regulated by *katF* (Mulvey, 1990) encoding a putative sigma factor (Mulvey, 1989).

Shellhorn *et al* (1988) examined the response to oxidative stress of double mutants defective in both superoxide dismutase and catalase. They showed that superoxide dismutase was more important than catalase in protecting cells from the stress of pure oxygen and from compounds that generate the superoxide anion. Unexpectedly,

E. coli strains overproducing superoxide dismutase were found to be hypersensitive to hyperbaric oxygen and paraquat, compounds that generate superoxide radicals (Scott, 1987). They proposed that high levels of the superoxide dismutase may lead to increased levels of hydrogen peroxide with subsequent production of reactive species, such as the hydroxyl radical, that are more reactive than the superoxide anion.

Verduyn *et al* (1988) presented evidence that, in a catalase-negative mutant of *Hansenula polymorpha* utilizing methanol, cytochrome-c peroxidase was a key enzyme in the detoxification of hydrogen peroxide. Catalase in this yeast is specifically induced during growth on substrates (methanol) that require the action of a hydrogen peroxide-producing oxidase. Kuyumdzhieva *et al* (1985) reported elevated levels of superoxide dismutase and catalase in various yeast (*Candida, Torulopsis, Hansenula* and *Pichia*) growing on methanol. It appears that the dismutase, catalase and cytochrome-c peroxidase have roles in the detoxification of hydrogen peroxide and other oxygen-derived reactive free radical species in micro-organisms.

In addition to protective enzymes, *E. coli* possess DNA repair enzymes which are required for the repair of oxidatively damaged DNA. These include the *recA* protein, the *recBCD* enzyme (Imlay, 1986), exonuclease III (Demple, 1983), endonuclease IV (Chan, 1987) and DNA polymerase I and III

(Hagensee, 1987).

The identification and characterization of proteins induced by various oxidative stresses is currently an area of active research. There is a variation in response among different species making it difficult to generalize from one organism to another. Furthermore there is extensive overlap among various regulons including heat shock, osmotic shock, acid stress and oxidative stress (Watson, 1990) complicating the study even further.

Catalase HPI of *E. coli*

E. coli produces two catalases which have been named HPI (Claiborne and Fridovich, 1979) and HPII (Claiborne *et al*, 1979). HPII is a monofunctional catalase which is active as a hexamer of identical subunits of 84,118 Da (von Ossowski, 1991), with one heme d-like group per unit (Chiu, 1989). The HPII subunit is encoded by *katE* at 37.8 min (Loewen, 1986) and affected by *katF* at 59.0 min (Loewen, 1984) which encodes a putative sigma transcriptional factor (Mulvey, 1989). HPII levels did not increase in response to hydrogen peroxide but its levels increase approximately twenty-fold during growth into stationary phase and during growth on TCA cycle intermediates (Loewen, 1985).

E. coli HPI is a bifunctional enzyme exhibiting both catalase and peroxidase activities. HPI has an optimum pH for the catalase activity at pH 7.5 and an optimum pH for

peroxidase activity at pH 6.5. The apparent Km for H_2O_2 in the catalase reaction is 3.9 mM. HPI was characterized as a tetramer of 81,000 Da monomers with two associated protoheme IX groups and was found to have a broad-spectrum peroxidase activity allowing it to use organic electron donors to reduce hydrogen peroxide (Claiborne & Fridovich, 1979). Purified HPI electrophoresed on non-denaturing polyacrylamide gels separated into two isoenzyme forms labelled HPI-A and HPI-B (Loewen, 1985). The significance of the two forms is unknown. Another bifunctional catalase-peroxidase has been isolated and characterized from *Rhodopseudomonas capsulata* (Hochman, 1987). The present work focuses on HPI which will be reviewed in more detail. The gene encoding HPI, *katG* has been mapped at 89.2 min on the *E. coli* chromosome (Loewen, 1985). Expression of *katG* following H_2O_2 induction is positively regulated by *oxyR* encoding a protein responsible for the positive regulation of a regulon involved in protection against oxidative stress (Christman, 1985).

The complete nucleotide sequence of *katG* has been determined (Triggs-Raine *et al*, 1988), revealing a 2181-bp open reading frame which predicts a 726 amino acid sequence for the HPI subunit with a size of 80,409 Daltons. The predicted sequence of HPI revealed 90 % similarity with catalase HPI sequences from *Salmonella typhimurium* (Loewen & Stauffer, 1990) and 48 % similarity with the peroxidase gene

perA of *Bacillus stearothermophilus* (Loprasert *et al*, 1989), but showed no similarity to other catalase sequences. Consequently, it was not possible to draw any conclusion about the three-dimensional structure of the enzyme. These observations suggest that the group of enzymes possessing both catalase and peroxidase activities is different from the typical catalases and may more closely resemble peroxidases (Loewen, 1990).

Several mutants lacking appreciable HPI have been generated by nitrosoguanidine and transposon Tn10 insertion. Of these, three generated by nitrosoguanidine have been characterized as to the lesions causing the mutations. In two cases, a G to A change in the coding strand resulted in glycine to aspartate changes at residues 119 and 314 respectively. In the third mutant, a C to T change resulted in a leucine to phenylalanine change at residue 139. The Phe139, Asp119 and Asp314 containing mutants exhibited low catalase and peroxidase activities attributable to the reduced heme content of enzyme (Loewen *et al*, 1990). The heme pocket in hemoglobin is quite hydrophobic and changes from a non polar to more polar residue such as Asp may reduce heme binding (Wireko *et al*, 1991).

Because the three dimensional structure of a peroxidase has not been determined, very little structural information is available. One approach to a study of structure-function relationships in HPI is to identify the locations and

effects of randomly generated mutations. Such a study would lead to the identification of possible regions of the protein involved in enzyme action. A number of such mutants, generated by nitrosoguanidine have been isolated and are available for use in such a study.

The Expression of *katG* Regulated by *oxyR*

How a gene is expressed in a selective manner in response to external or internal signals (stimuli) is a dominant theme in the regulation of gene expression. It is clear that the elements that ultimately respond to such stimuli are gene regulatory proteins which can act by binding to specific sites on DNA. The interaction between the stimulated gene regulatory protein and DNA often causes an activation or enhancement of transcription initiation by RNA polymerase. This is called positive control (Adhya *et al*, 1990).

In the case of oxygen damage control, cellular resistance to lethal effects of oxidizing agents can be induced by a previous exposure to a sublethal dose of H_2O_2. During such a treatment, the synthesis of 34 proteins is induced of which nine proteins are produced under the control of the activator protein, *oxyR* (Christman *et al*, 1985). Consequently, *E. coli* catalase HPI can be considered to be positively controlled by the *oxyR* protein. The molecular mechanism of *oxyR* activation by H_2O_2 for positive

control is not known, but both oxidized and reduced forms of *oxyR* bind to the *katG* promoter with only oxidized *oxyR*, not the reduced form, activating transcription (Storz *et al*, 1990). *OxyR*, a 34-kDa protein (Christman, 1989), does not seem to have a helix-turn-helix motif and does not behave like a dimer. The *oxyR* DNA binding site is quite large, about 45 bp, and does not have dyad symmetry and there is little similarity among the four DNA sequences to which *oxyR* binds. It is speculated that DNA secondary structure must dictate specificity (Tartaglia *et al*, 1989). The activator, oxidized *oxyR* protein, bound to DNA probably makes contact with RNA polymerase or alters the DNA conformation so that RNA polymerase can bind or function more efficiently (Adhya *et al*, 1990).

Amplifying Genes by Using Polymerase Chain Reaction (PCR)

The polymerase chain reaction involves synthesizing multiple copies of a gene, or a region of DNA, from oligonucleotide primers which bind to opposite strands, flanking the target sequences. Up to twenty to thirty cycles are required for amplification and each cycle in the reaction involves denaturation of the DNA and annealing the primers followed by extension by heat stable DNA polymerase. Each newly synthesized DNA segment, with the terminus consisting of the 5' end of the primer, now becomes a template for the next round, resulting in exponential

amplification of the original target DNA. Thus, in 20-30 cycles, it becomes possible to amplify the original sequence by a million-fold.

PCR is useful for more than just amplifying target sequences. It is also possible to use it to alter a particular nucleotide sequence. This is accomplished by adding new bases to the target via the primers (the reaction tolerates some degree of mismatch between the original target and the primer). Thus, it becomes possible to substitute, delete, or add nucleotides, as well as incorporate new restriction sites into one's initial gene (Brunt, 1990).

Basic PCR makes it possible to obtain large quantities of any DNA sequence as long as two short nucleotide sequences on either side of the target are known. However, two modifications make it possible to overcome even this requirement. Triglia *et al* (1988) described a procedure called inverted PCR to analyze sequences that lie outside the boundaries of known sequences. This approach requires inversion of the sequences of interest by circularization and re-opening at a different site, and followed by basic PCR. In order to analyze sequences that have variable termini, such as T cell antigen receptor or immumoglobins, Loh *et al* (1989) created "anchored PCR." In this procedure, mRNA is first transcribed with reverse transcriptase and a poly(dG) tail is added to the 3'-end of the strand with

terminal deoxynucleotidyl transferase. The product is then amplified with a specific primer corresponding to a known tract of nucleotides and another oligonucleotide consisting of poly(dC) attached to a sequence with a convenient restriction site, termed the anchor. Anchored PCR was developed for the study of genes that encode proteins for which partial sequences are known. The implications of inverted and anchored PCR for DNA sequencing are astonishing: enormous stretches of DNA can be sequenced once a tiny bit of sequence is known. Both techniques make it possible to proceed along the DNA, continually redefining the end to which synthetic primers can be bound and extended.

This thesis describes the cloning of a number of mutant *katG* genes using PCR, their sequence analysis, and the purification of proteins encoded by the mutant genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Bacteriophages

The bacterial strains used, all derivatives of *Escherichia coli* K-12, are listed in Table 1 with their genotypes and sources. Table 2 lists plasmids and bacteriophage used with their characteristics and sources.

Media

LB Medium:(Miller, 1972)

10.0 g Tryptone (Difco)

5.0 g Yeast Extract (Difco)

5.0 g NaCl.

Dissolved in 1.0 liter of double distilled deionized water and autoclaved for 20 minutes. Solid medium prepared with addition of 10 g agar. Ampicillin was added to 100 μg/ml as required.

M9 Minimal Medium:

0.5 g NaCl

6.0 g Na_2HPO_4

1.0 g NH_4Cl

3.0 g KH_2PO_4

per liter of distilled water.

For agar plates 10 g agar was added.

Table 1. Bacterial strains

Strain	Genotype	Source
UM308	HfrH *thi katE::*Tn*10 katG*	UM120. NG
UM309	HfrH *thi katE::*Tn*10 katG*	UM120. NG
UM311	HfrH *thi katE::*Tn*10 katG*	UM120. NG
UM312	HfrH *thi katE::*Tn*10 katG*	UM120. NG
NM522	*rec*$^{+}$(*supE thi* Δ(*lac-proAB*)	(Mead, 1985)
	hsd^{5},{F' *proAB LacI*^{Q} *lacZ*ΔM15})	
UM262	*recA katG::*Tn*10 pro leu rpsL*	(Loewen, 1990)
	hsdM hsdR endI lacY katE	

Table 2. Bacterial Plasmids and Bacteriophage

Plasmid	Characteristics	Source
Bluescript Phagemid SK+	Ap^R	Stratagene Cloning System
pBT22	Ap^R,Tc^S,*katG*$^+$	(Triggs-Raine, 1987)
M13 R408 helper phage		Stratagene Cloning System

After autoclaving, the medium was supplemented with

3.0 μM vitamin B1

1.0 mM $MgSO_4$

1.0 mL trace elements

Trace elements:

2.5 g $FeSO_4.H_2O$

2.9 g H_3BO_3

1.2 g $CoSO_4.7H_2O$

0.1 g $CuSO_4.5H_2O$

2.5 g $NaMoO_4.7H_2O$

2.1 g $ZnSO_4.7H_2O$

90 mg $MnCl_2.4H_2O$

5 mL conc. H_2SO_4

Other supplements, including 16.7 mM glucose and various amino acids to 0.16 mM were added as indicated.

SM Buffer (Miller, 1972)

0.02 M TRIS-HCl pH 7.5

0.01 M $MgSO_4$

0.01% gelatin

0.01 M NaCl

Autoclave.

TE Buffer (Maniatis et al., 1982)

10.0 mM TRIS-HCl pH 8.0

1.0 mM EDTA pH 8.0

Autoclave.

GTE Buffer:

50 mM Glucose

10 mM EDTA

25 mM TRIS-HCl pH 8.0

Ethanol

95% ethanol was distilled before use.

Phenol

All phenol was redistilled and buffered with

0.1 M NaCl

0.1 M TRIS-HCl pH 7.6

1.0 mM EDTA

5X Ligation Buffer (BRL)

0.25 M TRIS-Cl pH 7.6

50 mM $MgCl_2$

5 mM ATP

5 mM DTT

25% w/v PEG6000

10X TAE Buffer

48.4 g TRIZMA base

11.4 g glacial acetic acid

3.72 g EDTA

in 1 liter distilled H_2O.

Taq DNA polymerase (5000 Unit/mL) and AmpliTaq™ DNA Amplication Reagent Kit were bought from **Promega** (2800 Woods Hollow Road, Madison, WI 53711-5399, USA).

The following primers were purchased from the DNA Synthesis Laboratory, University of Calgary:

Primer13 5'-dACA TAA TCA AAA AAG CTT AATT-3' (Underline indicates *Hin*dIII site)

Primer14 5'-dACT CCA GAT AAG CTT GAG CAC-3'(Underline indicates *Hin*dIII site)

PrimerG1 5'-dATT CTA ATC GTT CTA AC-3'

PrimerG2 5'-dGTC GCC TGT TGT GGC CA-3'

PrimerG3 5'-dACC GTC TTC TGC GGC AG-3'

PrimerG4 5'-dTTC AAG TTT GAG TGG GT-3'

PrimerG5 5'-dAAG ATC TGA TCT GGC AA-3'

PrimerG6 5'-dCAT AGT GCT GGC TGG TG-3'

PrimerG7 5'-dCAA AAA CGG CGT CTT CA-3'

PrimerG8 5'-dCAC CGT CTT CCG GAA GC-3'

PrimerG9 5'-dTCA ATT TGA TCT ACA TC-3'

*Hin*dIII, *Bam*HI, *Eco*RI, *Cla*I endonucleases, Klenow fragment of DNA polymerase I and T4 Ligase were purchased from **Bethesda Research Laboratories, Life Technologies, Inc.** (Burlington, Ontario, CANADA L7P 1A1) or **Pharmacia LKB Biotechnology** (Uppsala, Sweden).

GeneClean Kit was bought from **BIO101, Inc.** (P.O. Box 2284, La Jolla, CA 92038-2284, USA).

1 kb Ladder size marker DNA, agarose, polyacrylamide, X-gal, and IPTG were purchased from **Bethesda Research Laboratories.**

Growth Conditions

Bacterial strains were grown in 10 ml volumes containing the desired medium and supplements at 37°C with aeration in a Dubnoff Metabolic Shaking Incubator (**Precision Scientific**, Inc., Chicago, Illinois, USA).

Cell densities were determined by using a Klett-Summerson colorimeter equipped with a blue filter.

Plates were incubated inverted at 37°C in a Fisher Isotemp Incubator. Liquid cultures were transferred aseptically using sterile pipettes and loops. Colonies were transferred with sterile toothpicks.

For long term storage of bacterial cultures, stationary

phase cultures were stored in 50% glycerol at -20°C and in 8% DMSO at -60°C.

Preparation of *E. coli* Chromosomal DNA for PCR

Chromosomal DNA was prepared from strains of *E. coli* carrying *katG* mutations. Bacteria were grown to mid-log phase in 50 mL of LB medium, harvested by centrifugation, and washed in 20 mL of 0.1 M TRIS-HCl, pH 8.0/0.1 M EDTA/0.15 M NaCl. Cells were resuspended in 1.5 mL of the above buffer and treated for 10 min with 0.2 mL of lysozyme (10mg/mL in 0.25 M TRIS-HCl, pH 8.0) at 37°C, followed by 0.2 mL of RNase A (2 mg/mL) for 15 min at 37°C. A 0.4 mL of 30% Sarkosyl was then added, and the cells were incubated for 20 min at 70°C, followed by 60 min at 37°C. This lysate was treated for 2 hrs at 37°C with 0.6 mL of 20 mg/mL DNase-free Pronase, dialyzed overnight at 37°C against TE buffer, extracted with phenol, precipitated with ethanol, dried by lyophilization, and suspended in 3 mL of 20 mM TRIS-HCl, pH 8.0/10 mM EDTA/50 mM NaCl with gentle shaking at 25°C. Further RNase treatment (two 0.2 mL additions over a 6-hour period) was followed by extractions with phenol (once) and with ether (four times), precipitation with ethanol, lyophilization, and extensive dialysis of the resuspended DNA against TE buffer. The dialyzed suspension was kept at 4°C.

Amplification of *katG* Genes by PCR

The PCR reaction was carried out in a mixture consisting of 10.5 μL HPLC H_2O, 3 μL 10X reaction buffer, 4 μL dNTP's mixture (50 mM for each dNTP), 3 μL primer13 (A_{260}=11), 2 μL primer14 (A_{260}=10), 2.5 μL chromosomal DNA (A_{260}=1.5). Mixtures were boiled for 2 minutes before 2-4 units Taq DNA polymerase was added and the mixture was overlaid with paraffin oil. The amplification reaction was carried out for 35 cycles in the DNA Thermal Cycler. The DNA was denatured at 93°C for 1 min, annealed at 50°C for 1 min and extended at 72°C for 5 min.

Agarose Gel Electrophoresis

The technique of agarose gel electrophoresis was used for analysing the amplified PCR products. The use of suitable molecular standards allowed estimation of the sizes of the DNA fragments. A Bio-Rad Subcell Horizontal Slab Gel system with a 15 cm X 15 cm or a 15 cm X 20 cm gel was used. The running buffer was prepared by diluting 10X TAE buffer 10-fold. The 1 kb molecular weight marker kit contained the following fragments, in addition to some smaller and larger ones: 7126, 6108, 5090, 4072, 3054, 2036, 1635, 1018, 517, 506 and 396 base pairs.

Preparation of Agarose Gels

0.8% (w/v) gels were prepared by dissolving 0.64 g agarose in 80 mL of 1X TAE Buffer. The slurry was boiled until the agarose dissolved. It was cooled to 55°C and poured into the gel mold. The well maker was placed at the top of the mold and the solution was allowed to solidify for 30 min. The well maker was removed and the gel was placed into the Bio-Rad SubCell apparatus and immersed in 1X TAE buffer.

Preparation of Samples and Electrophosis

Samples were mixed 6:1 with stop buffer.

6x Stop Buffer

100 mM EDTA

0.25% Bromophenol Blue

35% Glycerol

Molecular weight standards were prepared by mixing 2 μL of 1 μg/mL 1 kb Ladder with 8 μL sterile water and 3 μL stop buffer. A voltage of up to 80 V was applied until the dye marker had migrated an appropriate distance.

Staining and Photography of DNA in Agarose Gels

After completion of electrophoresis the gel was placed in a 0.5 μg/mL solution of ethidium bromide for 30 min. The DNA was then visualized under ultraviolet light and photographed with a Polaroid MP-4 Land Camera, using a Kodak 22 A filter and Polaroid Type 667 film. Exposure time was usually 3 sec at an aperture setting of 8 but was occasionally varied depending on the intensity of the DNA bands. The size of the fragments was determined by comparison with the mobilities of the 1 kb ladder observed in the photograph.

Extraction of DNA from Agarose

The amplified DNA fragments were usually between 2 kb and 3 kb. Once located under UV light, and the segments of gel containing the fragments were cut out. The gel was dissolved in 3 volumes of NaI solution supplied with the GeneClean kit, followed by incubation at 55°C for 5 min. Following resuspension, 5 μL of glassmilk was added. The suspension was mixed thoroughly and placed on ice for 5 min. The mixture was centrifuged for 5 seconds and supernatant was discarded. The white pellets were washed three times with 400 μL of the New Wash solution. The New Wash supernatant was removed by aspiration and a 30 sec centrifugation followed by aspiration to remove all of the third wash. The pellet was resuspended in 5 μL of TE buffer

and the suspension was incubated at 55°C for 5 min. The mixture was centrifuged for 30 seconds and the clear supernatant containing PCR DNA was transferred into a new Eppendorf tube. The tube was stored at -20°C.

Integration of PCR Amplified DNA into Bluescript Plasmid

The Bluescript plasmid DNA was cut with *Hin*dIII endonuclease in a mixture consisting of 2 μl Bluescript plasmid (A_{260}=35), 16 μL sterile distilled water, 2 μL No.2 reaction buffer (500 mM TRIS-HCl pH 8.0/100 mM $MgCl_2$/500 mM NaCl) and 2 μL *Hin*dIII (20,000 Unit/mL). The mixture was incubated at 37°C for 5 hours and heated at 65°C for 15 min.

The PCR DNA was cut with *Hin*dIII endonuclease in a mixture consisting of 5 μL PCR product, 19 μL sterile distilled water, 3 μL No. 2 reaction buffer (500 mM TRIS-HCl pH 8.0/100 mM $MgCl_2$/500 mM NaCl) and 3 μL *Hin*dIII. The mixture was incubated at 37°C for 6 hours and heated at 65°C for 15 min.

The two DNAs cut with *Hin*dIII, Bluescript plasmid 2 μL (A_{260}=4.5) and PCR DNA 30 μL, were mixed and 3 μL 5X ligase buffer was added along with 2 μL ligase (6000 unit/mL). The mixture was placed at 37°C for 3 hours.

Transformation with Bluescript Vector

10 mL *E. coli* NM522 cultures were grow at 37°C with aeration to mid-log phase. The cells were harvested by centrifugation for 5 min at 8000 g, resuspended in 1 mL of cold sterile 0.1 M $CaCl_2$ and placed on ice for a minimum of 15 min. Then, 0.1 mL of $CaCl_2$-processed competent cells and 37 μL ligation mixture were mixed in an Eppendorf tube and placed on ice for 15 min or longer, followed by heating for 90 seconds at 42°C and the addition of 0.4 mL LB Broth. The mixture was incubated at 37°C for 1 hour and spread on LB plates containing 0.1 mg/mL ampicillin.

Color Selection of Clones Containing Inserts

Plates for color selection of inserts were prepared by spreading 50 μL of 200 mM IPTG dissolved in sterile distilled water and 50 μL of 2% X-gal dissolved in the N, N,-dimethylformamide on an LB plate containing 0.1 mg/mL ampicillin. After growth for 12-18 hours at 37°C, colonies containing plasmids with no inserts turned blue. Colonies containing plasmids with inserts remained white.

Detection of the Clones Containing *katG* Gene Inserts

White colonies were grown in 10 mL LB broth containing 0.1 mg/mL ampicillin at 37°C overnight. Cells were harvested by centrifugation at 8000g for 5 min, suspended in 1 mL GTE buffer (50mM Glucose/10 mM EDTA/25mM TRIS-HCl, pH 8.0) and

centrifuged in an Eppendorf tube. The pellet was resuspended in 200 μL of GTE buffer containing 4 mg/mL lysozyme and let sit at room temperature for 5 min. Then 400 μL of 0.2 M NaOH/1% SDS was added and mixed gently by inversion and rotation until mixture became clear. The clear lysate was placed on ice for 5 min. Finally 300 μL 7.5 M ammonium acetate pH 7.5 was added, mixed gently and placed on ice for 10 min. The mixture was centrifuged for 15 min at 12000g and the supernatant was transferred to a new tube for a second centrifugation lasting 10 min. The supernantant was transferred into a new Eppendorf tube, to which 550 μL isopropanol was added. The solution was mixed and, after sitting at room temperature for 10 min, it was centrifuged for 15 min at 12000g. The supernatant was discarded and pellet was washed twice with 70% ethanol at -20°C. The pellet was dried in a desiccator for 30 min, resuspended in 50 μL TE buffer and stored at -20°C.

For characterization, the chimeric plasmids were first digested with *Hin*dIII to produce the desired 2.5 kb (*katG*) and 2.96 kb (linear plasmid) fragments. Digestion with *Eco*RI and *Cla*I was used to determine the orientation of the insert.

Enzyme Assays

Catalase activity was determined by the method of Rorth and Jensen, 1967 using a Gilson oxygraph equipped with a Clarke electrode. One unit of catalase is the amount of enzyme catalyzing the decomposition of 1.0 μmole of H_2O_2 per minute at 37°C. All assays were carried out using a final H_2O_2 concentration of 60 mM. Protein concentration was estimated by the method of Lane (1957).

Purification of Hydroperoxidase I

Bluescript plasmids containing the mutant *katG* gene were transformed into UM262 in which the only *katG* HPI protein produced would be expressed from the plasmid because of the transposon in the chromosomal *katG*. Cells were grown in LB containing 0.05 mg/mL ampicillin to stationary phase at 37°C. The procedure for purification of the protein was essentially that of Claiborne and Fridovich (1979), as applied to the purification of HPII (Loewen and Switala 1986). All manipulations were carried out at 4°C. The crude extract was prepared using the Aminco French press at 20,000 psi (1 psi=6.894 kPa) and fractionated using 2.5% streptomycin sulfate precipitation. The supernatant was subsequently fractionated by ammonium sulfate precipitation at 20%, 30% and 40% saturation with solid ammonium sulfate. At each step the solution was stirred gently for 15 min to ensure complete salt dissolution and protein precipitation.

The precipitate was collected by centrifugation and additional ammonium sulfate was added to the supernatant. *KatG* protein was normally precipitated in the 20% pellets and was subjected to further purification by an additional ammonium sulfate fractionation. The pooled ammonium sulfate fractions were dialyzed for 24 hours against 2 liters of 50 mM potassium phosphate, pH 7.0 and bound to a 2.8 X 55 cm column of DEAE-Sephadex A50 equilibrated in the same buffer. The column was washed until the absorbance of the elute at 280 nm had dropped below 0.05. *KatG* protein was eluted with a linear gradient prepared by mixing 50 mM potassium phosphate, pH 7.0 with 0.5 M NaCl in the same buffer. The peak of catalase activity was pooled, concentrated by ultrafiltration (Amicon) and assayed for specific activity (Unit/mg).

Determination of the *katG* Protein Absorbance Spectrum

Purified *katG* protein as diluted appropriately with 50 mM phosphate buffer, pH 7.0 and the absorbance determined using a Milton Roy MR3000 Spectrophotometer.

Polyacrylamide Gel Electrophoresis and Staining

Gel electrophoresis of the purified proteins was carried out under denaturing conditions on SDS-polyacrylamide gels (Weber, 1972). The protein bands were located by staining with Coomassie brilliant blue R.

Running Buffer:

1 g SDS

3 g TRIZMA base

14g glycine.

dissolved in 1 liter distilled water.

Sample Buffer:

2% SDS

0.2 M 2-mercaptoethanol

8% glycerol

68 mM TRIS, pH 6.8

Staining Solution:

0.1 g Commassie Brilliant Blue R

60 mL Ethanol.

20 mL Acetic acid.

mixed with 120 mL distilled water.

Destaining Solution:

(1) 30% ethanol, 10% acetic acid for 1 hour.

(2) 30% methanol, 10% acetic acid for 2-3 hours.

(3) 2% glycerol, 7% acetic acid for 30 min.

An 8% polyacrylamide, 0.1% SDS running gel was prepared by mixing 9.6 mL of 30% acrylamide, 12 mL of 1.0 M TRIS pH 8.8, and 13.7 mL of distilled H_2O. This solution was

degassed and mixed with 0.7 mL of 5% SDS, 15 mg of ammonium persulfate, and 15 μL TEMED. The solution was poured between two 15 X 15 cm vertical gel plates. Distilled H_2O was layered onto the mixture and gel was allowed to polymerize for 1 hour.

A stacking gel was prepared by mixing 2.4 mL of 30% acrylamide, 1.9 mL of 1 M TRIS, pH 6.8, and 10 mL distilled H_2O. This mixture was degassed and then mixed with 0.3 mL of 5% SDS, 10 mg of ammonium persulfate and 10 μL of TEMED. The stacking gel was layered on top of the running gel and a well maker was placed into it to the desired depth. After polymerization, the well maker was removed and the wells were washed once with distilled H_2O before adding a solution of running buffer.

Single Strand DNA Preparation

Cells grown on glucose minimal plates were inoculated into 2.5 mL of LB medium supplemented with 25 μL of 25 mg/mL ampicillin and then were grown for about 2 hours to a Klett reading of 30. 10 μL of helper phage (PFU=10^{12} per mL) was added and the cells were grown for 6 to 8 hours. The solution was poured into 1.5 mL Eppendorf tube and centrifuged for 2 min. The supernatant was centrifuged for another 2 min and poured into another tube containing 300 μL of 1.5 M NaCl/20% PEG. The solution was gently mixed by inversion and incubated at room temperature for 15 min. The

mixture was centrifuged for 15 min and the supernatant was removed by aspiration. The tube was centrifuged for an additional 2 min and the liquid was carefully removed without disturbing the pellet.

The small white pellet was resuspended in 200 μL of TE buffer and 150 μL of phenol was added. The mixture was mixed using a vortex mixer for 30 sec and centrifuged for 2 min. The upper aqueous phase was transferred into another tube and 150 μL of water-saturated chloroform was added. After mixing and centrifugation, the upper aqueous phase was transferred into a new tube and 150 μL of 7.5 M ammonium acetate pH 7.5 and 600 μL of cold 95% ethanol were added. The mixture was placed at -60°C for 20 min, and then centrifuged for 15 min. The supernatant was removed by aspiration and the pellet was washed two times with 70% ethanol. After sitting for a few minutes, any residual liquid was removed by aspiration, the tube was dried in a desiccator overnight and stored at -20 °C. The pellet was resuspended in 25 μL HPLC water just prior to use.

Preparation of Sequencing Reagents

10X Klenow Buffer

100 mM TRIS-HCl pH 8.0

50 mM $MgCl_2$

Dideoxy nucleotide working solutions:

0.1 mM ddATP (Pharmacia)

0.1 mM ddCTP (Pharmacia)

0.15 mM ddGTP (Pharmacia)

0.5 mM ddTTP (Pharmacia)

Deoxynucleotide working solutions:

Each nucleotide was diluted from 10 mM stocks to 0.5 mM. The nucleotide mixtures were prepared as followed:

Mixture	A°	C°	G°	T°
0.5 mM dCTP stock	20 µL	1 µL	20 µL	20 µL
0.5 mM dGTP stock	20 µL	20 µL	1 µL	20 µL
0.5 mM dTTP stock	20 µL	20 µL	20 µL	1 µL
50 mM Tris pH 8.0	5 µL	5 µL	5 µL	5 µL

Primer was dissolved in HPLC water to a concentration of 2 mg/mL.

Formamide Dye

0.03 g xylene cyanol FF

0.03 g Bromophenol blue

0.75 g EDTA

dissolved in 100 mL of deionized formamide.

[α-^{32}P]dATP was diluted with 0.125 mM cold dATP (1 μL for every 4 μL of label) to a final specific activity of 300 Ci/nM.

Preparation of Sequencing Gels

10X TBE buffer:

108 g TRIZMA base (Sigma)

55 g boric acid

9.3 g EDTA

dissolved in 1 liter distilled water.

40% Acrylamide:

38 g acrylamide (BRL)

2 g N,N-methylene *bis*acrylamide (BRL)

The solution was filtered and stored at room temperate in the dark.

Silanizing Solution:

2% dichloromethylsilane (Aldrich)

(v/v) in carbon tetrachloride

Gel Mixture (for 1 gel)

21 g Urea (BRL)

21 mL distilled water

5 mL 10x TBE

7.5 mL 40% acrylamide

Two 38.5 X 20 cm plates were washed with an SOS pad and rinsed with distilled water. One plate was silanized by wiping with a tissue wetted with 2% dichlorodimethylsilane in CCl_4 on the inside face and then rinsed with distilled water. The plates were placed together separated by two 0.05 mm spacers and taped together. The gel mixture was prepared and 40 μL of TEMED (BRL) was added along with 15 mg of ammonium persulfate (BRL). The mixture was poured between the two glass plates using a 50 mL syringe. A shark's tooth comb (flat side in) was placed into the glass plates to form a trough. The gel was left to polymerize and could be stored overnight at 5 °C.

Sequencing Protocol

The shark's tooth comb in the polymerized gel was removed and the trough was rinsed with distilled water. All tape was removed and the plates were clamped into the sequencing apparatus. The sharks tooth comb was reinserted with the teeth just touching the acrylamide. The gel was prerun in 1X TBE at 1200v for 30 min before samples were loaded.

The following reaction mixtures were prepared:

	A	C	G	T
dNTP	4μl **A°**	4μl **C°**	4μl **G°**	4μl **T°**
ddNTP	4μlddATP	4μlddCTP	4μlddGTP	4μlddTTP

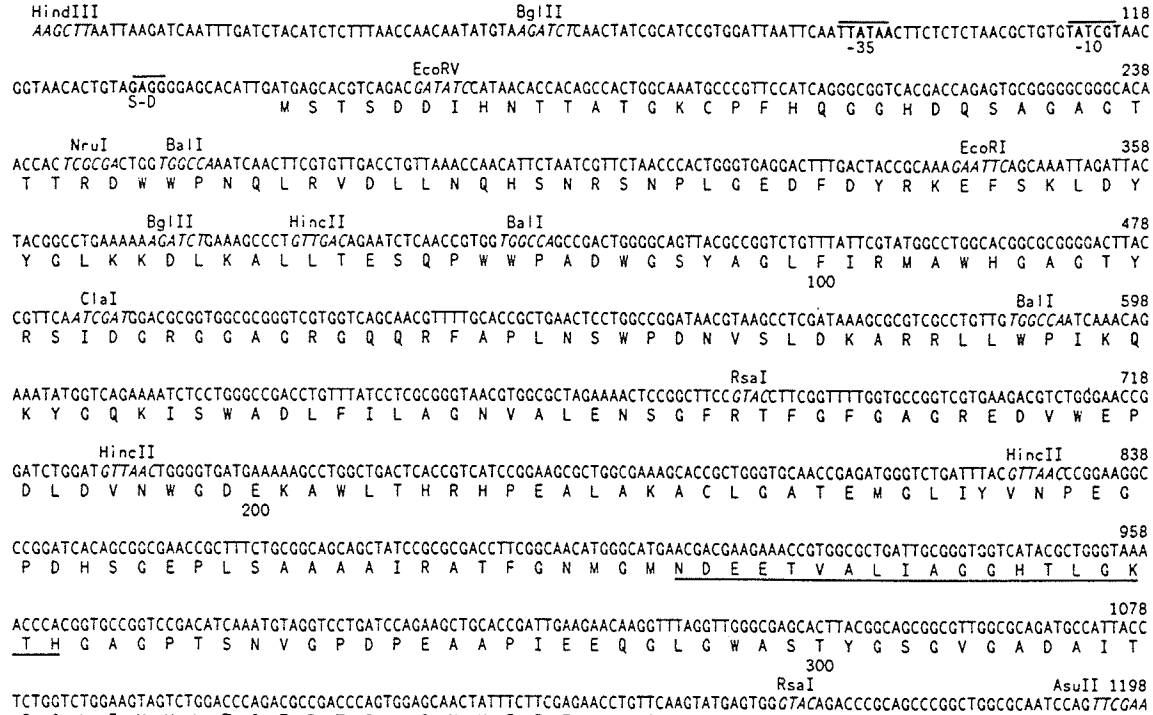
A mixture containing 7 μL single stranded DNA, 3 μl primer and 1.6 μl 10X Klenow buffer were mixed, heated at 65°C for 5 min and annealed at room temperature for 15 min. To this mixture was added 5 μl 1X Klenow buffer, 1.2 μl 0.125 mM dATP, 2.5 μl Klenow fragment and 6 μl [α-^{32}P]dATP. Finally 3.8 μL of this mixture was transferred into each of the corresponding A, C, G and T nucleotide tubes followed by quick spin and incubation at 50°C water bath for 20 min. To stop the reaction, 3.5 μL of formamide-dye was added to each reaction tube followed by heating at 100°C for 2 min. The sample wells in the gel were rinsed out and 2 to 3.5 μL of samples in the order A, C, G, T were loaded. The gel was run at 19-21 mA for an appropriate length of time and temperature was maintained at 50°C.

Once the samples had run the desired distance, the gel was removed from the apparatus, the plates were separated, the gel was transferred to Whatman 3MM paper, the gel was

covered with Saran Wrap and dried under vacuum for 30 min at 80°C. The gel was exposed to Kodak X-Omatic AR X-ray film for an appropriate time at room temperature. Developing was achieved by placing the exposed film in developer for 3 min, rinsing with water and finally placing in fixer for 3 min. The film was rinsed in water and dried before being analyzed.

RESULTS

Figure 1. Nucleotide sequence of the *katG* gene and predicted amino acid sequence. Potential -35, -10, Shine-Dalgarno and terminator sequences are indicated by solid line over nucleotide sequences. Cyanogen bromide-generated peptides from purified HPI underlined were sequenced by Edman degradation are and confirm the predicted amino acid sequence (Triggs-Raine *et al*, 1988).


HindIII
BglII
118
-35
-10
EcoRV
238
S-D
M S T S D D I H N T T A T G K C P F H Q G G H D Q S A G A G T
NruI
BalI
EcoRI
358
T T R D W W P N Q L R V D L L N Q H S N R S N P L G E D F D Y R K E F S K L D Y
BglII
HincII
BalI
478
Y G L K K D L K A L L T E S Q P W W P A D W G S Y A G L F I R M A W H G A G T Y
100
ClaI
BalI
598
R S I D G R G G A G R G Q Q R F A P L N S W P D N V S L D K A R R L L W P I K Q
RsaI
718
K Y G Q K I S W A D L F I L A G N V A L E N S G F R T F G F G A G R E D V W E P
HincII
HincII
838
D L D V N W G D E K A W L T H R H P E A L A K A C L G A T E M G L I Y V N P E G
200
958
P D H S G E P L S A A A A A I R A T F G N M G M N D E E T V A L I A G G H T L G K
1078
T H G A G P T S N V G P D P E A A P I E E Q G L G W A S T Y G S G V G A D A I T
300
RsaI
AsuII 1198
S G L E V V W T Q T P T Q W S N Y F F E N L F K Y E W V Q T R S P A G A I Q F E
AccI
BamHI
BglII 1318
A V D A P E I I P D P F D P S K K R K P T M L V T D L T L R F D P E F E K I S R
1438
R F L N D P Q A F N E A F A R A W F K L T H R D M G P K S R Y I G P E V P K E D
400
BglII
ClaI
NruI
1558
L I W Q D P L P Q P I Y N P T E Q D I I D L K F A I A D S G L S V S E L V S V A
1678
W A S A S T F R G G D K R G G A N G A R L A L M P Q R D W D V N A A A V R A L L
500
EcoRV
RsaI 1798
V L E K I Q K E S G K A S L A D I I V L A G V V G V E K A A S A A G L S I H V P
1918
F A P G R V D A R Q D Q T D I E M F E L L E P I A D G F R N Y R A R L D V S T T
SphI
RsaI
2038
E S L L I D K A Q Q L T L T A P E M T A L V G G M R V L G A N F D G S K N G V F
600
AsuII
2158
T D R V G V L S N D F F V N L L D M R Y E W K A T D E S K E L F E G R D R E T G
HaeIII
2278
E V K F T A S R A D L V F G S N S V L R A V A E V Y A S S D A H E K F V K D F V
700
2398
A A W V K V M N L D R F D L L L END
terminator
726
2518
DraI
2638
2758
SspI NruI 2805

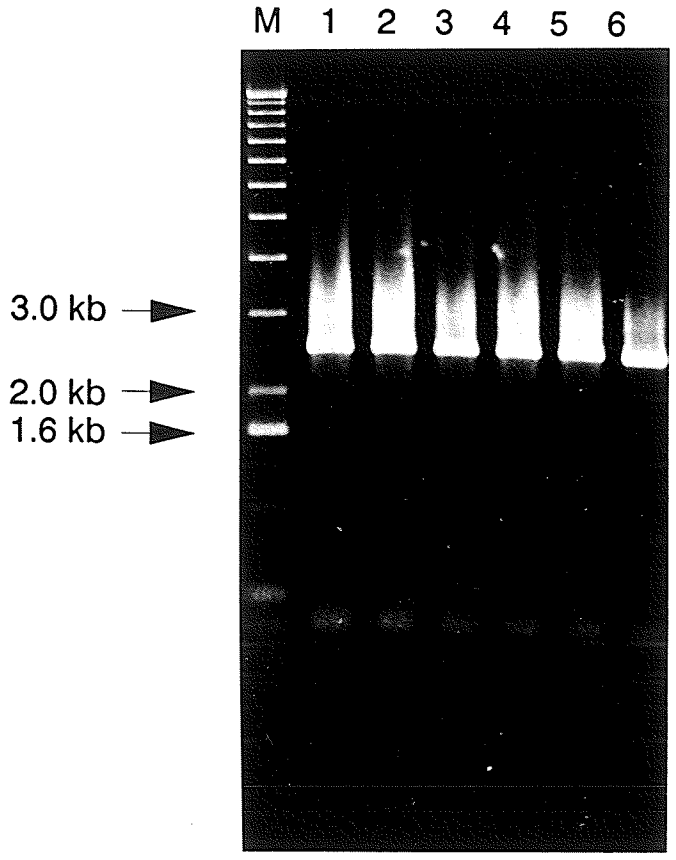

M 1 2 3 4 5 6
3.0 kb
2.0 kb
1.6 kb

Figure 3. The strategy for cloning *katG* PCR DNA. The PCR product with tailored *Hin*dIII sites on each end was inserted in the single *Hin*dIII site of the multiple cloning site of the Bluescript SK+ vector.

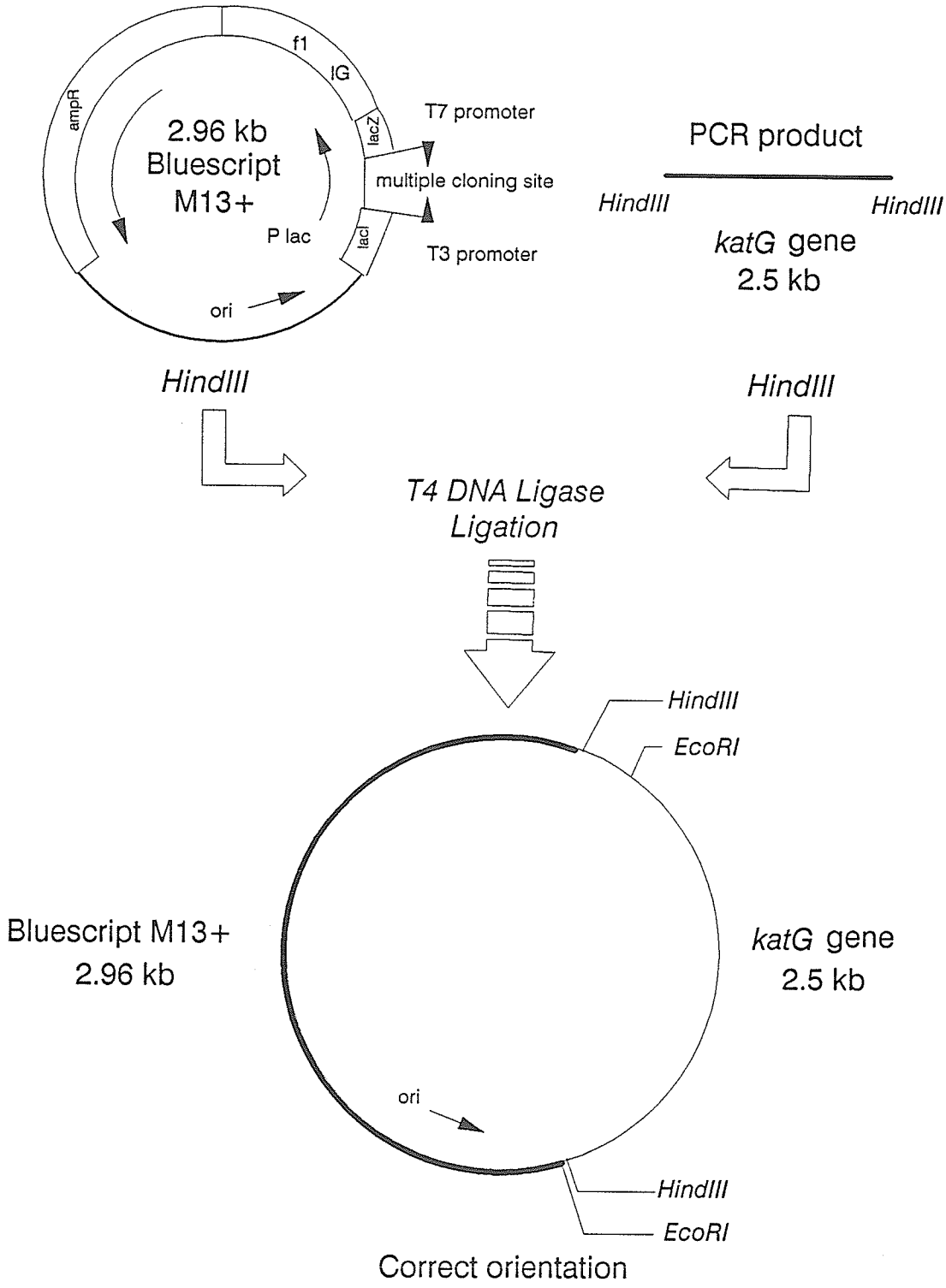

f1
IG
ampR
lacZ
2.96 kb
Bluescript
M13+
P lac
lacI
ori
T7 promoter
multiple cloning site
T3 promoter
PCR product
HindIII
HindIII
katG gene
2.5 kb
HindIII
HindIII
T4 DNA Ligase
Ligation
HindIII
EcoRI
Bluescript M13+
2.96 kb
katG gene
2.5 kb
ori
HindIII
EcoRI
Correct orientation

Figure 4. Chimeric plasmids digested with *Hin*dIII separated on agarose gels. Lane M contains a 1 kb ladder marker (**BRL**). Lane 1 was from the digestion of UM308,SK+ chimeric plasmids with *Hin*dIII. Lane 2 was from the digestion of UM309,SK+ chimeric plasmids with *Hin*dIII. Lane 3 was from the digestion of UM311,SK+ chimeric plasmids with *Hin*dIII. Lane 4 was from the digestion of UM312,SK+ chimeric plasmids with *Hin*dIII.

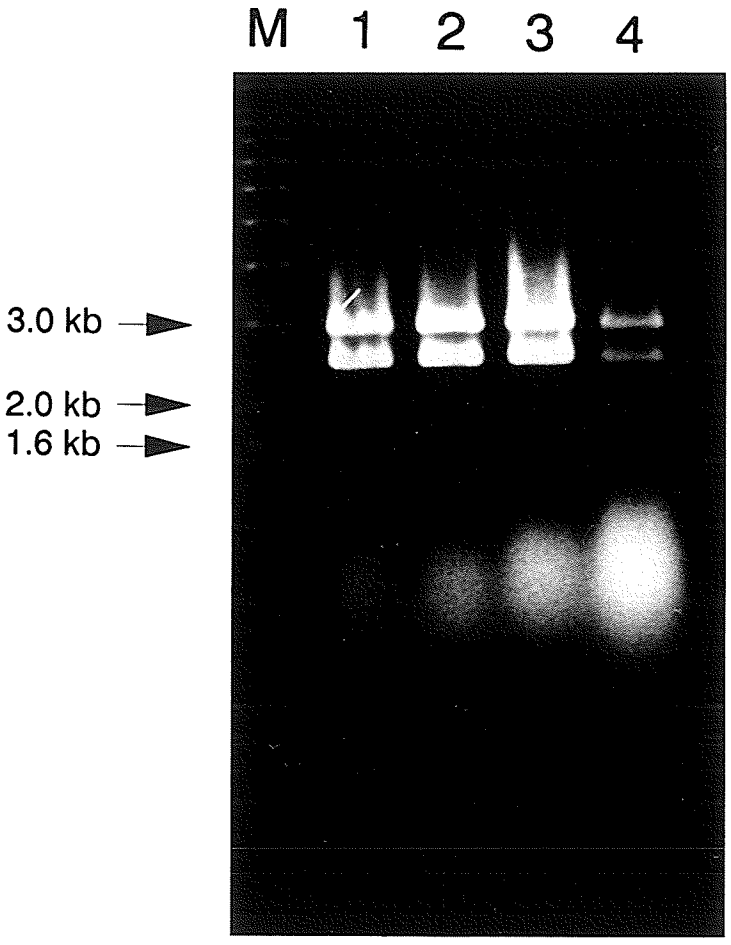
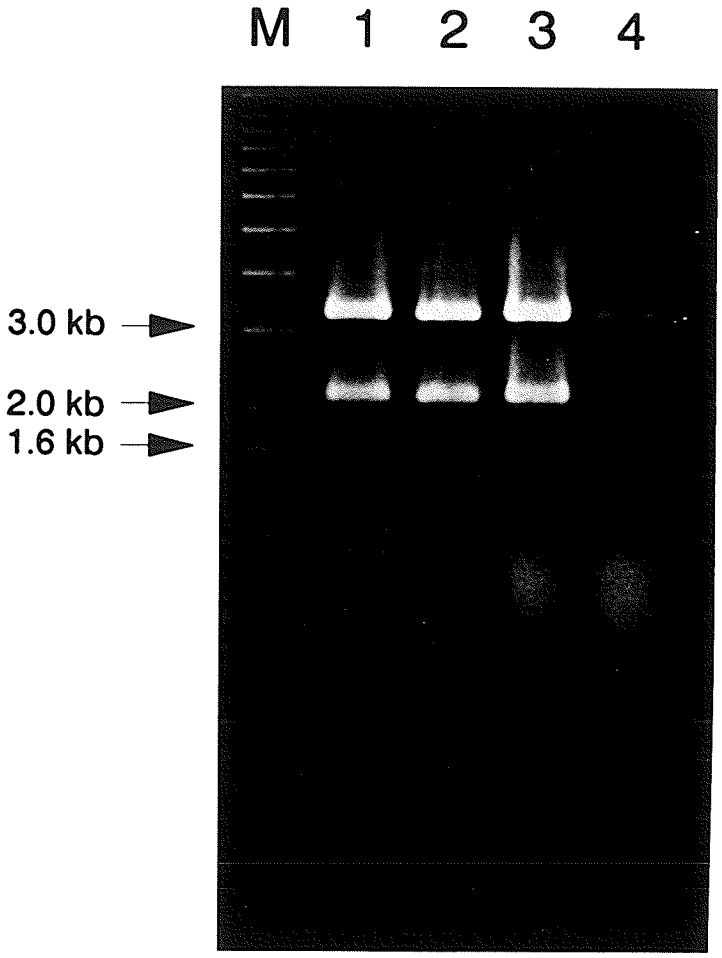

M
1
2
3
4
3.0 kb
2.0 kb
1.6 kb

Figure 5. Determination of insert orientation by *Eco*RI digestion. Lane M contains a 1 kb ladder marker (**BRL**). Lane 1 was from the digestion of UM308,SK+ chimeric plasmids with *Eco*RI. Lane 2 was from the digestion of UM309,SK+ chimeric plasmids with *Eco*RI. Lane 3 was from the digestion of UM311,SK+ chimeric plasmids with *Eco*RI. Lane 4 was from the digestion of UM312,SK+ chimeric plasmids with *Eco*RI.


M
1
2
3
4
3.0 kb
2.0 kb
1.6 kb

Purification of mutant HPIs

Recombinant plasmids containing a *katG* insert from UM308, UM309, UM311 or UM312 were subsequently transformed into UM262 which contains a transposon in *katG* and will not produce any HPI protein. In this strain, all HPI protein produced is derived from the plasmid. In order to generate wild-type HPI from a comparable plasmid background, a 3.8 kb fragment containing *katG*$^{+}$ from pBT22 was integrated into the SK+ vector, followed by transformation into UM262. A summary of the yields and specific activities of the various mutant enzymes is contained in Table 3. It can be seen that all mutant enzymes exhibited reduced catalase specific activity throughout the purification protocol as compared with the wild-type enzyme. Since UM262 still produces some active HPII catalase and these HPII will interfere the catalase assay, the fluctuation of specific activity of the mutant catalase HPI was observed in the Table 3.

Table 3. Purification of wild-type and mutant HPIs.

Fraction	Wild-type	UM308	UM309	UM311	UM312
Catalase (U/mg protein)					
Crude extract	640	7.9	5.7	3.2	3.8
Streptomycin sulfate	682	6.8	4.2	3.4	4.5
Ammonium sulfate	1525	2.8	1.0	0.7	0.9
DEAE-Sephadex A50	1354	10	0.4	1.8	1.6

Characterization of mutant HPIs

The UV-visible absorption spectra were determined for all of the mutant enzymes and found to differ from the spectrum of the wild-type principally in having a much lower Soret absorption peak (Figure 6). Using the peak at 280 nm as a measure of the protein concentration and the Soret peak at 407 nm as a measure of the protoheme IX content, there was just 10-15% of wild-type amounts of protoheme IX in each of the mutant HPIs (Table 4). In spectroscopy, the Soret bands, the absorption bands in the region around 400 nm, are the characteristic of conjugated tetrapyrroles.

Electrophoresis of the wild-type and mutant HPIs on denaturing SDS polyacrylamide gels resulted in the wild-type HPI migrating as a double band which previously had never been observed. The size of the two bands was estimated to be 81 kDa and 80 kDa. The mutant HPIs all migrated as single bands with the same molecular size as the larger of the two wild-type bands (Figure 7).

Figure 6. Comparsion of absorption spectra of HPIs isolated from UM262 harboring clones of *katG* from wild-type, UM308, UM309, UM311 and UM312. All samples were dissolved in 50 mM potassium phosphate buffer pH 7.0.

a. Wild-type

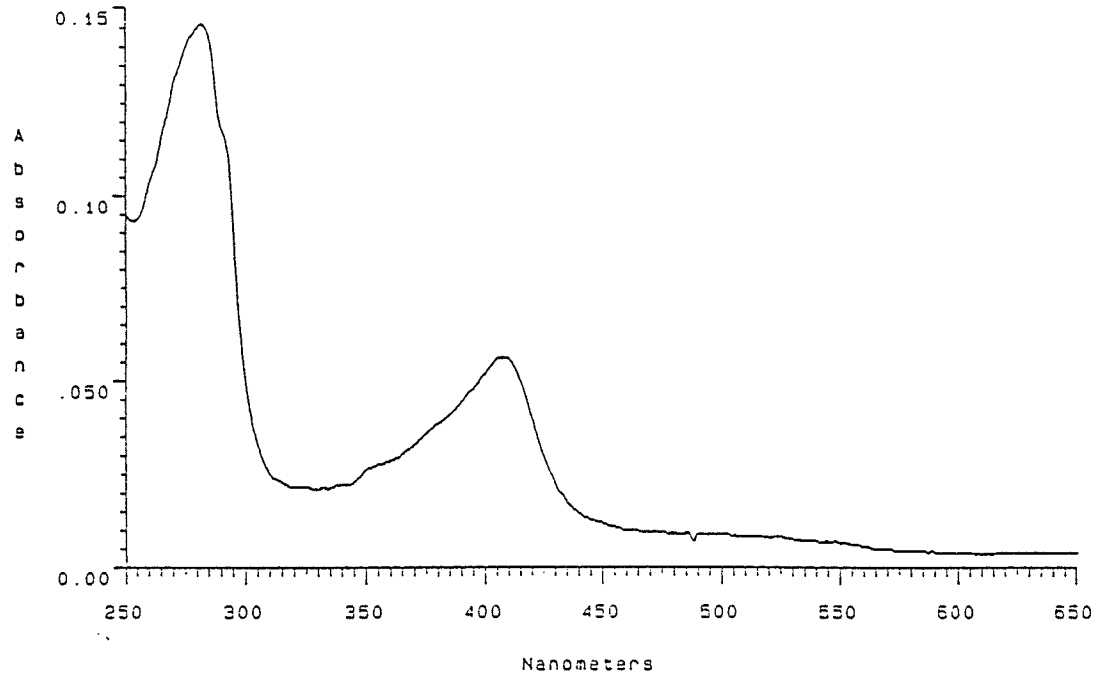
b. UM308

c. UM309

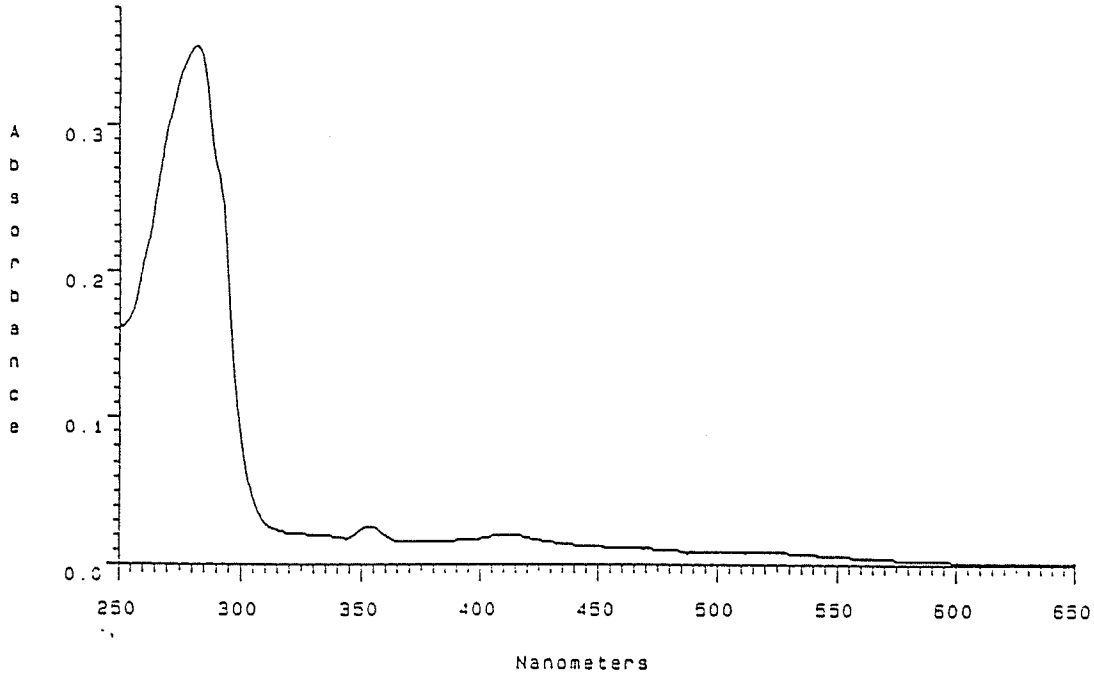
d. UM311

e. UM312

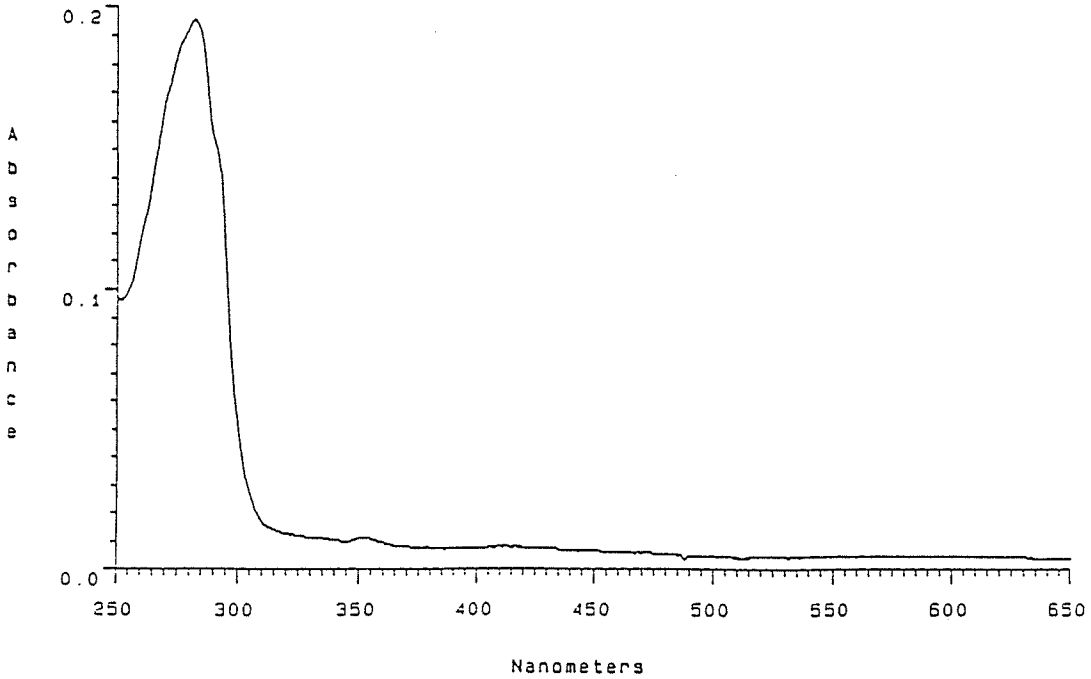
a.


0.15
0.10
.050
0.00
Absorbance
250
300
350
400
450
500
550
600
650
Nanometers

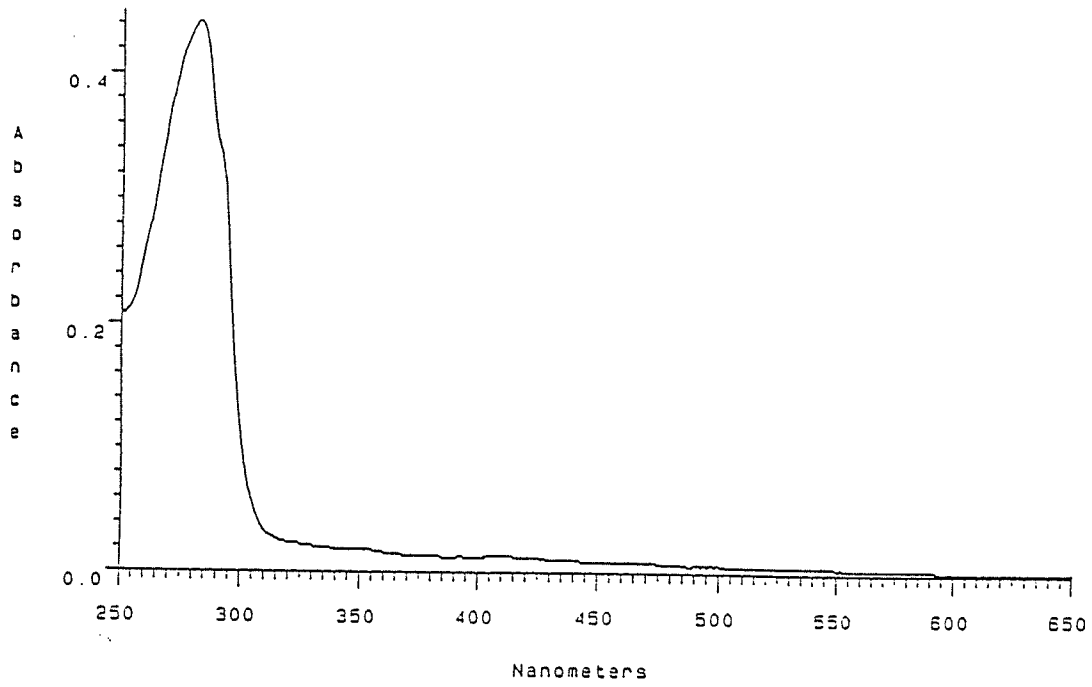
b.


Absorbance
0.3
0.2
0.1
0.0
250
300
350
400
450
500
550
600
650
Nanometers

c.


Absorbance
0.2
0.1
0.0
250
300
350
400
450
500
550
600
650
Nanometers

d.


Absorbance
0.4
0.2
0.0
250
300
350
400
450
500
550
600
650
Nanometers

e.

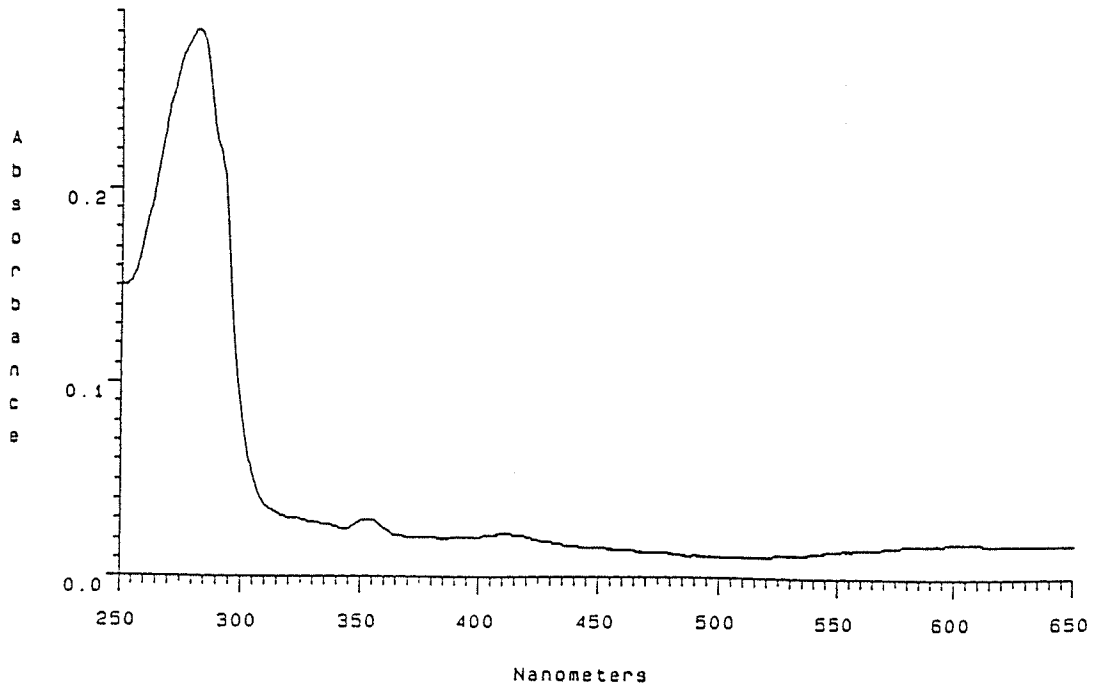
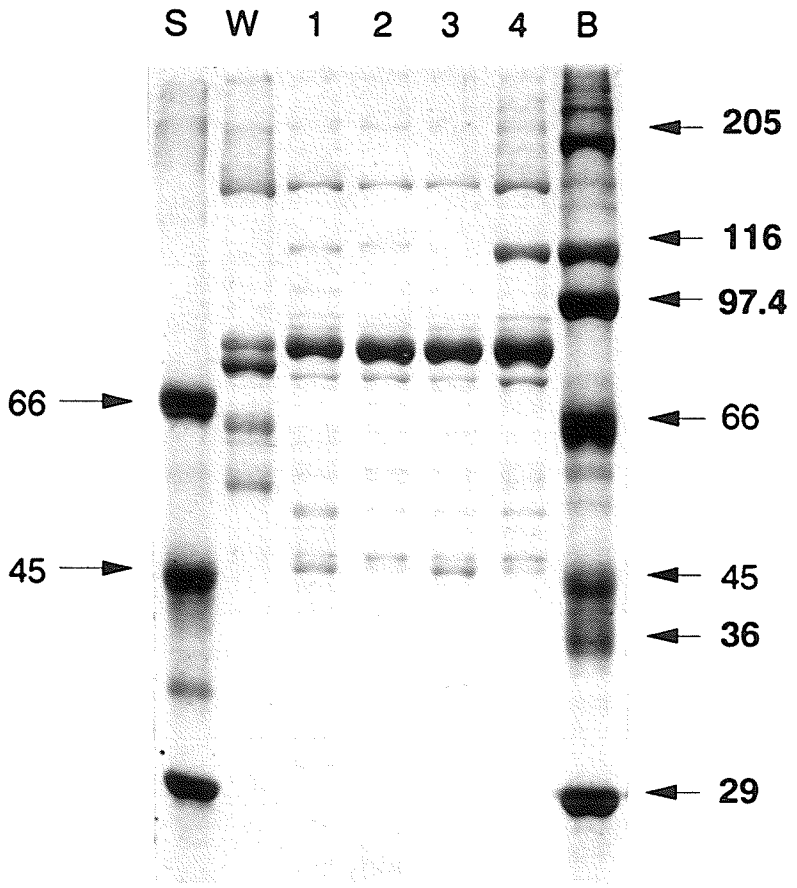

Absorbance
0.2
0.1
0.0
250
300
350
400
450
500
550
600
650
Nanometers

Table 4. Spectral characteristics of the wild-type and mutant HPIs.

	Wild-type	UM308	UM309	UM311	UM312
Wavelength (nm)					
280	0.145	0.352	0.194	0.437	0.273
407	0.057	0.02	0.01	0.017	0.024
Calculated values					
$Ratio_{OD407/OD280}$	0.393	0.057	0.052	0.039	0.088
$Ratio_{Heme/tetramer}$	1.572	0.23	0.21	0.16	0.35

Figure 7. SDS polyacrylamide gel electrophoresis of partially purified wild-type HPI and mutant HPIs. The gel was run under denaturing conditions (Weber *et al*, 1972). The numbers at the left and right indicate the molecular weights ($X10^3$) used for size estimation. Lane S contained SDS molecular weight marker from MW-SDS-70L Kit (**Sigma**). Lane W contained 5 μg of partially purified wild-type HPI. Lane 1 contained 5 μg of partially purified UM308 HPI. Lane 2 contained 5 μg of partially purified UM309 HPI. Lane 3 contained 5 μg of partially purified UM311 HPI. Lane 4 contained 5 μg of partially purified UM312 HPI. Lane B contained SDS molecular weight marker from MW-SDS-200 Kit (**Sigma**). The following molecular weight standards were used: 205 kDa, myosin; 116 kDa, ß-galactosidase; 97.4 kDa, phosphorylase B; 66 kDa, bovine plasma albumin; 45 kDa, egg albumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase; and 29 kDa, carbonic anhydrase.


S
W
1
2
3
4
B
205
116
97.4
66
66
45
45
36
29

Sequencing analysis of mutant *katG* gene

The mutant genes were sequenced using a series of nine oligonucleotide primers complementary to the *katG* sequence at 270 bp intervals (Figure 1). These primers were used in the Sanger sequencing method such that the sequence from each primer overlapped the succeeding primer, ensuring sequence analysis of the complete gene. Sequencing of *katG* from UM311 revealed the following changes as shown in Figure 8: a T to C change at 539 changing Ser132 to Pro; a G to A change at 954 changing Gly270 to Asp; an A to G change at 2268 changing Lys708 to Arg, and an A to G change at 2107 which did not change Lys654. Subsequent sequence analysis of *katG* from UM308, UM309 and UM312 revealed the same mutations indicating that it was unlikely they were from separate isolates.

Figure 8. Autoradiograms of sequencing gels revealing base changes in the UM311 mutant *katG* gene.

(1) The sequence of wild-type *katG* between bases 524 and 554 (TTTGCACCGCTGAAC**T**$_{539}$CCTGGCCGGATAACG) is shown on the left and the sequence on the right covers the same region for the mutant. The T to C change at base 539 of UM311 mutant is indicated on the right side.

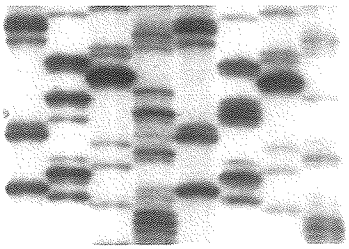
(2) The sequence of wild-type *katG* between bases 939 and 969 (GTGGTCATACGCTGG**G**$_{954}$TAAAACCCACGGTGC) is shown on the left and the sequence on the right covers the same region for the mutant. The G to A change at base 954 of UM311 mutant is indicated on the right side.

(3) The sequence of wild-type *katG* between bases 2096 and 2119 (TACGAGTGGAA**A**$_{2107}$GCGACCGACGAA) is shown on the left and the sequence on the right covers the same region for the mutant. The A to G change at base 2107 of UM311 mutant is indicated on the right side.

(4) The sequence of wild-type *katG* between bases 2253 and 2283 (ACGAGAAGTTTGTTAA$_{2268}$AGACTTCGTGGCGGC) is shown on the left and the sequence on the right covers the same region for the mutant. The A to G change at base 2268 of UM311 mutant is indicated on the right side.

(1) Wild-type UM311

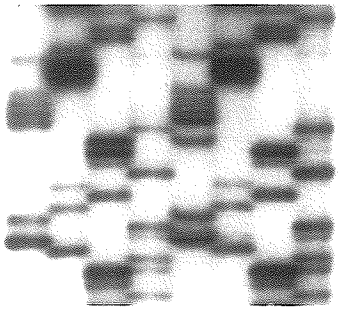
A C G T A C G T



T ⟶ C 539

(2) Wild-type UM311

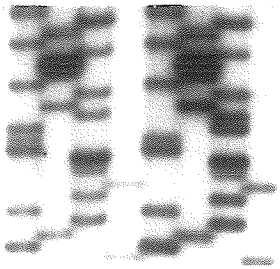
A C G T A C G T



G ⟶ A 954

(3) Wild-type UM311

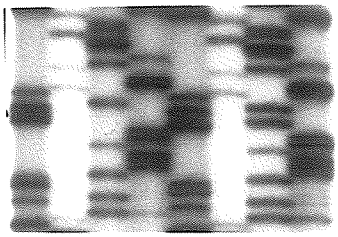
A C G T A C G T



A ⟶ G 2107

(4) Wild-type UM311

A C G T A C G T



A ⟶ G 2268

DISCUSSION

Polymerase Chain Reaction (PCR)

The ability of PCR to generate specific DNA fragments in large amounts can simplify the procedures necessary to clone a single-copy gene from genomic DNA into a vector, eliminating the need to construct phage libraries and screen large numbers of recombinant clones.

Over the past few years PCR has been used very extensively in eucaryotic systems. However, up to now not many papers using PCR for the study of procaryotic systems have appeared in the literature. Unquestionably, no single PCR protocol will be appropriate for all situations. Consequently, each new PCR application is likely to require optimization. I encountered some problems including a low yield of the desired product, an unstable PCR reaction, the presence of nonspecific background bands due to mispriming or misextension of the primers (Figure 2) and possible artificial mutations due to misincorporation.

PCR generated DNA containing terminal restriction endonuclease recognition sites have been found to be difficult to cut with the target restriction endonucleases despite 3 or 4 extra 5' bases flanking that site; for e.g. *Not*I and *Xba*I incorporated into the termini of PCR products. Three possible explanations have been proposed (Jung *et al*, 1990): (1) *Taq* polymerase is inefficient for certain terminal sequences, producing frayed ends that cannot be cleaved by the restriction endonuclease. (2) Broken of

terminal sequences prevents stable association of the restriction endonucleases with the terminal site. (3) The extra 3-4 bases at the terminal restriction endonuclease recognition site are insufficient to allow for stable association with, and cutting by, certain restriction endonucleases.

Initially, I used a combination of primer-12 and primer-13 for PCR amplification. Primer-12 generated a *Hin*dIII site at the 5'-end but this product was found to be a poor substrate for *Hin*dIII as determined by unsuccessful cloning. I subsequently turned to a combination of primer-14 and primer-13 which also generated a *Hin*dIII site but 9 bases from the 5'-end. Using equal concentrations of primer-13 and primer-14, the yields of the desired 2.5 kb product were low and four strong bands of 0.4, 0.8, 1.2 and 1.7 kb were observed, probably the result of mispriming. Decreasing the concentration of primer-14 resulted in better yields of the 2.5 kb product and eliminated the smaller bands although there was a faint smear of background products. Subsequent work to clone the PCR products into Bluescript SK+ vector using the tailored *Hin*dIII sites at each end was successful. The clones were successfully used for sequencing and for overexpression of the mutant enzymes. Surprisingly all four mutant clones, from UM308, UM309, UM311 and UM312, contained the same base changes at four locations. It is unlikely that the PCR elongation would give rise to such reproducible

misincorporation. Consequently, we must assume that all four clones were derived from the same original mutant clone. In other words UM308, UM309, UM311 and UM312 were not independent isolates as originally thought.

A useful application of PCR is the ability to amplify DNA from one organism by using primers homologous to another related organism. *E. coli* and *Salmonella typhimurium* are about 150 million years apart in the evolutionary scale and the divergence of their DNA at silent sites is 58% (Ochman, 1987). In data not described, I was able to amplify *katG* from *S. typhimurium* using primers from *E. coli* confirming that this approach is feasible for closely related organisms. It may be possible to amplify DNA from organisms even further removed than these, perhaps by utilizing longer primers (Shyamala, 1989).

The Characterization of Mutant HPIs

The information content for protein function is inherent in the primary amino acid sequence. By comparing the properties of the mutant and wild-type forms of the protein, it may be possible to identify domains or individual amino acid residues that are essential for the structural integrity or biological function of the protein.

The object of the study, HPI catalase, is active as a tetramer of identical subunits presumably associated through specific hydrophobic or hydrophilic contacts. The ability to

form this quaternary structure requires that the individual subunits be folded in a particular conformation.

Mutant HPI catalases expressed from cloned genes were purified and all exhibited low catalase specific activity. The four base substitutions identified in the mutant clones include a T to C change at 539, a G to A change at 954, an A to G change at 2268, and an A to G change at 2107. These changes resulted in amino acid changes including serine to proline at residue 132, glycine to aspartic acid at residue 270 and lysine to arginine at residue 708, and no change at the fourth mutation, respectively.

It is possible to predict that the change from a hydrophilic serine to a hydrophobic proline, which would also disrupt an α-helix section, and the change from a hydrophobic glycine to a hydrophilic aspartate would have the greatest effect on structure and activity. The change from lysine to arginine would not affect the charge of the protein, nor would it significantly change the spatial relationship with other residues.

From previous work, the primary identifiable physical effects of three amino acid changes including Leu to Phe139, Gly to Asp119 and Gly to Asp314 were putative conformational changes and a reduction in the amount of heme bound (Loewen *et al*, 1990). Because of the absence of crystallographic data for HPI and conjecture about the location of the heme, it was not possible to conclude whether the mutations

directly prevented the binding of protoheme IX or simply resulted in a conformational change which in turn prevented binding. Similarly in this work it is not possible to conclude whether it is conformation or charge effects that are affecting heme binding and activity. Two main structural differences were observed between the wild-type and mutant enzymes. First, the absorption spectra of the mutant proteins revealed that they all contained much less protoheme IX than the wild-type HPI; about 15% of the wild-type amount (Table 4). Second, the subunit of the mutant HPIs migrated as a single band on SDS-polyacrylamide gels as compared to the wild-type HPI which migrated as two bands. It is not clear why the wild-type exhibited two bands in these preparation because this had not been observed previously although non-denaturing gels revealed two charge variant forms. Possibly the two denatured subunit sizes observed here are related to the two charge variant forms and previous work simply had not succeeded in separating the two denatured subunit sizes. This would suggest that the difference in charge on non-denaturing gels has arisen from processing of the subunit to produce two subunit sizes.

It has been observed that the heme pocket of hemoglobin is quite hydrophobic and it would be reasonable to assume that the heme binding site in HPI would be hydrophobic. The Gly to Asp changes noted in the earlier work, and in this work, would decrease the hydrophobic nature of the protein

which in turn might reduce heme binding. Because three amino acid changes were founded in the four mutant *katG*s investigated in this study, it is not possible conclude whether all three changes were having an effect on heme binding and protein folding or just one residue was responsible. This problem might be resolved if the three-dimensional shape of the wild-type enzyme were known or by subcloning of individual mutations.

Unfortunately a prediction of the structure of *katG* HPI protein is not possible at this time because:

(1) the HPI amino acid sequence has little similarity to other catalases, peroxidases or heme proteins for which X-ray crystallography and NMR information is available;

(2) there is not enough information about the roles of various amino acids in activity, heme binding and 3-D structure;

(3) *E. coli* HPI protein has not been crystallized making it impossible to commence an X-ray crystallographic study.

REFERENCES

Adhya, S. and S. Garges, 1990. Positive control, J. Biol. Chem. 265:10797-10800.

Brunt, J. V., 1990. Amplifing Gene: PCR and its Alternatives, Bio/technology Vol.8:291-294.

Chan, E. and B. Weiss, 1987. Endonuclease IV of *Escherichia coli* is induced by paraquat, Proc. Natl. Acad. Sci. USA 84:3189-3193.

Chiu, J. T., P. C. Loewen, J. Switala, R. B. Gennis and R. Timkovich, 1989. Proposed structure for the prosthetic group of the catalase HPII from *Escherichia coli*, J. Am. Chem. Soc. 111:7046-7050.

Christman, M. F., R. W. Morgan, F. S. Jacobson and B. N. Ames, 1985. Positive control of a regulon for defenses against oxidative stress and some heat shock proteins in *Salmonella typhimurium*, Cell 41:753-762.

Christman, M. F., G. Storz and B. N. Ames, 1989. *OxyR*, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins, Proc. Natl. Acad. Sci. USA 86: 3484-3488.

Claiborne, A. and I. Fridovich, 1979. Purification of the o-dianisidine peroxidase from *Escherichia coli* B, J. Biol. Chem. 254: 4245-4252.

Claiborne, A., D. P. Malinowski and I. Fridovich, 1979. Purification and characterization of hydroperoxidase II of *Escherichia coli* B, J. Biol. Chem. 254:11664-11668.

Conn, E. E. and P. K. Stumpf, 1976. Outlines of Biochemistry, 4 rd., p:400. John Wiley & Sons, Inc. New York.

Demple, B., J. Halbrook and S. Linn, 1983. *Escherichia coli xth* mutants are hypersensitive to hydrogen peroxide, J. Bacteriol. 153:1079-1082.

Fridovich, I., 1975. Superoxide dismutase, Ann. Rev. Biochem. 44:147-159.

Hagensee, M. E., S. K. Bryan and R. E. Moses, 1987. DNA polymerase III requirement for repair of DNA damage caused by methyl methanesulfonate and hydrogen peroxide, J. Bacteriol. 169:4608-4613.

Halliwell, B. and J. M. Gutteridge, 1986. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts, Archives of Biochemistry and Biophysics 246:501.

Hochman, A. and A. Shemesh, 1987. Purification and characterization of a catalase-peroxidase from the photosynthetic bacterium *Rhodopseudomonas capsulata*, J. Biol. Chem. 262:6871-6876.

Imlay, J. A. and S. Linn, 1986. Biomodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide, J. Bacteriol. 166:797-799.

Innis, M. A., D. H. Gelfand, J. J. Sninsky and T. J. White, 1990. PCR Protocals: A Guile to Methods and Applications, Academic Press, Inc.

Jung, V., S. B. Pestka and S. pestka, 1990. Efficient cloning of PCR generated DNA containing terminal restriction endonuclease recognition sites, Nucleic Acid Res. Vol. 18. No. 20. p.6156.

Kuyumdzhieva-Savova, A., V. A. Savov, L. K. Genova and S. P. Peikova, 1985. Superoxide dismutase in methyloyrophic yeasts, FEMS Microbiology Letters 27:103.

Latne, E. 1957. Spectrophotomeric and turbidmetric methods for easuring proteins, Methods Enzymol. 3:447-454.

Loewen, P. C. and G. V. Stauffer, 1990. Nucleotide sequence of *katG* of *Salmonella typhimurium* LT2 and characterization of its product, hydroperoxidase I, Mol. Gen. Genet. 224:147-151.

Loewen, P. C. and J. Switala, 1986. Purification and characterization of Catalase HPII in *Escherichia coli* K12, Biochem. Cell Biol. 64:638-646.

Loewen, P. C., B. L. Triggs-Raine, G. C. Carolyn and B. E. Hrabarchuk, 1985. Genetic mapping of *katG*, a locus that affect synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in *Escherichia coli*. J. Bacteriol. 162:661-667.

Loewen, C. P., J. Switala, M. Smolenski and B. L. Triggs-Raine.1990. Molecular characterization of three mutation in *katG* affecting the activity of hydroperoxidase I of *Escherichia coli*, Biochem. Cell Biol. 68:1037-1044.

Loewen, P. C. and B. L. Triggs, 1984. Genetic mapping of *katF*, a locus that with *katE* affects the synthesis of a second catalase species in *Escherichia coli*, J. Bacteriol. 160:668-675.

Loewen, P. C., J. Switala and B. L. Triggs-Raine, 1985. Catalase HPI and HPII in *Escherichia coli* are induced independently, Arch. Biochem. Biophys. 243:144-149.

Loh, E. Y., J. F. Elliott, S. Cwirla, L. L. Lanier and M. M. Davis. 1989. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain, Science 243: 217.

Loprasert, S., S. Negoro and H. Okada, 1989. Cloning, Nucleotide Sequence, and Expression in *Escherichia coli* of the *Bacillus stearothermophilus* Peroxidases Gene (perA), J. Bacteriol. 171:4871-4875.

Maniatis, T., E. F. Fritsch and J. Sambrook, 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Maniatis, T., E. F. Fritsch and J. Sambrook, 1989. Molecular cloning: a laboratory manual. 2nd., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Mead, D. A., E. S. Skorupa and B. Kemper, 1985. Single stranded DNA SP6 promoter plasmids for engineering mutant RNAs and proteins: synthesis of a stretched preprothyroid hormone, Nucl. Acids. Res. 13:1103-1118.

Miller, J. H. 1972. Expermints in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz and B. N. Ames, 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins, Proc. Natl. Acad. Sci. USA 83: 8059-8063.

Mulvey, M. R., J. Switala, A. Borys and P. C. Loewen, 1990. Regulation of transcription of *katE* and *katF* in *Escherichia coli*. J. Bacteriol. 172:6713-6720.

Mulvey, M. R. and P. C. Loewen. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests *katF* protien is a novel sigma transcriptional factor, Nucleic Acid Res. 17:9979-9991.

Mulvey, M. R., A.S. Pamela, B. L. Triggs-Raine and P.C. Loewen, 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*, Gene 73:337-345.

Ochman, H. and A. C. Wilson, 1987. Evolutionary history of enteric bacteria. p. 1649-1654. in F. C. Neidhardt (ed.). *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, American Society for Microbiology, Washington, D. C.

Rorth, M. and P. K. Jensen, 1967. Determination of catalase Activity by means of the Clark electrode, Biochim. Biophys. Acta. 139:171-173.

Sakamoto, H. and D. Touati, 1984. Cloning of the iron superoxide dismutase gene (*sodB*) in *Escherichia coli* K-12, J. Bacteriol. 170:5141-5145.

Schellhorn, H. E. and H. M. Hassan, 1988. Canadian Journal of Microbiology 34:162.

Schiavone, J. R. and H. M. Hassan, 1988. The role of Redox in the Regulation of Manganese-containing Superoxide Dismutase Biosynthesis in *Escherichia coli*, J. Biol. Chem. 263:4269-4273.

Scott, M. D., S. R. Meshnick and J. Eaton, 1987. Superoxide dismutase-rich bacteria, J. Biol. Chem. 262:3640.

Shyamala V. and G. F. L. Ames, 1989. Amplification of bacterial genomic DNA by the polymerase chain reaction and direct sequencing after Asymmetric amplification: application to the study of periplasmic permeases, J. Bacteriol. 171:1602-1608.

Sies, H., 1986. Biochemistry of oxidative stress, Angew. Chem. Int. Ed. Engl. 25:1058-1071.

Storz, G., L. A. Tartaglia and B. Ames, 1990. Transcriptional Regulator of Oxidative Stress-Inducible Genes: Direct Activation by Oxidation, Science 248:189-194.

Tartaglia, L., G. Storz and B. Ames, 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress, J. Mol. Biol. 210:709-719.

Touati, D. 1983. Cloning and mapping of the manganese superoxide dismutase gene (*sodA*) of *Escherichia coli* K-12, J. Bacteriol. 155:1078-1087.

Triggs-Raine B. L., B. W. Doble, M. R. Mulvey, P. A. Sorby and P. C. Loewen, 1988. Nucleotide sequence of *katG*, encoding catalase HPI of *Escherichia coli*. J. Bacteriol. 170:4415-4419.

Triggs-Raine, B. L. and P. C. Loewen. 1987. Physical characterization of *katG*, encoding catalase HPI of *E. coli*, Gene 52: 121-128.

Triglia, T., M. G. Peterson and D. J. Kemp, 1988. A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences, Nucleic Acid Res. v. 16, No. 18:8186.

von Ossowski, I., M. R. Mulvey, P. A. Leco, A. Borys and P. C. Loewen. 1991. Nucleotide Sequence of *Escherichia coli katE*, Which encodes catalase HPII, J. Bacteriol. 173:514-520.

Verduyn, C., M. L. Giuseppin, W. A. Scheffer and J. van Dijken, 1988. Hydrogen peroxide metabolism in yeasts, Applied and Environmental Microbiology 54:2086.

Watson, K., 1990. Microbial Stress Proteins. In: Advances in Microbial Physiology, Vol.31:197-202.

Weber, K., J. R. Pringle and M. Osborn, 1972. Measurement of molecular weights by electrophoresis on SDS-polyacrylamide gels, Methods Enzymol. 26: 3-27.

Wireko, F. C. and D. I. Abraham, 1991. X-ray diffraction study of the binding of the antisickling agent 12C79 to human hemoglobin, Proc. Natl. Acad. Sci. USA 88:2209-2211.