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Cloning and Partial Characterization of *katE* Encoding HPII Catalase in *Escherichia coli*.

by

Pamela Ann Sorby

A Thesis

submitted to the Faculty of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

Department of Human Genetics

The University of Manitoba

Winnipeg, Manitoba

1989

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

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To my Dad.

You never said no

when it was "for school".

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"If I ever get out of here..."

"Band on the Run"

1.0 Abstract

The *Escherichia coli* gene *katE* involved in the synthesis of catalase HPII was cloned in a multistep procedure into a high copy number plasmid pAT153. The plasmid was shown to produce HPII on transformation into UM255 (Kat G Kat E) and UM120 (*katE*::Tn10) complementing the *katE* mutations. In maxicell analysis pAMkatE6 was shown to encode a 93000 dalton protein corresponding to the subunit size of HPII and *katE* was thus determined to be the structural gene for HPII. The region of DNA containing the gene was prepared for single-stranded sequencing by the construction of subclones of pAMkatE72 fragments inserted into the Bluescript M13 phagemids.

List of Abbreviations

ATP	adenosine 5′-triphosphate
bp	base pairs
BSA	bovine serum albumin
BRL	Bethesda Research Labs
dATP	2′-deoxyadenosine 5′-triphosphate
dCDP	2′-deoxycytidine 5′-triphosphate
dGTP	2′-deoxyguanosine 5′-triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dTTP	2′-deoxythymidine 5′-triphosphate
EDTA	ethylenediaminetetraacetic acid
HPI	hydroperoxidase I
HPII	hydroperoxidase II
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase pairs
MSH	mercaptoethanol
mw	molecular weight
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
SOD	superoxide dismutase

List of Abbreviations (continued)

SDS	sodium dodecyl sulphate
SSC	sodium citrate and NaCl solution
TCA	tricarboxylic acid cycle
TE	Tris-EDTA
TEMED	N,N,N^1,N^1-tetramethylethylenediamine
Tn	transposon
Tris	Tris(hydroxymethyl)aminomethane
U	units
UV	ultraviolet
wt	weight

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LITERATURE REVIEW

2. Literature Review

2.1 Oxygen

2.1.1 Introduction

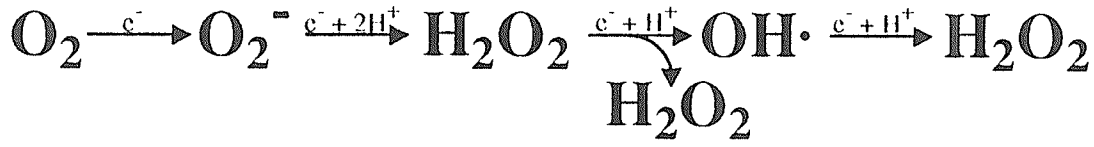
The current conditions on the outer surface of the earth with respect to its elemental composition are to a large extent due to the activities of living organisms. Oxygen as O_2 was probably missing from the prebiotic atmosphere and appeared after oxygenic photosynthesis evolved. Atmospheric oxygen is a product of life rather than a prerequisite to it. The concentration of oxygen in the early atmosphere would have been initially very low due to competition between the oxygen utilizers and producers. The present concentration of O_2 in the atmosphere of 20% was probably established only 2.5 billion years ago (Fridovich, 1978).

As organisms evolved during the increasing oxygenation of the atmosphere they acquired the mechanisms for utilization of oxygen and protection from its effects or were forced to remain in environments impenetrable to molecular oxygen. Elemental oxygen is a ubiquitous component of cells and is always provided in the major nutrient water. In the elementary composition of microbial cells oxygen makes up 20% of dry weight. The physiological functions of oxygen within the cell are as a constituent of cellular water and organic materials and as O_2 utilized as an electron acceptor in respiration of aerobic organims.

2.1.2 Oxygen Toxicity

Relatively high concentrations of oxygen have been known to be toxic to plants, animals and aerobic microorganisms such as *E. coli*. Oxygen toxicity relative to humans in the areas of underwater and space

Figure 2.1 The univalent dismutation of molecular oxygen to water. The reactions catalyzed by the enzymes responsible for scavenging the toxic oxygen intermediates. (Fridovich, 1978)

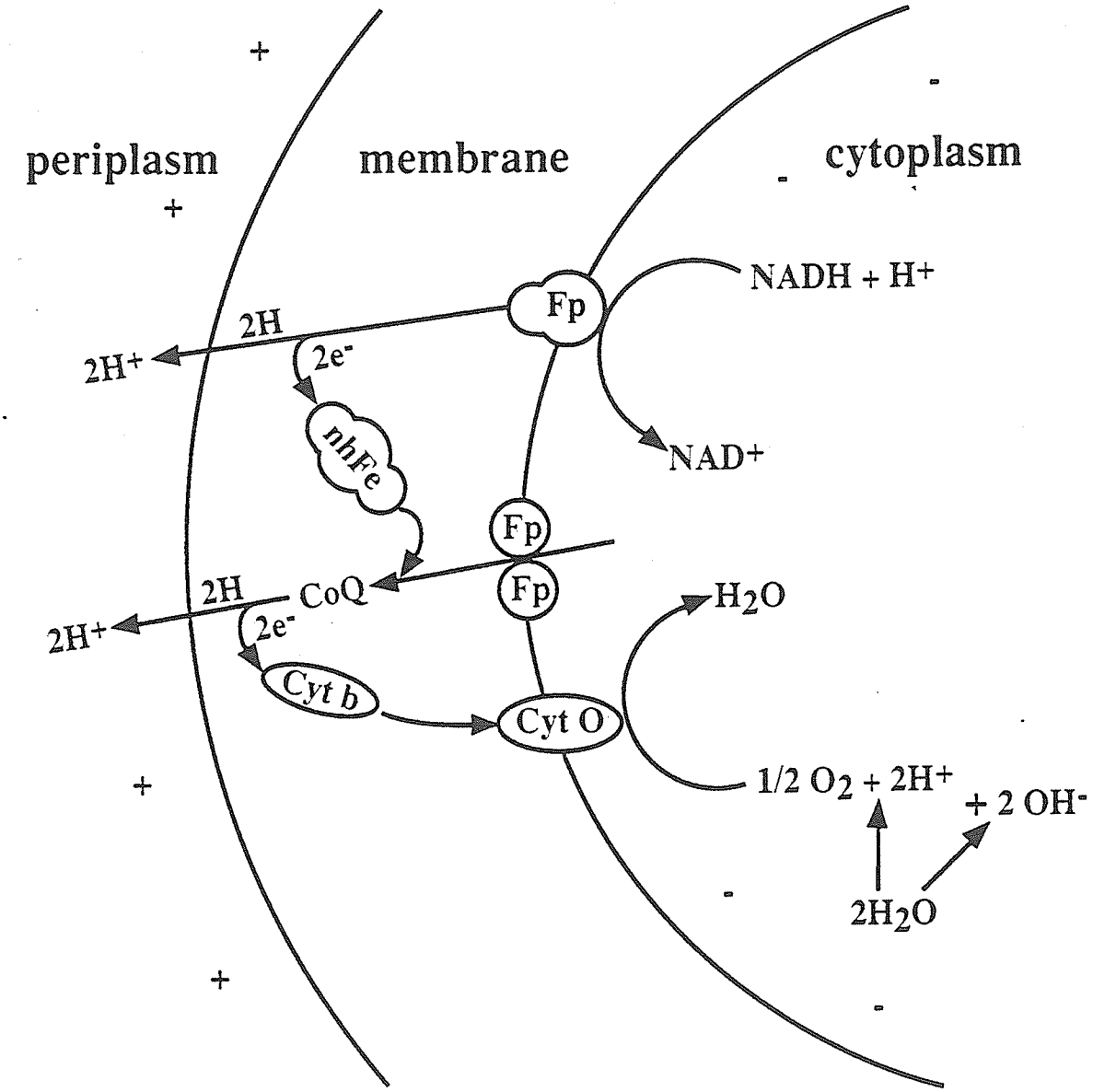


$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$ *(Superoxide Dismutases)*

$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2$ *(Catalases)*

$H_2O_2 + RH_2 \longrightarrow 2H_2O + R$ *(Peroxidases)*

Figure 2.2 The *E. coli* electron transport chain. Fp - flavoprotein; nhFe - non-heme iron; CoQ - Coenzyme Q; Cytb - Cytochrome b; CytO - Cytochrome O. (in Biology of Microorganisms, Thomas D. Brock ed., Prentice-Hall Inc. 1984)


periplasm
membrane
cytoplasm
+
+
+
+
-
-
-
Fp
NADH + H+
NAD+
2H
2H+
2e-
nhFe
Fp
Fp
2H
CoQ
2H+
2e-
Cyt b
Cyt O
H2O
1/2 O2 + 2H+
+ 2 OH-
2H2O

activity, cancer treatment, autoimmune disease, aging and ischemia and reperfusion have heightened investigations into the damaging effects of oxygen (reviewed by Halliwell and Gutteridge, 1985; Przyklenk & Kloner, 1989). Molecular oxygen is not toxic because of its own reactivity but because of the highly reactive intermediates formed in the sequential reduction of oxygen to water (Fridovich, 1977). Molecular oxygen in the ground state or most stable state contains two unpaired electrons of parallel spin in two different electron orbitals. The Pauli exclusion principle states that electrons occupying the same orbital must have equal and opposite spin. Complete reduction of oxygen to water requires four electrons but electrons cannot be passed to oxygen from the reducing agent in pairs as they would have antiparallel spin in the electron donor. This criterion restricts oxygen to a univalent reduction with the subsequent production of toxic oxygen species (Fig. 2.1) (Halliwell & Gutteridge, 1985). Formation of toxic oxygen species is largely due to the process of aerobic respiration. In aerobic respiration ATP is generated from the energy released through an oxidation-reduction cycle by transporting electrons through a chain of carrier molecules with fixed orientation in the cell membrane. The components and complexity of electron transport chains vary but are composed of carrier molecules able to undergo reversible oxidation and reduction. In the *E. coli* ETC (Fig. 2.2) NADH acts as the initial donor and O_2 the terminal electron acceptor. For every four electrons introduced into the ETC one molecule of oxygen is reduced to two molecules of water. The final electron "shuttle" in the *E. coli* ETC, cytochrome oxidase, appears to bind the partially reduced oxygen intermediates and the presence of toxic oxygen species in free solution

does not originate at this point (Antonini *et al.* 1970). Another component of the ETC, coenzyme Q, is less efficient than cytochrome oxidase and can "leak" electrons to O_2 while passing the greater portion onto the following molecule (Halliwell & Gutteridge 1985). The result is the presence of the superoxide radical O_2^-, in free solution. Other examples of O_2^- production include oxidative enzymes and flavoprotein hydrogenases and although there are many biochemical sources of O_2^- the major biological sources of O_2^- remain unidentified (Fridovich 1977, Halliwell and Gutteridge 1985).

Another intermediate of oxygen reduction and the one considered most stable is hydrogen peroxide. Hydrogen peroxide can be formed through divalent reduction of O_2, and through univalent reduction of O_2 and dismutation of O_2^-. Some cellular oxidases produce H_2O_2 as the result of O_2 reduction without the production of O_2^-, such as glycollate oxidase, D-aminoacid oxidase and urate oxidase (Halliwell & Gutteridge, 1985). Hydrogen peroxide is a weak oxidizing agent and is indeed cytotoxic in high concentrations. It can react with thiol groups of proteins causing enzyme inactivation or lipid peroxidation of polyunsaturated fatty acids. The greater danger of intracellular H_2O_2 lies in its ability to give rise to the highly reactive hydroxyl radical.

The hydroxyl radical $OH\cdot$ is the strongest oxidizing agent known. Haber and Weiss concluded that the production of $OH\cdot$ could result from the interaction of O_2^- and H_2O_2 which react readily when catalyzed by reduced metal compounds as in the Fenton reaction. Systems which produce O_2^- will also produce H_2O_2 by dismutation of O_2^- and the two compounds will react to give $OH\cdot$. It is this reaction of H_2O_2 and O_2^- which gives

rise to the damage done to DNA, membrane lipids and other cellular constituents when reduced metal ions are present (Fridovich 1977, Finlay & Linn 1988).

Other sources of external oxidative stresses which cause toxic effects such as γ-radiation, near U.V. radiation, ozone, peroxides and radiometric drugs all act through the formation of or have their damaging effects increased by oxygen reduction intermediates (Imlay & Linn 1988, Halliwell & Gutteridge 1985).

2.1.3 Defence Against Oxygen Damage

Evolutionary adaptation to atmospheric oxygenation resulted in classification of subsets of organisms based on their ability to tolerate oxygen. Facultative anaerobes can grow in the presence or absence of O_2. One subgroup can utilize O_2 as a terminal electron acceptor when it is present or shift to a fermentative mode of energy production when it is not. The other subgroup is exclusively fermentative but insensitive to the presence of O_2. Obligate anaerobes obtain energy only through fermentation, and molecular oxygen is not a nutrient but a toxic substance which inhibits growth or can result in cell death. At the opposite end of the physiological spectrum lie the obligate aerobes. These organisms require molecular oxygen for respiration and contain defences against toxic oxygen species.

Toxic oxygen protection occurs at two levels, predamage and postdamage mechanisms. Predamage mechanisms include the superoxide dismutases, peroxidases, and catalases which scavenge toxic oxygen species. Because the greatest danger to the cell is $OH\cdot$ these enzymes act to remove H_2O_2 and O_2^- before they can react to form $OH\cdot$ and cause cellular damage.

Figure 2.3 The two-step conversion of H_2O_2 to water and molecular oxygen carried out in the catalase reaction.

$$H_2O_2 + Fe\text{-}E \rightarrow H_2O + O{=}Fe\text{-}E \qquad \textit{(Compound I)}$$

$$H_2O_2 + O{=}Fe\text{-}F \rightarrow H_2O + O_2 + Fe\text{-}E \qquad \textit{(Compound II)}$$

There are three known types of superoxide dismutases which all catalyze the same reaction. Prokaryotic organisms characteristically have the manganese or iron containing SOD (MnSOD or FeSOD) and eukaryotes the SOD containing both copper and zinc (CuZn SOD) as well as MnSOD. SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2 (Fig. 2.1).

SOD is present in nearly all aerobes but the absence of SOD in *E. coli sodA* and *sodB* mutants shows SOD to be nonessential to some aerobically growing organisms (Farr *et al.* 1986).

Peroxidases (Fig. 2.1) are present in many forms and are important in scavenging not only hydrogen peroxide but a large range of biologically toxic peroxidases (Fridovich, 1977). In some cases organisms which lack catalase can compensate by an increase in peroxidase (Fridovich, 1977).

The catalase reaction (Fig. 2.1) is a dismutation of H_2O_2 to water and molecular oxygen in two steps (Fig. 2.3). The H_2O_2 reacts with the haem group of catalase to form H_2O and Compound I which reacts with a second H_2O_2 molecule to form H_2O and molecular oxygen. Some catalases are capable of peroxidase-type reactions where Compound I can react with other organic donors such as alcohols or acids (Halliwell and Gutteridge, 1985). Although most aerobic organisms contain catalase some do not. This and the presence of catalase mutants in *E. coli* (Loewen, 1984) shows that catalase is nonessential for aerobic growth in some organisms.

DNA is believed to be damaged oxidatively by $OH\cdot$ through a Fenton reaction involving DNA bound metal and H_2O_2 as well as by O_2^- in a mechanism unrelated to Fenton chemistry. The postdamage defences against oxygen radical damage in *E. coli* can be shown through mutants defective

in DNA polymerase I, recombination and sugar fragment excision. Mutations in *xthA*, *recA*, *nfo*, *polA* and *lexA* all show increased rates of killing by H_2O_2 (Imlay and Linn, 1988).

2.2 Catalases in *E. coli*

2.2.1 The Proteins

There are two proteins in *E. coli* with catalase activity. Hydroperoxidase I (HPI) is a bifunctional catalase (H_2O_2:H_2O_2 oxidoreductase EC1.11.16) with an associated broad spectrum peroxidase (donor: H_2O_2 oxidoreductase EC1.11.1.7) activity, and hydroperoxidase II (HPII) is a monofunctional catalase (Claiborne, 1978; Claiborne and Fridovich, 1979; Claiborne *et al*, 1979; Loewen and Switala, 1986). A third protein, KatF protein, is associated with HPII production but in a regulatory not structural fashion (Mulvey *et al.*, 1988).

HPI is composed of four identical subunits of 81,000 daltons with two protoheme IX groups (Claiborne and Fridovich, 1979) and separates into two isozyme forms HPI-A and HPI-B when electrophoresed on native polyacrylamide gels run in Tris-glycine buffer (Loewen *et al.*, 1985). The enzyme is similar in structure to other purified catalases (Halliwell & Gutteridge, 1985) but is relatively resistant to inhibition by 3-amino-1,2,4-triazole which acts on compound I (Fig. 2.3) in the presence of H_2O_2. The enzyme has a pH optimum at 6.8, has an apparent Km for H_2O_2 of 3.7 mM at this pH and is heat labile at 70°C (Meir and Yagil, 1985). It has been found that following the synthesis of HPI the active protein is associated with the inner membrane on the periplasmic side (Heimberger and Eisenstark, 1988).

HPII is a hexamer of identical 93,000 dalton subunits with one haem group per unit (Loewen and Switala, 1986). The haem in HPII has been

determined to be an isomer of haem d (Timkovich & Loewen, unpublished). HPII is heat stable at 70°C and unlike HPI is sensitive to inhibition by 3-amino-1,2,4-triazole. The enzyme has two pH optima at pH 6.8 and pH 10.5 with corresponding Km values of 18.2 mM and 10 mM (Meir and Yagil, 1985). HPII has been shown to be an immunologically distinct protein from HPI (Loewen and Triggs, 1984; Claiborne *et al.*, 1979) and to be located cytoplasmically rather than membrane associated (Heimberger and Eisenstark, 1988).

The Kat F protein is 42,000 daltons and is believed to act as a positive regulator in the expression of genes required for protection from cellular stress (Mulvey and Loewen, 1989; Sammartano *et al.*, 1986).

2.2.2 The Genes

The gene affecting HPI synthesis in *E. coli* has been mapped to 89.2 min on the chromosome between *ppc* and *metB* and designated *katG* (Loewen *et al.*, 1985). The gene has been sequenced predicting a 726-amino-acid protein with no homology to other known catalases (Triggs-Raine *et al.*, 1988). The HPII protein is produced by *katE* (Mulvey *et al.*, 1988) which maps to 37.8 min on the *E. coli* chromosome between *pfkB* and *xthA* (Loewen, 1984). The third gene involved in *E. coli* catalase production is *katF* which maps to 59.0 min on the chromosome between *mutS* and *cys* (Loewen and Triggs, 1984). The *katF* gene has been sequenced and shows homology to the sigma subunit of RNA polymerase and the heat shock regulatory protein encoded by *htpR* (Mulvey and Loewen, 1989).

2.2.3 Regulation

Catalase levels in *E. coli* are influenced by growth medium components, growth phase, presence of H_2O_2 and genetic control (Yosphe-Purer and Henis, 1976; Christman *et al.*, 1985; Loewen *et al.*,

1985; Richter and Loewen, 1982). The synthesis of HPI and HPII and their levels of activity however are controlled separately (Loewen *et al.*, 1985).

HPI levels increase during logarithmic growth but not into the stationary phase of the culture. HPI levels increase in response to ascorbate and to oxidative stress when H_2O_2 is added to the culture (Loewen *et al.*, 1985; Richter and Loewen, 1982). HPI is found to be one of 30 proteins induced during H_2O_2 adaptation and overproduced in *oxyR* mutants (Christman *et al.*, 1985). The *oxyR* gene product was subsequently found to be a diffusible protein which acts as a positive regulator of *katG* (Christman *et al.*, 1985) at the transcriptional level (Morgan *et al.*, 1986). Although the presence of glucose in growth medium lowers the levels of catalase (Schellhorn & Hassan, 1988), the involvement of catabolite repression has been refuted (Richter and Loewen, 1982). The role of glucose in lowering catalase levels may be related to the ability of glucose to scavenge $OH^{\cdot}$ but another effect of glucose is due to semi-anaerobic growth of cells on glucose with little use of TCA cycle intermediates resulting in lower levels of HPII.

The increase in catalase activity as a culture enters stationary phase is due to the production of HPII (Loewen *et al.*, 1985). HPII is not inducible by ascorbate or H_2O_2 (Loewen *et al.*, 1985), and expression is lower under anaerobic as compared to aerobic conditions (Schellhorn & Hassan, 1988). Levels of HPII are not influenced by the activity of electron transport but are 5-10 fold higher during growth on TCA cycle intermediates in mid-log phase (Loewen *et al.*, 1985). Schellhorn and Hassan (1988) have also found using a *katE* gene fusion product that *katE* is not part of the *oxyR* regulon and confirmed that *katE* is positively regulated by *katF* agreeing with the findings of

Mulvey *et al.* (1988).

2.2.4 Role of Catalase in *E. coli*

E. coli is equipped with a network of inducible responses against cellular stress which are controlled in multiple regulatory pathways. *E. coli* produces entire sets of proteins when challenged with heat shock (Yamomori & Ura, 1982), glucose starvation (Jenkins *et al.*, 1988) H_2O_2 or oxidative stress (Christman *et al.*, 1988) or redox-cycling agents (Greenberg and Demple, 1989). Subsets of proteins are involved in more than one of these pathways (Christman *et al.*, 1985; Jenkins *et al.*, 1988) and the *katG*, *katE* and *katF* proteins are among them. The hydroxyl radical is responsible for DNA strand scission (Brawn and Fridovich, 1981), initiation of autoxidation of polyunsaturated fatty acids (Mead, 1976), and covalently bound protein aggregates or spontaneous fragmentation and proteolytic susceptibility of proteins (Davies *et al.* 1987). As earlier stated the production of $OH\cdot$ is largely dependent on the presence of O_2^- and H_2O_2 derived from molecular oxygen. Mechanisms that produce H_2O_2 and its associated cellular damage correlate with those that induce production of or are inhibited by the presence of catalase or whose effects are increased by the lack of catalase (Hassan and Fridovich, 1979; Schellhorn and Hassan, 1988; Mead, 1976; Greenberg and Demple, 1988; Mackey and Seymour, 1987; Christman *et al.*, 1975; Greenberg and Demple, 1989; Heimberger and Eisenstark, 1988). The value of *E. coli* catalases appears to lie in their ability to remove H_2O_2 from the cell aiding in the decrease of formation of $OH\cdot$ and in this manner reducing the damaging effects of oxidative stress.

The HPI and HPII catalases have been compared to known catalases (Triggs-Raine, 1987) and found to have some atypical features but do

share these features with yet catalases.

The bifunctional activity, inducible nature and membrane associated location of HPI and the constitutive synthesis and cytoplasmic location of the monofunctional HPII indicate different physiological roles for the two enzymes but the definition of these roles is as yet unknown.

2.3 Purpose

Although *katE* has been mapped to 37.8 min on the *E. coli* chromosome and was known to affect HPII synthesis, at the outset of this work the gene had neither been isolated nor characterized.

The intention of this work was to isolate and clone *katE*, determine whether it encoded the protein HPII, subclone the gene into small fragments, and sequence the gene. To date the gene has been cloned, partially characterized and subcloned as will be described in the following sections.

MATERIALS AND METHODS

3. Materials and Methods

3.1 Bacterial Strains, Plasmids and Bacteriophage

The bacterial strains used, all derivatives of *Escherichia coli* K-12, are listed in Table 3.1 with their genotype and source.

Table 3.2 lists plasmids and bacteriophage with their relevant characteristics and source.

3.2 Media

3.2.1 LB Medium (Miller, 1972)

10.0 g tryptone

5.0 g yeast extract

5.0 NaCl

in 1.0 litre distilled H_2O.

Solid medium prepared with addition of 10.0 g agar, supplemented with 0.2% maltose and 10 mM $MgCl_2$ for preparation of bacteriophage.

Ampicillin added to 10 μg/ml and tetracycline to 15 μg/ml as required.

For color selection, 50 μl of 2% X-gal and 50 μl of 100 mM IPTG were spread on individual plates.

3.2.2 R-Top Agar (Miller, 1972)

10.0 g tryptone

1.0 g yeast extract

8.0 g NaCl

8.0 g agar

in 1.0 litre H_2O, supplemented after autoclaving with

2 mM $CaCl_2$

16.6 mM glucose

Table 3.1 Bacterial Strains

Strain	Genotype	Source
HB101	*recA, ramC, pro, gal, rpsL, leu, hsdM, TnsdR, endI, lacY*	Boyer & Roulland-Dussoix (1969)
NM522	*supE, thi,* Δ(*lac-proAB*), *hsd5* {F′, *proAB*, *lacI*^{Q}, *lacZ*, ΔM13}	Mead *et al.* (1985)
JM101	*supE, thi,* Δ(*lac-proAB*) [F′, *traD36*, *proA*$^{+}$, *proB*$^{+}$	Yanisch-Perron *et al.* (1985)
Q359	P2 lysogen, *hsdR*, *hsdM*$^{+}$	Elledge and Walker (1985)
MP180	*thi-1* HfrH	Pearson (1972)
UM120	as MP180 but *katE*::Tn10	Loewen *et al.* (1985)
UM122	as MP180 but *katF*::Tn10	Loewen *et al.* (1985)
UM202	as MP180 but *katG17*::Tn10	Loewen *et al.* (1985)
UM255	*pro, leu, rpsL, hsdM, hsdR endI, lacY, katG2, katE12*::Tn10 *recA*	Mulvey *et al.* (1988)
UM258	*pro, leu, rpsL, hsdM, hsdR endI, lacY, katG2* *katF13*::Tn10, *recA*	Mulvey *et al.* (1988)

Table 3.2 Bacterial Plasmids and Bacteriophage

Plasmid	Characteristics	Source
pAT153	Ap^R Tc^R	Twigg & Sherratt (1980)
pKK232-8	Ap^R Cm^S	Brosius (1984)
Bluescript KS M13+	Ap^R	Stratagene Cloning Systems
Bluescript KS M13-	Ap^R	Stratagene Cloning Systems
Bluescript SK M13+	Ap^R	Stratagene Cloning Systems
Bluescript SK M13-	Ap^R	Stratagene Cloning Systems
pAMkatE2	Ap^R	5.6 kb *Hin*dIII fragment from λkatE6 in pAT153
pAMkatE6	Ap^R	4.8 kb *Cla*I fragment from λkatE6 in pAT153
pAMkatE22	Ap^R	4.0 kb *Cla*I fragment from pAMkatE2 in pAT153
pAMkatE72	Ap^R	3.1 kb *Pst*I-*Cla*I fragment from pAMkatE22 in Bluescript KSM13+

Bacteriophage	Source
λEMBL3	Frischauf *et al.* (1983)
λkatE6	16 kb fragment from MP180 in λEMBL3

3.2.3 **M9 Minimal Medium** (Miller, 1972)

0.5 g NaCl

6.0 g Na_2HPO_4

1.0 g NH_4Cl

3.0 g KH_2PO_4

in 1.0 litre distilled H_2O. Solid media prepared with addition of 10.0 g agar.

Supplemented after atuoclaving with 3 μM vitamin B1, 1.0 mM $MgSO_4$ and 1.0 ml trace elements.

Trace Elements

2.5 g $FeSO_4 \cdot H_2O$

2.9 g H_3BO_4

1.2 g $CoSO_4 \cdot 7H_2O$

0.1 g $CuSO_4 \cdot 5H_2O$

0.09 g $MnCl_2 \cdot 4H_2O$

2.5 g $Na_2MoO_4 \cdot 2H_2O$

2.1 g $ZnSO_4 \cdot 7H_2O$

5.0 ml conc. H_2SO_4

in 1.0 liter distilled H_2O.

Glucose minimal medium prepared as M9 minimal medium supplemented with 16.7 mM glucose after autoclaving.

3.2.4 **K Medium** (Rupp *et al.*, 1971)

M9 minimal medium with 1.0% (w/v) casamino acids. Supplemented after autoclaving with 0.3 μM vitamin B1 and 16.6 mM glucose.

3.2.5 **Hershey's Salts** (Worcel and Burgi, 1974)

5.4 g NaCl

3.0 g KCl

1.1 g NH_4Cl

0.2 mg $MgCl_2 \cdot 6H_2O$

87 mg KH_2PO_4

12.1 g Trizma Base

in 2.0 litre distilled H_2O, pH adjusted to 7.4 with HCl.

Supplemented after autoclaving with

15 mg $CaCl_2 \cdot 2H_2O$

0.2 mg $FeCl_3 \cdot 6H_2O$

Hershey's medium prepared with addition of 3 mM vitamin B1, 22.2 mM glucose and 0.16 mM amino acids as required, after autoclaving.

3.3 Solutions and Buffers

3.3.1 **SM Buffer** (Miller, 1972)

0.02 M Tris-HCl pH 7.5

0.01 M $MgSO_4$

0.01% gelatin

0.01 M NaCl

autoclaved.

3.3.2 **TE Buffer** (Maniatis *et al.*, 1982)

10.0 mM Tris-HCl pH 8.0

1.0 mM EDTA pH 8.0

autoclaved.

3.3.3 **Ethanol**

95% ethanol was distilled before use.

3.3.4 Phenol

All phenol was redistilled and buffered with

0.1 M NaCl

0.1 M Tris-HCl pH 7.6

1.0 mM EDTA

3.3.5 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 PEG8000

3.3.6 GTE Buffer (Morelle, 1989)

50 mM glucose

25 mM Tris-HCl pH 8.0

10 mM EDTA

3.3.7 STE Buffer

100 mM NaCl

20 mM Tris-HCl pH 7.5

10 mM EDTA

3.3.8 TBE Buffer (10x)

108 g Trizma base

55 g boric acid

8.5 g EDTA

in 1.0 litre distilled H_2O.

3.3.9 TAE Buffer (10x)

48.4 g Trizma base

11.4 ml glacial acetic acid

3.72 g EDTA

in 1.0 litre distilled H_2O

3.3.10 Tracking Dye

200 mM EDTA

35% glycerol

25 mg Bromophenol Blue

in 10 ml distilled H_2O.

3.3.11 Denaturing Solution

1.5 M NaCl

0.5 M NaOH

3.3.12 Neutralizing Solution

1.0 M Tris-HCl pH 8.0

1.5 M NaCl

3.3.13 20x SSC

3.0 M NaCl

3.0 M sodium citrate

3.3.14 5x Nick Translation Buffer

0.1 mM dCTP

0.1 mM dGTP

0.1 mM dTTP

0.05 mM $MgCl_2$

0.5 mM DTT

0.25M Tris-HCl pH 7.6

250 μ/ml BSA

in HPLC grade H_2O.

3.3.15 50x Denhardt's Solution (Maniatis *et al.*, 1982)

10.0 g Ficoll

10.0 g polyvinylpyrrolidone

10.0 g BSA

in 1.0 litre distilled H_2O.

3.3.16 Prehybridization Solution (Maniatis *et al.*, 1982)

6x SSC

0.5% SDS

5x Denhardt's solution

100 mg/ml salmon sperm DNA

Hybridization Solution

As prehybridization solution with addition of 0.01 M EDTA.

3.3.17 BAL 31 Incubation Buffer

20 mM Tris-HCl pH 8.0

600 mM NaCl

12 mM $MgCl_2$

12 mM $CaCl_2$

1 mM EDTA

3.3.18 Cell Extract Sample Buffer

2% SDS

8% glycerol

0.2 M MSH

63 mM Tris-HCl pH 6.8

3.4 Bacterial Growth and Storage

Precultures were prepared by inoculating 10 ml LB broth with a loop of glycerol stock culture or from a storage plate culture. Cultures for use were inoculated with 1% of volume of preculture. Liquid cultures were grown at 37°C with aeration, solid cultures at 37°C, inverted.

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

Cultures were stored at -20°C in 50% glycerol.

3.5 Preparation of Bacteriophage DNA

Bacteriophage DNA was prepared by the method of Kaslow (Kaslow, 1986) with the following change. 1.0 litre flasks containing infected cells were incubated for a minimum of 8-1/2 hours at 37°C with vigorous shaking rather than 4-1/2 hours.

3.6 Restriction Enzymes and Buffers

Restriction enzymes were obtained from Boehringer Mannheim, Pharmacia, or BRL. Restriction enzyme buffers used were as supplied by Boehringer Mannheim and BRL.

Restriction enzyme digestions were carried out at 37°C for a minimum of 1 hour, with the exceptions of *Sma*I and *Taq*I which were done at 30°C and 65°C respectively.

3.7 Agarose and Polyacrylamide Gel Electrophoresis of DNA

3.7.1 Agarose Gels

0.7% (w/v) agarose gels were prepared in 1x TBE or TAE buffer for separation and identification of DNA fragments as small as 2.0 kb.

1.2% (w/v) agarose gels were prepared in 1x TBE or TAE buffer for separation and identification of DNA fragments as small as 200 base pairs.

Tracking dye was added to DNA samples to be electrophoresed to a final concentration of 12%. Gels were run in a BIO-RAD DNA SUB CELL electrophoresis apparatus submerged in 1x TBE or TAE buffer. Gels were run at 80V constant voltage for 4-6 hours or 30V constant voltage for 15-18 hours.

3.7.2 Visualization of DNA on Polyacrylamide Gels

8% polyacrylamide gels were used for separation and identification of DNA fragments as small as 25 base pairs.

20 ml 30% acrylamide

30 ml 1x TBE

Degas

20 μl TEMED

30 mg ammonium persulphate

The gel was cast between 15x15 cm vertical glass plates. DNA samples containing 12% tracking dye solution were loaded in the buffer-washed wells. Gels were run in a BIO-RAD Protean Dual Slab Cell at 100V constant voltage for four hours in 1x TBE.

3.7.3 Characterization of Gels

Gels were immersed in 1x TBE or TAE buffer containing 0.5 mg/l ethidium bromide for 20 minutes after electrophoresis. Gels were

photographed under indirect UV light source with a Polaroid MP4 Land Camera and a Kodak 22A Wratten filter and Polaroid type 667 film. DNA fragment sizes were determined from the photograph by construction of a standard curve. The standard curve was plotted with the distance travelled by the standard size markers as a function of the natural logarithm (ln) of their size (base pairs). Standard markers used were λDNA cut with *Hin*dIII for larger fragments and pBR322 DNA cut with *Hae*III for small fragments.

3.8 Preparation of Plasmid DNA

3.8.1 Large Scale Plasmid DNA Preparation

E. coli containing the required plasmid was grown in 500 ml LB broth containing 50 mg ampicillin at 37°C overnight with aeration. The cells were collected in 250 ml aliquots by centrifugation and washed with 20 ml of GTE buffer. The cells were again collected by centrifugation and resuspended in 7 ml GTE. GTE, 1.0 ml with 4 mg/ml lysozyme was added and the suspension was incubated at room temperature for 5 min. To this 16 ml of 0.2 M NaOH - 1% SDS was added and incubation continued, on ice, for 10 min. To precipitate high molecular weight proteins, genomic DNA, and cellular DNA 12 ml of 7.5 M ammonium acetate was added. After 15 min of incubation on ice the preparation was centrifuged at 5,000 rpm for 30 min. The supernatant was transferred to smaller tubes and centrifuged for 3 min at 12,000 rpm. To the clear supernatant 24 ml of isopropanol was added and incubated for 15 min at room temperature. The DNA was collected by centrifugation at 5,000 rpm for 30 min and washed twice with 70% ethanol. The dried pellet was resuspended in 12 ml of H_2O and 12 g of CsCl and 0.5 ml of 10 mg/ml ethidium bromide were added. The mixture was centrifuged at 8,000 rpm

for 10 min to remove protein-ethidium bromide complexes. The supernatant was transferred to 16x76 mm polyallomer Quick-Seal (Beckman) ultracentrifuge tubes and carefully balanced. The tubes were placed in an 80Ti rotor and centrifuged at 50,000 rpm for 20 hrs in a Beckman L8-80 ultracentrifuge. The tubes were removed and the lower plasmid band was collected with an 18 gauge needle. The ethidium bromide was extracted with isoamyl alcohol. The clear DNA solution was dialyzed overnight in 2-3 changes of TE buffer.

The desalted solution was precipitated with ethanol, washed and dried. The pellet was resuspended in sterile distilled H_2O and stored at -20°C. DNA concentration was estimated by applying a small sample to an agarose gel along with a standard marker of known concentration and comparing the intensities under UV illumination.

3.8.2 Mini Plasmid DNA Preparation

Small quantities of plasmid DNA were prepared as described by Holmes *et al* (1981) unchanged, or Morelle (1989) with the following alteration. The mixture was centrifuged for 15 min after addition of 7.5 M ammonium acetate. The supernatant was transferred to a clean tube and the centrifugation was repeated twice more. The DNA was then precipitated by addition of an equal volume of isopropanol.

3.8.3 Quick Screening of Plasmid DNA

A rapid isolation of plasmid DNA was done to compare supercoiled plasmids in order to determine the presence of an insert or deletion. The method consisted of scraping the *E. coli* colony from the LB plate with a toothpick and resuspending the cells in 40 μl STE buffer. 40 μl of 1:1 phenol/chloroform was added and the tube vortexed for 10 sec then centrifuged for 2 min. Fifteen μl of the aqueous phase was mixed with 3

μl of tracking dye and the preparation was applied to an agarose gel for electrophoresis. This preparation did not yield plasmid DNA of quality for restriction enzyme digestion.

3.9 DNA Cloning

3.9.1 Extraction of DNA Fragments from Agarose Gels

The DNA band required was cut from the ethidium bromide stained gel under UV illumination. The DNA was eluted from the agarose as described in the GENECLEAN kit (BIO 101 Inc.). A NaI solution was used to dissolve the agarose. The DNA was attached to a silica matrix, washed, and eluted from the matrix in H_2O.

3.9.2 Ligations

Plasmid DNA was digested with the appropriate restriction enzymes and precipitated with ethanol, or the enzymes were heat inactivated at 65°C for 10-20 minutes. Plasmid DNA and isolated insert DNA were mixed at a 1:2 ratio in 1x BRL ligation buffer. One unit of T4 DNA ligase (BRL) was added to each reaction. Ligations were incubated for 2-4 hours at room temperature.

3.9.3 Transformation of *E. coli*

A 10 ml mid-log phase culture of *E. coli* grown in LB broth was collected by centrifugation. The cells were washed with 0.1 M $CaCl_2$ and placed on ice for 20 min. The cells were again collected by centrifugation and resuspended in 0.5 ml 0.1 M $CaCl_2$. Aliquots of cells, 0.1 ml, were mixed with the DNA to be transformed and placed on ice for 45 min. The cells were placed at 42°C for 1-1/2 min and diluted with 0.3 ml of LB broth. The cells were allowed to recover for 1 hour and plated on selection plates in 0.2 ml aliquots.

3.9.4 Selection of Recombinants

3.9.4.1 pAT153 Recombinants

The vector pAT153 is 3.6 kb in size and has genes conferring resistance to the antibiotics tetracycline and ampicillin. These genes contain unique restriction enzyme sites into which DNA fragments can be inserted, interupting the expression of the gene, resulting in a selection characteristic. Fragments were cloned into the various sites within the antibiotic resistance genes. The resulting UM255 transformants were selected for on LB-ampicillin or LB-tetracycline plates. The colonies were streaked with sterile toothpicks onto replica plates, LB-ampicillin and LB-tetracycline. Recombinants were scored by the inability to grow on both antibiotics and selected colonies were recovered from the replica plate. These colonies were then screened for catalase activity to determine whether the fragment contained the entire *katE* gene. This was done by applying a drop of H_2O_2 to the edge of the colony which resulted in the evolution of oxygen bubbles, if catalase was present.

3.9.4.2 pKK232-8 Recombinants

The pKK232-8 vector was used to determine which part of the insert containing *katE* contained the promoter. This vector contains a gene conferring ampicillin resistance as well as a promoterless CAT gene flanked by a multiple cloning site (Brosius, 1984). Various fragments from *katE2* plasmids were cloned into the *Hin*dIII site of pKK232-8 in both orientations. The transformants were selected on LB-ampicillin plates and recombinants scored for their ability to grow on increasing concentrations of chloramphenicol indicating the presence of the *katE* promoter.

3.9.4.3 Bluescript M13 Recombinants

The Bluescript M13 phagemids are 3.0 kb and contain a selection gene conferring ampicillin resistance and a *lacZ* gene, flanked by a multiple cloning site, for chromophore production as a scorable marker for recombinants. Transformants were selected on LB-ampicillin and resulting colonies streaked on LB-ampicillin spread with the chromophore X-Gal and the inducer IPTG. Recombinants remain white in color while nonrecombinants, with the uninterrupted *lacZ* gene, are blue. Recombinant phagemid DNA was isolated by the method of Morelle (1989) and inserts were cut out with suitable restriction enzymes to verify their identity. Four derivatives of the phagemid were utilized to produce subclones of *katE* for use in single-stranded dideoxy-chain termination sequencing. Bluescript KS+ and KS- contain the multiple cloning site in the *Kpn*I and *Sac*I orientation and secrete opposite strands in the preparation of single-stranded DNA. Bluescript SK+ and SK- contain the multiple cloning site in the alternate orientation and secrete opposite strands.

3.10 DNA/DNA Hybridization

3.10.1 Southern Blots

Southern blots were prepared as described by Maniatis *et al.* (1982). DNA samples were electrophoresed, stained and photographed as before. The gels were trimmed to a suitable size and placed in denaturing solution for 1 hr at room temperature. The gel was then placed in neutralizing solution for 1 hr. The gel was placed on a piece of filter paper soaked in 10x SSC which was placed on a glass plate held above a solution of 10x SSC with the ends of the filter paper in the buffer. A piece of nitrocellulose filter, prewetted in 2x SSC, or nylon filter was placed on the gel. This was covered with 2-3 layers of

filter paper of similar size. A stack of paper towels was placed on top of this and weighted to increase efficiency of transfer of buffer through the gel to the towels. The transfer of the DNA from the agarose gel to the filter via capillary action was carried out overnight. The nitrocellulose filters were air dried then baked at 80°C for 2 hrs. Nylon filters were removed from the blotting apparatus, immediately wrapped in Saran Wrap to prevent drying, and exposed to UV light for 3 minutes on a transilluminator to cross-link the DNA to the filter.

3.10.2 Nick Translation

Radioactive labelled DNA probes were prepared by Maniatis *et al.* (1982). In a final volume of 50 μl a solution containing 1 μg of DNA, 1x nick translation buffer, 5-10 units of DNA polymerase, 1 μl (0.1 μl/ml) of DNAse I and 5 μl (1 mCi/ml) of α^{32}P-dATP was incubated at 16°C for 1 hr.

The reaction mixture was loaded on a 5 ml Sephadex G-50 column, prepared in TE buffer (pH 8.0) to separate the labelled DNA from the unincorporated nucleotides. The passage of the radioactive signal through the column was monitored with a Geiger counter as TE buffer (pH 8.0) was applied to the column. When the signal was separated into two peaks, the first peak containing the labelled DNA and the second peak containing the free nucleotides, the first peak was collected in a volume of 1.5 ml and a fraction was assayed for specific activity in a Beckman LS-230 liquid scintillation counter. The labelled DNA was stored at -20°C.

3.10.3 Hybridization

The nitrocellulose Southern blots were first wetted and soaked in 6x SSC for 2 min. While nylon filters were placed directly in 50 ml of prehybridizaiton solution. The filters were prehybridized at 65°C for

2-4 hrs to allow the salmon sperm DNA to bind nonspecific sequences. The prehybridization solution was removed and replaced with 50 ml of hybridization solution. The α^{32}P-labelled DNA was boiled to denature it and a volume containing 6 x 10^6 cpm was added to the hybridization solution. The hybridization was incubated at 65°C overnight. The filters were removed from the solution and washed with 2x SSC and 0.5% SDS at room temperature for 5 min, 2x SSC and 0.1% SDS at room temperature for 15 min, and 0.1x SSC and 0.5% SDS for 2 hrs at 65°C (repeated). The filters were air dried, wrapped in Saran Wrap and exposed to Kodak x-ray film in a Picker Source One cassette containing one Cronex Quantaill intensifying screen (DuPont) at -70°C for 8-72 hrs.

3.11 BAL 31 Deletion Mutagenesis (Frey *et al.* 1984)

BAL 31 is an exonuclease with the capacity for degrading double stranded DNA. It can be used to delineate the functional region of a fragment of DNA containing a gene of interest. Exonuclease BAL 31 digestion was done on pAMkatE6 initiating at a unique SalI site.

Ten μg of pAMkatE6 DNA was digested with *SalI* in a 40 μl sample volume. A 3 μl sample was electrophoresed on an agarose gel to determine complete digestion. The remaining DNA was precipitated by addition of 0.1 volumes of 3M sodium acetate pH 4.8 and 30 μl isopropanol. The DNA was collected by centrifugation, washed with 80% ethanol and dried.

The DNA pellet was dissolved in 125 μl BAL 31 incubation buffer and warmed to 30°C before adding the enzyme. Samples of 25 μl were taken after 0, 2, 5, 10 and 15 minute intervals of enzyme digestion and immediately placed in 5 μl of 0.2M EDTA, on ice, and 80 μl of ice cold H_2O was added. Samples were precipitated as before and redissolved in 10 μl TE buffer.

Aliquots of 5 μl from time intervals 0 and 15 minutes were electrophoresed on an agarose gel to verify BAL 31 activity. Ligations were carried out as previously described but at 14°C and were diluted with 90 μl of ligation buffer at 2 hours to prevent intermolecular ligation. The ligations were transformed into UM255 and selected on LB-ampicillin. Colonies were screened for catalase activity by H_2O_2 application and catalase negative colonies were recovered for further analysis. Plasmid DNA was prepared by the method of Holmes *et al.* (1981) and restriction enzyme analysis was done to compare the extent of deletion between catalase positive and negative clones.

3.12 Maxicell Identification of Plasmid Encoded Proteins

The proteins produced from the plasmid pAMkatE6 were identified in maxicell extracts as described by Sancar *et al.* (1979) with modifications. The *E. coli* strain UM255, containing the plasmid pAMkatE6 or pAT153 was grown to early mid-log phase in K medium with ampicillin. The cells were irradiated in a sterile Petri dish with a Mineralight 115v UV lamp from a distance of 54 cm for 140 sec. This resulted in >99% killing.

The irradiated cells were placed in a sterile 125 ml Erlenmeyer flask and shaken at 37°C for 1 hr. Cycloserine was dissolved at 100 mg/ml in 0.1 M phosphate buffer pH 8.0 and added to the cells at a final concentration of 100 μg/ml. The cells were incubated at 37°C with shaking overnight. The cells were collected by centrifugation and washed twice with Hershey's salts. The final pellet was resuspended in 5.0 ml Hershey's Medium and 15 μCi of ^{14}C-labelled mixed amino acids (NEN) was added. The culture was incubated at 37°C for 1.5 hrs with shaking. The cells were collected by centrifugation and washed with SM buffer. The

cells were resuspended in 40 μl cell extract sample buffer and placed at -20°C for storage.

A 15 μl aliquot of each sample was mixed with 2 μl bromophenol blue (0.25%) tracking dye and boiled for 3 min. The samples were loaded on a 0.1% SDS - 8% polyacrylamide gel and electrophoresed at 40 mA for 4 hrs, stained with Coomassie Brilliant Blue and destained until suitable resolution of bands resulted. The gel was prepared for fluorography as described by Boulnois and Timmis (1984). After destaining the gel was immersed in DMSO and gently agitated for 30 min. The DMSO was replaced with fresh DMSO to ensure removal of all water from the gel and agitated for another 30 min. The DMSO was then replaced with a solution of 20% PPO (w/v) in DMSO and soaked with agitation for 1 hr. This solution was drained and the gel washed several times with water, very gently, to precipitate the PPO within the gel. The gel was removed from the water and dried onto filter paper. The dried gel was exposed to Kodak X-ray film in a Picker Source One cassette containing two Cronex Quantaill intensifying screens (DuPont) at -70°C for 3 weeks.

3.13 Assay of Catalase Activity

3.13.1 Qualitative Colony Catalase Assay

A drop of 30% H_2O_2 was applied with a high gauge needle to the edge of a bacterial colony. Cells containing catalase evolved oxygen which appeared as bubbles.

3.13.2 Quantitation of Catalase Activity

Catalase activity was quantitated using cultures in liquid medium by the method of Rorth and Jensen (1967) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is that amount which decomposes 1 μmol of H_2O_2 in 1 min at 37°C. Assays were done using a

final concentration of H_2O_2 in the reaction chamber of 60 mM. Dry cell weight was determined as 100 Klett units representing 0.14 mg/ml dry cell weight.

3.13.3 Visualization of Catalase Activity on Polyacrylamide Gels

Crude extracts were prepared with cells collected by centrifugation from 250 ml stationary phase cultures. The cells were resuspended in 1 ml of SM and sonicated for four 30 sec cycles incubating the suspension on ice between cycles. Cell debris was removed by centrifugation. Aliquots of the cell free extract were mixed with glycerol and bromophenol blue dye and loaded onto an 8.5% acrylamide non-denaturing gel. The gels were run at 25 mA until the dye reached the bottom.

The gels were soaked for 45 min in 50 mM potassium phosphate pH 7.0 containing 0.4 mg/ml of diaminobenzidine hydrochloride and 0.05 mg/ml of horseradish peroxide. The gel was rinsed with water and soaked for 2 hrs in a solution of 20 mM H_2O_2 in 50 mM potassium phosphate pH 7.6. Catalase bands appeared white on a brown background.

RESULTS

4. Results

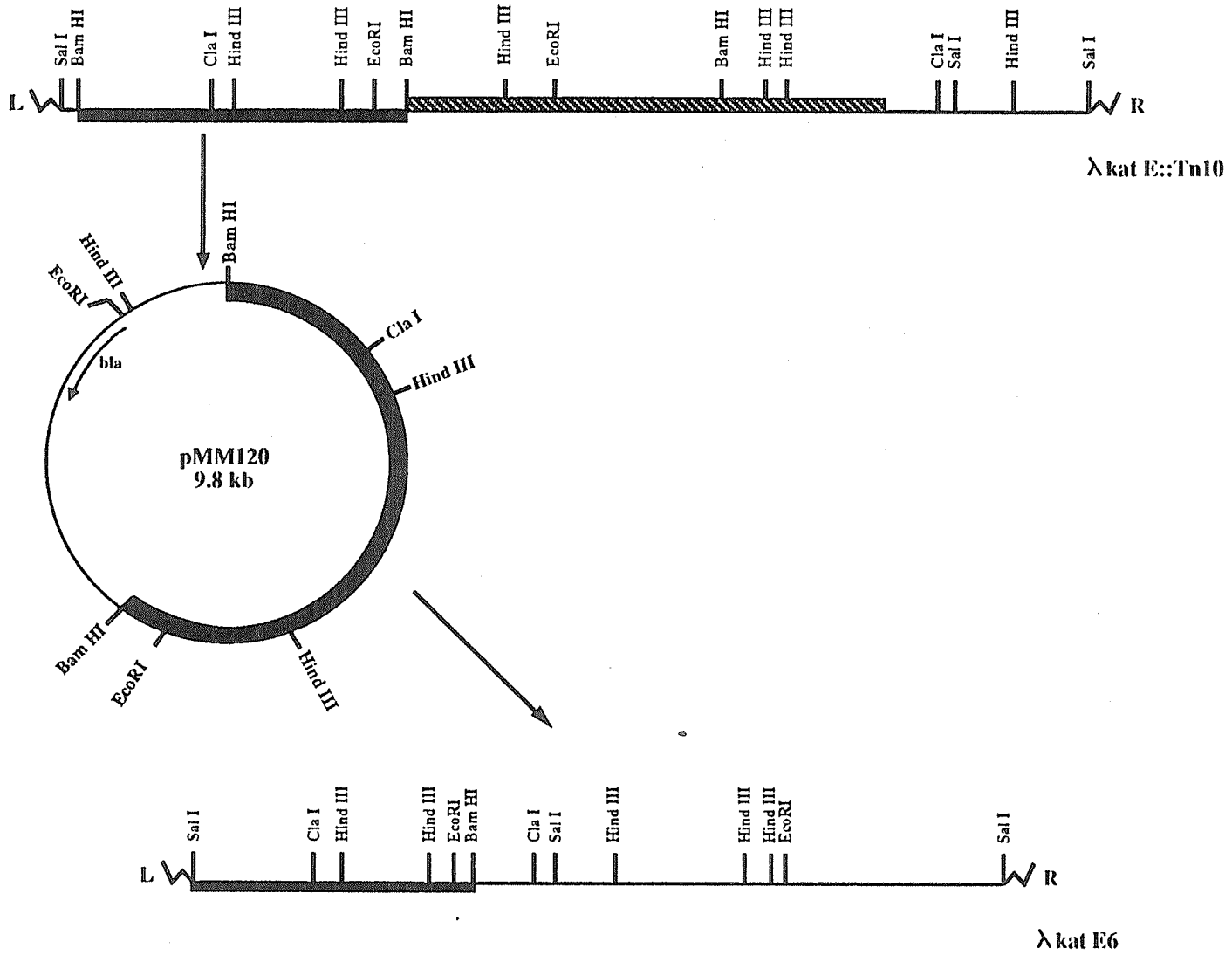
4.1 Cloning of *katE*

Due to the location of restriction enzyme sites within the gene, initial attempts to clone *katE* directly from wild-type genomic DNA digested with *Hin*dIII, *Bam*HI and *Eco*RI were unsuccessful. A protocol was designed in which the gene could be isolated through identification of the transposon Tn10 inserted in UM120. The transposon was known to have inserted in the chromosome at 37.8 min resulting in inactivation of *katE* expression (Loewen, 1984). Genomic DNA from UM120 partially digested with *Sau*IIIA was inserted into λEMBL3 to prepare a library. This library was screened with the Tn10 containing plasmid pBT107 (Moyed *et al.*, 1983). A Tn10 containing phage, λkatE::Tn10, was isolated and phage DNA prepared. The region of DNA flanking the transposon was isolated and subcloned into pAT153 resulting in pMM120. This plasmid was used to identify λkatE6, an EMBL3 clone from a wild-type *E. coli* genomic library (Fig. 4.1).

4.2 Subcloning of *katE*

Restriction analysis of λkatE::Tn10 revealed that the transposon Tn10 in UM120 was inserted near a *Bam*HI site. Because this insertion caused inactivation of *katE*, it was concluded that the *Bam*HI site lay within the gene. Restriction digests of λkatE6 using enzymes *Cla*I, *Pvu*II, *Sal*I, *Eco*RI, *Hin*dIII and *Eco*RV were done individually and in double enzyme digests with *Bam*HI. DNA bands from the single enzyme digests that were seen to be cleaved with *Bam*HI in the double digests were isolated from the agarose gels and cloned into compatible restriction sites of the high copy number vector pAT153. Plasmids were

Figure 4.1 Strategy for the cloning of *katE*. The UM120 λ clone obtained with pBT107 showing the transposon (hatched area) and the adjacent fragment isolated in pMM120 (solid) which was used to probe the MP180 library to obtain λkatE6 containing *katE*.


Sal I
Bam HI
Cla I
Hind III
Hind III
EcoRI
Bam HI
Hind III
EcoRI
Bam HI
Hind III
Hind III
Cla I
Sal I
Hind III
Sal I
L
R
λkat E::Tn10
Bam HI
Hind III
EcoRI
bla
Cla I
Hind III
pMM120
9.8 kb
Bam HI
EcoRI
Hind III
Sal I
Cla I
Hind III
Hind III
EcoRI
Bam HI
Cla I
Sal I
Hind III
Hind III
Hind III
EcoRI
Sal I
L
R
λkat E6

determined to carry an inserted fragment by scoring transformants for tetracycline sensitivity and observing Rapid plasmid DNA isolations on agarose gels. All plasmids containing inserts were transformed into UM255 to test for complementation of the katE12::Tn10. Catalase activity was scored by applying 30% H_2O_2 to transformant colonies.

Two catalase positive clones were identified (Fig. 4.2). The plasmid pAMkatE2 contained a 5.6 kb fragment generated from a partial *Hin*dIII digest inserted into the HindIII site of pAT153 to produce pAMkatE6. A 4.0 kb region was common to both pAMkatE2 and pAMkatE6. This region was obtained using the *Cla*I site in the vector portion of pAMkatE2 and a *Cla*I site in the insert. The plasmids pAMkatE22 and pAMkatE24 contain the 4.0 kb *Cla*I fragment in opposite orientations.

4.3 Identification of *katE* Clones

4.3.1 Complementation by pAMkatE6

Qualitative determination of catalase activity was used to select pAMkatE6. Complementation analysis of pAMkatE6 was subsequently done quantitatively. The plasmid was transformed into MP180 (wild-type), UM120 (*katE*::Tn10), UM122 (*katF*::Tn10) and UM202 (*katG*::Tn10). Cultures of transformed and nontransformed strains were grown to mid-log phase and stationary phase. Cell density was measured to ensure equivalent cell growth in all comparisons.

All transformed strains showed slightly elevated catalase levels in early mid-log phase. In stationary phase MP180 [pAMkatE6] showed catalase levels 25 fold higher than non-transformed MP180, UM120 [pAMkatE6] showed a 94 fold increase and UM202 [pAMkatE6] a 35 fold increase in activity. UM122 [pAMkatE6] showed a slightly lower level of activity (Table 4.1).

Figure 4.2 Isolation of *katE* plasmids. pAMkatE6 was obtained with a *Cla*I fragment from λkatE6 and pAMkatE2 with a *Hin*dIII fragment generated from a partial digest of λkatE2. The pAMkatE22 plasmids were constructed to contain that portion of DNA common to both pAMkatE6 and pAMkatE2 (solid lines) contained within the boundaries of a *Cla*I site in the insert and a *Cla*I site from pAT153.

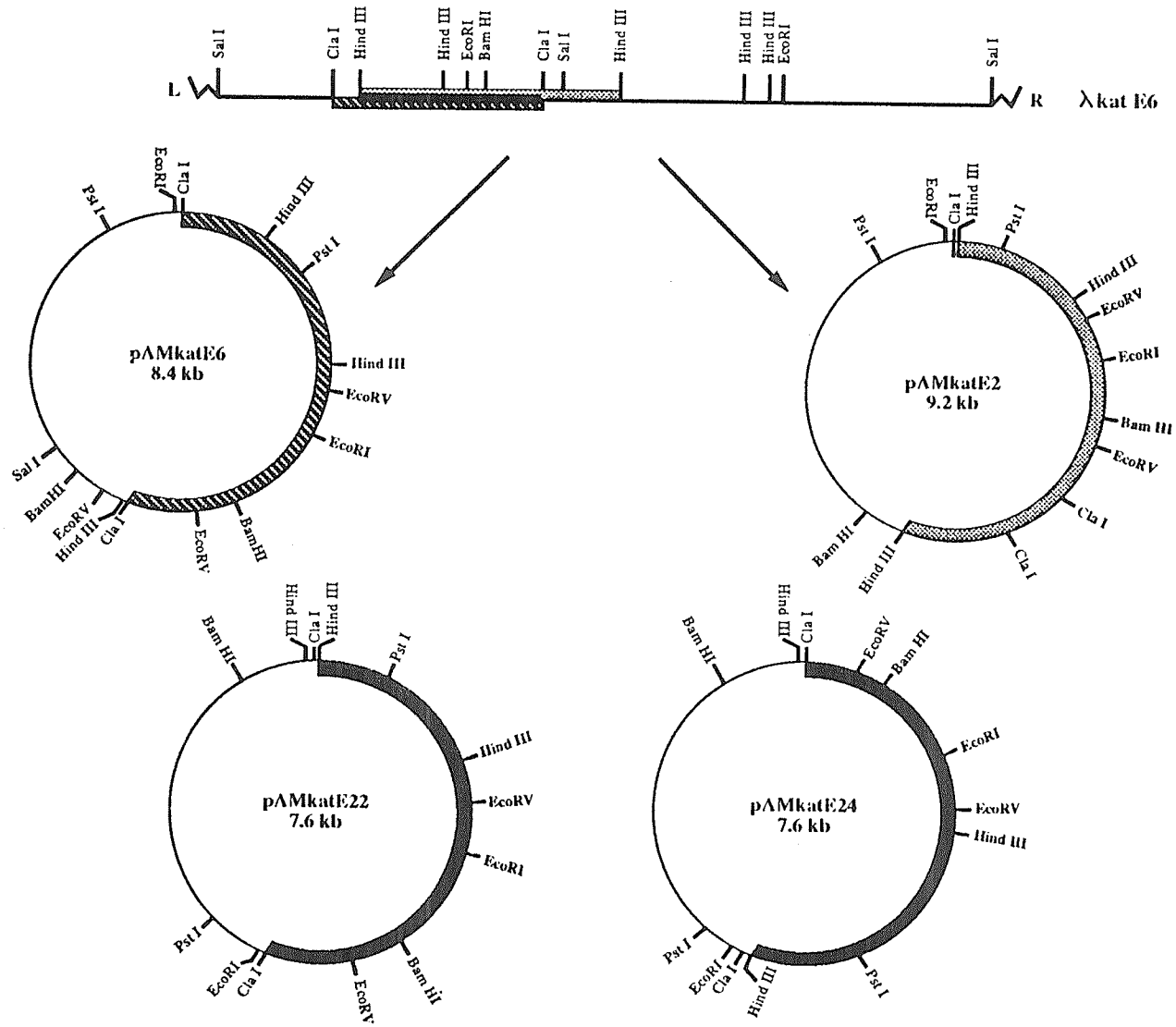

L
Sal I
Cla I
Hind III
Hind III
EcoRI
Bam HI
Cla I
Sal I
Hind III
Hind III
Hind III
EcoRI
Sal I
R
λkat E6
pΛMkatE6
8.4 kb
pΛMkatE2
9.2 kb
pΛMkatE22
7.6 kb
pΛMkatE24
7.6 kb

Table 4.1 Catalase Levels

Strain	Catalase (units) mg dry cell weight)	
	Mid-log phase	Stationary Phase
MP180 (wild type)	5.7	58.8
MP180[pAMkatE6]	13.1	1478.0
UM120(*katE*::Tn10)	8.8	17.3
UM120[pAMkatE6]	11.3	1603.0
UM122(*katF*::Tn10)	4.3	17.8
UM122[pAMkatE6]	5.3	12.4
UM202(*katG*::Tn10)	2.0	33.7
UM202[pAMkatE6]	3.9	1060.0

4.3.2 Visualization of HPII

Having transformed pAMkatE6 into MP180 (wild-type), UM120 (katE::Tn10), UM122 (katF:Tn10) and UM202 (katG::Tn10) it was possible to visualize catalase activities following gel electrophoresis of extracts. Cell extracts were prepared from transformed and nontransformed cells at mid-log and stationary phase. The extracts were run on nondenaturing polyacrylamide gels and stained for catalase activity. The gels from mid-log and stationary phase revealed similar patterns of catalase activity except that the samples from mid-log phase showed relatively less expression of HPII. In MP180 [pAMkatE6] the greater portion of activity was due to the upper band, HPII, while the lower doublet, HPI, showed greater amounts in MP180. The HPI doublet was the only catalase present in UM120 but UM120 [pAMkatE6] showed an intense upper HPII band. Extracts of UM122 contained a greater proportion of HPI relative to HPII but UM122 [pAMkatE6] contained approximately equivalent amounts of HPI and HPII, UM202 and UM202 [pAMkatE6] both produced only one source of catalase activity, an intense upper band of HPII (Fig. 4.3).

4.3.3 Maxicell Analysis of pAMkatE6

Proteins encoded by pAMkatE6 were analyzed using the maxicell procedure of Sancar *et al.* using strain UM255. Maxicell extracts of UM255, UM255 [pAT153] and UM255 [pAMkatE6] were run on SDS-PAGE and labelled protein bands were located by autoradiography giving rise to the autoradiogram shown in Fig. 4.4. No bands are present in the extract of UM255 except for cellular debris at the bottom of the gel. The pattern of proteins in extracts of UM255 [pAT153] was consistent with the pattern of proteins expected from pAT153. The proteins involved in

Figure 4.3 Visualization of catalase activities following electrophoresis on an 8.5% acrylamide non-denaturing gel. Extracts of various strains with (+) and without (-) pAMkatE6, grown to stationary phase.

A

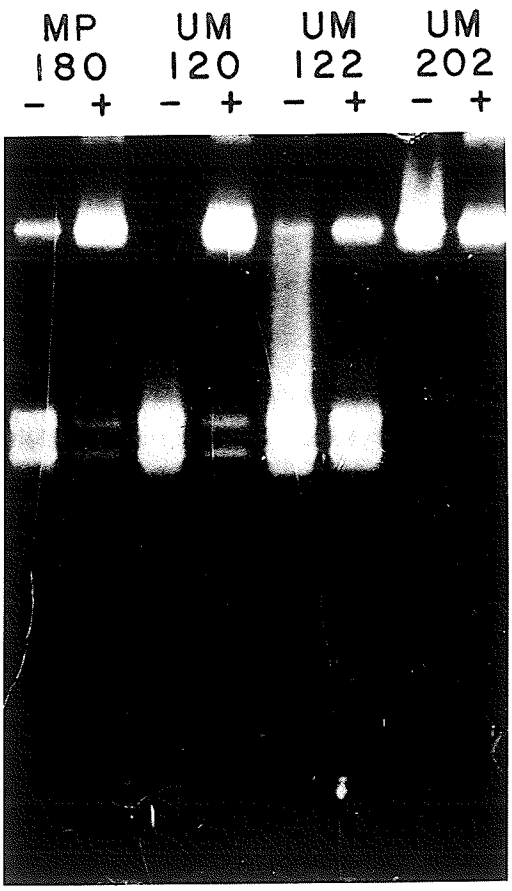
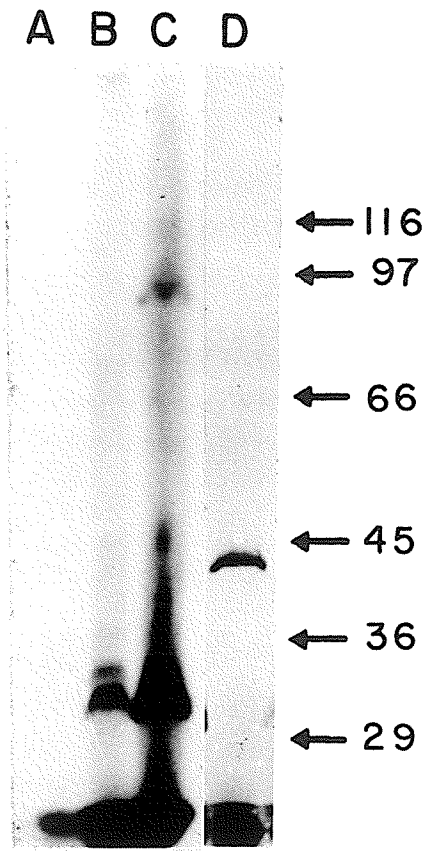


Figure 4.4 Maxicell analysis of plasmid-encoded proteins. Lanes: A, UM255; B, UM255[pAT153]; C, UM255[pAMkatE6]; D, UM258[pMMkatF2]. The numbers along the side indicate the locations of molecular weight standards ($x10^3$).


A B C D
116
97
66
45
36
29

ampicillin resistance appear as a doublet at 31,000 and 28,000 daltons and the protein involved in the tetracycline resistance is approximately 37,000 daltons but is produced in small amounts and is very faint in the autoradiogram. The UM255 [pAMkatE6] proteins include the ampicillin resistance doublet and a protein with molecular weight of 93,000 daltons, the size of the HPII subunit.

4.4 Confirmation of Chromosomal Location of Tn10 in *katE*

The transposon Tn10 was originally utilized to map the chromosomal location of *katE* (Loewen, 1984), and to construct HPII deficient *E. coli* strains. The following protocol was used to confirm that the *katE* clones were from the same region of the chromosome as the Tn10 insertion in UM120 genomic DNA. Genomic DNA from MP180 and UM120 was digested with *Cla*I and digests were electrophoresed on agarose gels for blotting to nylon filter. The blots were then probed with ^{32}P-labelled pAMkatE6. The patterns obtained are shown in Fig. 4.5. The MP180 genomic DNA shows a fragment hybridized to pAMkatE6 which contains a 4.8 kb *Cla*I insert obtained from the wild-type library. The hybridization pattern of UM120 shows two bands. These fragment sizes correspond to the Tn10 insertion near the *Bam*HI site in UM120 as described in fig. 4.6 and contain both Tn10 and chromosomal DNA.

4.5 Characterization of HPII Production and Activity

E. coli produces two catalases HPI and HPII which are independently regulated. The HPI protein produced by *katG* is believed to be regulated by the *oxyR* regulon (Christman *et al.*, 1985). HPI levels increase gradually during logarithmic growth but do not increase into stationary phase (Loewen *et al.* 1985). HPII synthesis is affected by *katE* and *katF* with *katE* being the structural gene and *katF* being required for *katE*

Figure 4.5 Autoradiogram of the change in hybridization pattern when genomic DNA with or without a transposon Tn10 insertion in *katE* was digested with *Cla*I and probed with α^{32}P-labelled pAMkatE6. Lanes: A, MP180 genomic DNA digested with *Cla*I; B, UM120(*katE*::Tn10) genomic DNA digested with *Cla*I. The numbers along the side indicate the sizes in kb of fragments generated by the digestion of λDNA with *Hin*dIII.

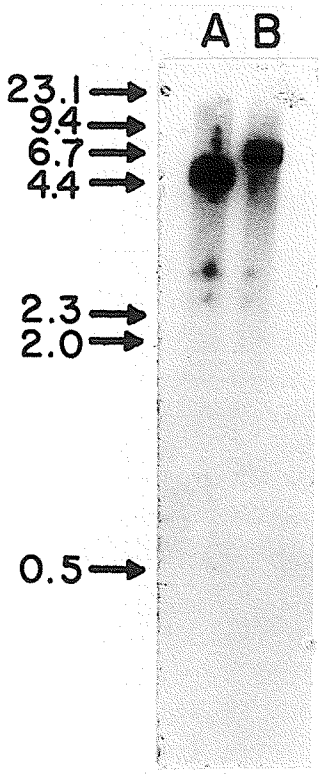
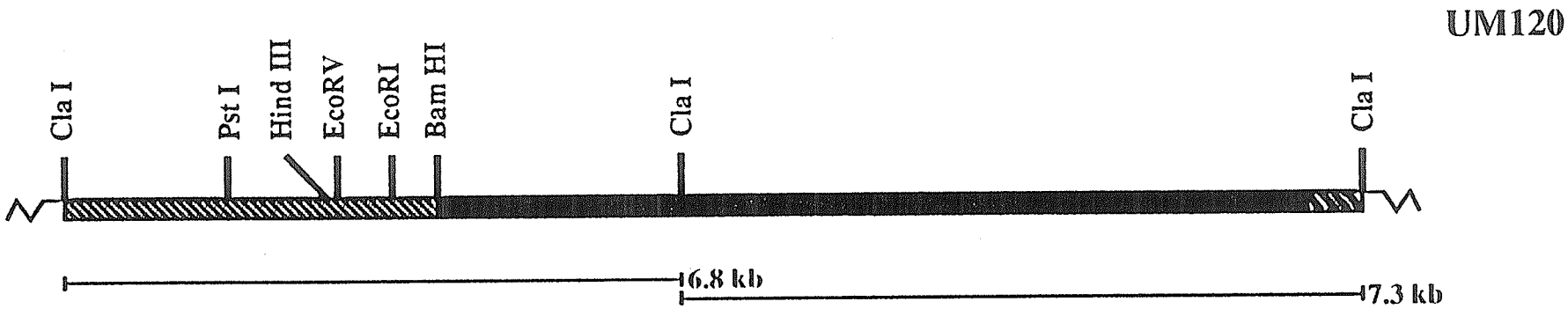
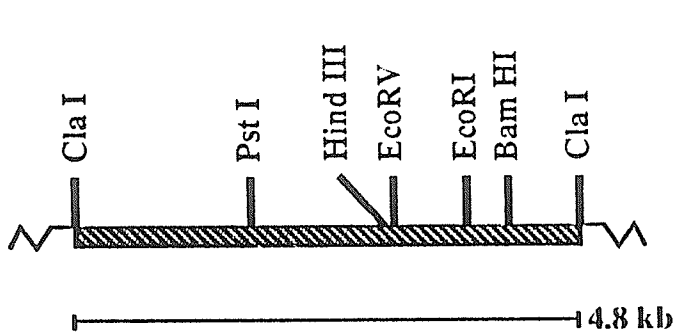

A B
23.1
9.4
6.7
4.4
2.3
2.0
0.5

Figure 4.6 Schematic representation of the location of transposon Tn10 in UM120. The solid line indicates the transposon and the hatched segment the genomic DNA. Upper diagram indicates the two fragments generated from UM120 DNA digested with *Cla*I and the lower diagram the 4.8 kb *Cla*I fragment that hybridized to pAMkatE6.


UM120
Cla I
Pst I
Hind III
EcoRV
EcoRI
Bam HI
Cla I
Cla I
6.8 kb
7.3 kb


MP180
Cla I
Pst I
Hind III
EcoRV
EcoRI
Bam HI
Cla I
4.8 kb

expression (Mulvey *et al.*, 1988). HPII levels are lower than HPI levels in mid-log phase but HPII levels increase eight fold in stationary phase (Loewen *et al.*, 1985). The *katE* containing plasmid pAMkatE6 was transformed into UM255 (KatG-, KatE-) to study the expression of *katE* when localized on a high copy number plasmid. Cultures of UM255 and UM255 [pAMkatE6] were grown for three hours. At this point any HPII contributed by the cells in the inoculum had effectively been diluted out. These three hour cultures were then used to inoculate either a fresh culture of UM255 or UM255 [pAMkatE6]. The UM255 had a slower growth rate than UM255 [pAMkatE6] but soon reached equivalent cell density. The levels of HPII in the transformed strain were relatively low at early and mid-log phases and increased dramatically in stationary phase with maximum levels being reached at 15-17 hrs of growth (Fig. 4.7). These results indicated that in the high copy number situation although greater amounts of protein are produced the regulation of *katE* is equivalent to the single copy gene residing on the chromosome.

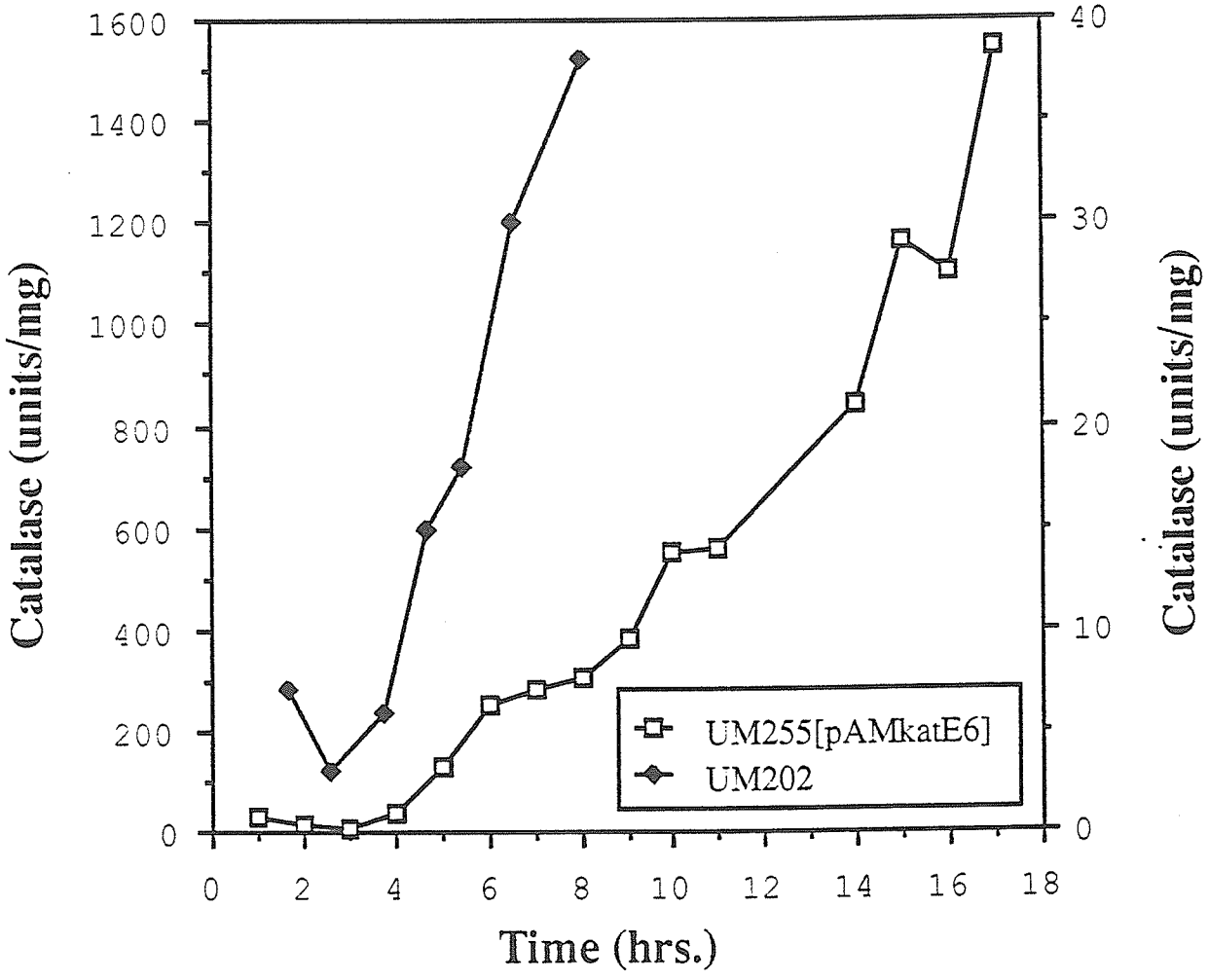
4.6 Definition of the Coding Region of *katE*

4.6.1 Restriction Enzyme Deletions

The plasmid pAMkatE22 contains the 4.0 kb *Hin*dIII-*Cla*I fragment common to pAMkatE6 and pAMkatE2 and encodes HPII activity. A protein the size of the HPII subunit should require only 2.5 kb of DNA for synthesis and attempts were made to further reduce the size of the insert to identify the *katE* coding region by deleting portions of pAMkatE6 and pAMkatE22. A *Nru*I site located 850 bp from the *Cla*I site of the original insert DNA in pAMkatE6 and a *Nru*I site located in the vector resulted in a deletion of 2.1 kb from the plasmid. This deleted plasmid did not complement the *katE* mutation in UM255. A similar attempt using the *Bam*HI

Figure 4.7 Comparison of HPII catalase levels expressed by a single copy gene in UM202(*katG*), and by a multicopy gene (UM255[pAMkatE6]). Left axis describes catalase activity from UM255[pAMkatE6] and right axis catalase activity from UM202.

HPII Production



sites in pAMkatE6 caused a deletion of 2.0 kb, approximately 150 bp less than the *Nru*I deletion (Fig. 4.10), and this plasmid also did not complement the *katE*::Tn10 mutation in UM255.

In order to reduce the size of the insert from the opposite end the only restriction sites yielding a fragment large enough to contain the entire coding region without cutting within that region were *Pst*I and *Cla*I in pAMkatE22. These sites resulted in a 3.1 kb fragment that was cloned into the Bluescript KS M13-vector using the *Pst*I and *Cla*I sites. The resulting plasmid complemented the UM255 mutation upon transformation. This HPII encoding plasmid was named pAMkatE72 (Fig. 4.8). The 2.4 kb *Dra*I fragment of this plasmid when cloned into Bluescript M13 did not complement *kat*E mutants.

4.6.2 BAL31 Deletion Mutagenesis

Because the restriction pattern did not allow for a more precise definition of the gene location, the generation of deletions using BAL31 exonuclease was attempted. The only unique restriction site available as a BAL31 initiation point was the *Sal*I site in the vector region of pAMkatE6. Due to the proximity of the *Sal*I site to the origin of replication of the plasmid and the bidirectionality of exonuclease BAL31, only small deletions could be obtained. This was useful in that the few deletions which could be isolated varied only slightly in their length allowing for finer mapping. It was found that deletions which retained the *Eco*RV site approximately 550 bp into the insert also retained activity. Deletions eliminating the *Eco*RV site did not exhibit catalase activity. These catalase negative deletions were further characterized and all were found to contain the *Bam*HI site 257 bp from *Eco*RV site. If the limit of *katE* required for activity lies near the

*Eco*RV site and the length of the entire gene is assumed to be approximately 2.5 kb, then the functional portion of *katE* should be contained in the segment extending between the *Pst*I and *Eco*RV sites as shown in Fig. 4.8.

4.6.3 Promoter Cloning

The promoter cloning vector pKK232-8 contains six restriction enzyme sites within the multiple cloning region. Small DNA fragments could not be cloned because the orientation of these restriction sites is not compatible with the orientation of restriction sites within any of the *katE* containing plasmids. However several attempts were made to clone large fragments of pAMkatE2 and pAMkatE72 into pKK232-8. The resulting clones were assayed for promoter activity on increasing concentrations of chloramphenicol. A 3.6 kb *Hin*dIII fragment from pAMkatE2 in pKK232-8 allowed transformants to grow on >60 μg/ml chloramphenicol. The orientation of this fragment indicated a promoter activity directed into the insert towards the two adjacent *Cla*I sites (Fig. 4.9A). A 1.2 kb *Bam*HI-*Hin*dIII fragment (Fig. 4.9B) from pAMkatE72 cloned into pKK232-8 also enabled transformants to grow on >60 μg/ml chloramphenicol. This fragment indicated a promoter activity directed from the *Pst*I site into the insert. A third clone constructed from a 1.3 kb *Bam*HI-*Hin*dIII of pAMkatE72 (Fig. 4.9C) (part of the 3.6 kb *Hin*dIII fragment previously used) indicated a promoter activity directed from the *Bam*HI site in the direction of the *Eco*RI site. The resulting transformants grew on 40 μg/ml chloramphenicol. These conflicting results which indicate promoters oriented in both directions from 3 different sources are most likely due to the large fragment sizes inserted into the vector and may not indicate true promoters.

Figure 4.8 Schmatic representation of pAMkatE72 the smallest resulting plasmid containing the entire *katE* gene. The solid line indicates the insert.

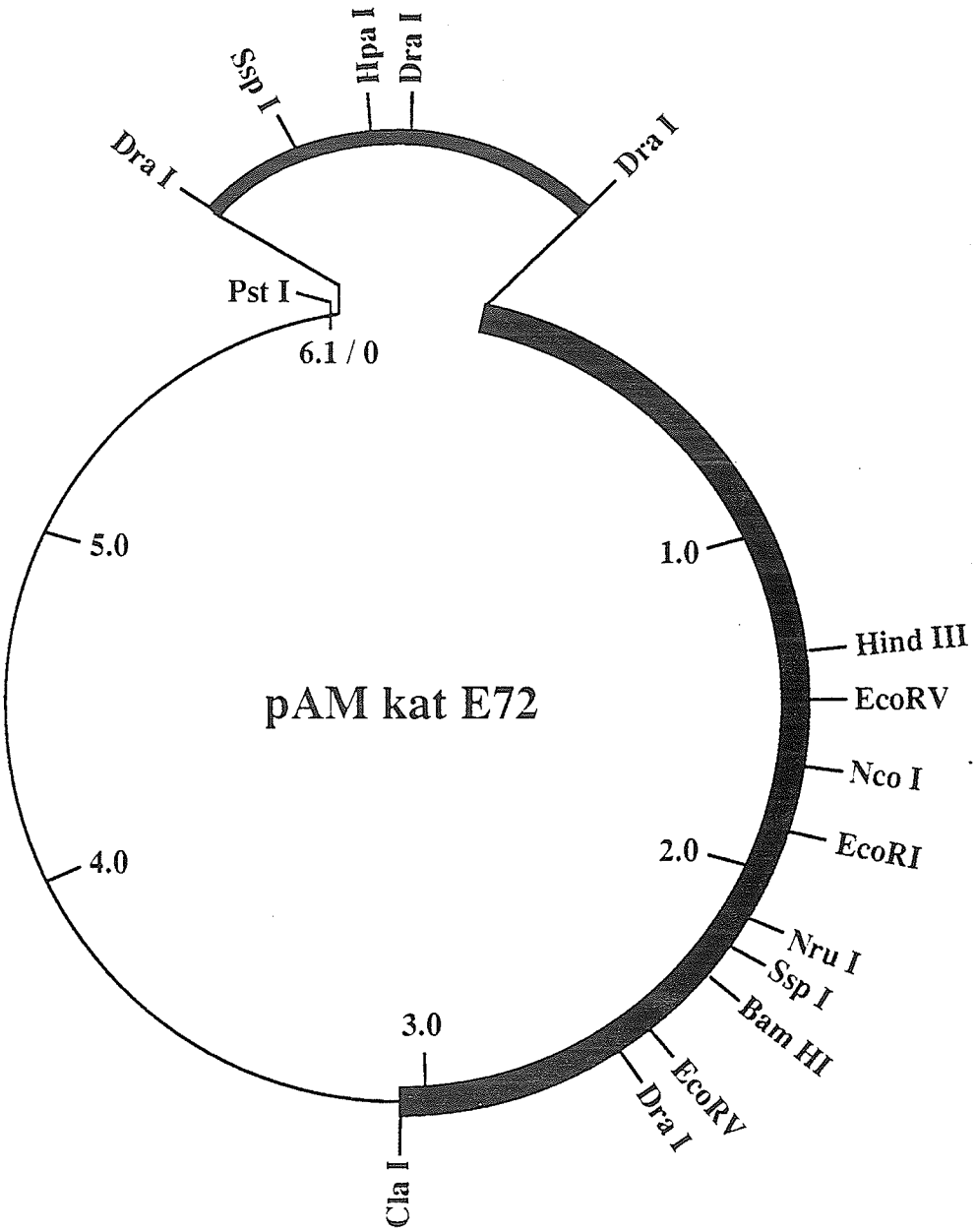
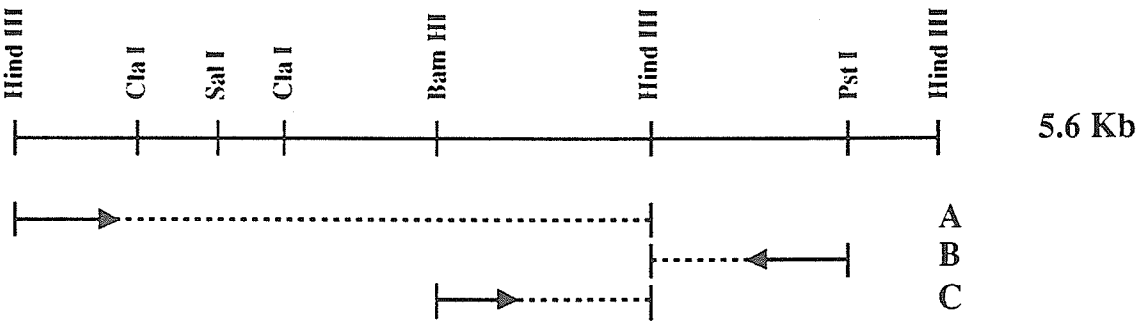
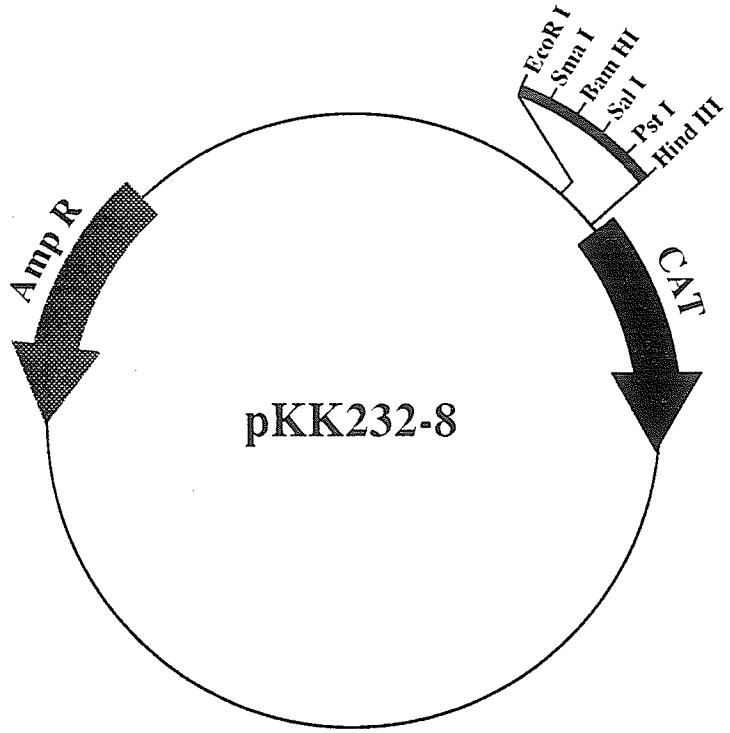

pAM kat E72
Ssp I
Hpa I
Dra I
Dra I
Dra I
Pst I
6.1 / 0
1.0
2.0
3.0
4.0
5.0
Hind III
EcoRV
Nco I
EcoRI
Nru I
Ssp I
Bam HI
EcoRV
Dra I
Cla I

Figure 4.9 Generation of pKK232-8 promoter clones. The pKK232-8 plasmid is represented (lower) showing restriction sites in the multilinker. The inserted fragments are shown (upper) delineated with vertical bars and the direction of promoter activity indicated by arrows.


Hind III
Cla I
Sal I
Cla I
Bam HI
Hind III
Pst I
Hind III
5.6 Kb
A
B
C


EcoR I
Sma I
Bam HI
Sal I
Pst I
Hind III
Amp R
CAT
pKK232-8

Consequently the data are inconclusive and the location of the *katE* promoter remains unknown.

4.7 Subcloning of *katE* for Sequencing

In order to sequence *katE* the gene had to be subcloned into smaller fragments. The sequencing protocol chosen was that using the Stratagene Bluescript vectors. These phagemids could be utilized as plasmids for the cloning procedure and single-stranded DNA for sequencing recovered through utilization of a helper phage. The fragments isolated for subcloning are shown in Fig. 4.10. Subclones isolated during the course of this work are listed in Table 4.2.

Figure 4.10 Construction of *katE* subclones. The 3.1 kb fragment isolated in pAMkatE72 is shown at the top of the figure. The fragments isolated are shown as bars with their size in bp noted above. The corresponding plasmid identification number given to each fragment is to the right of the bar.

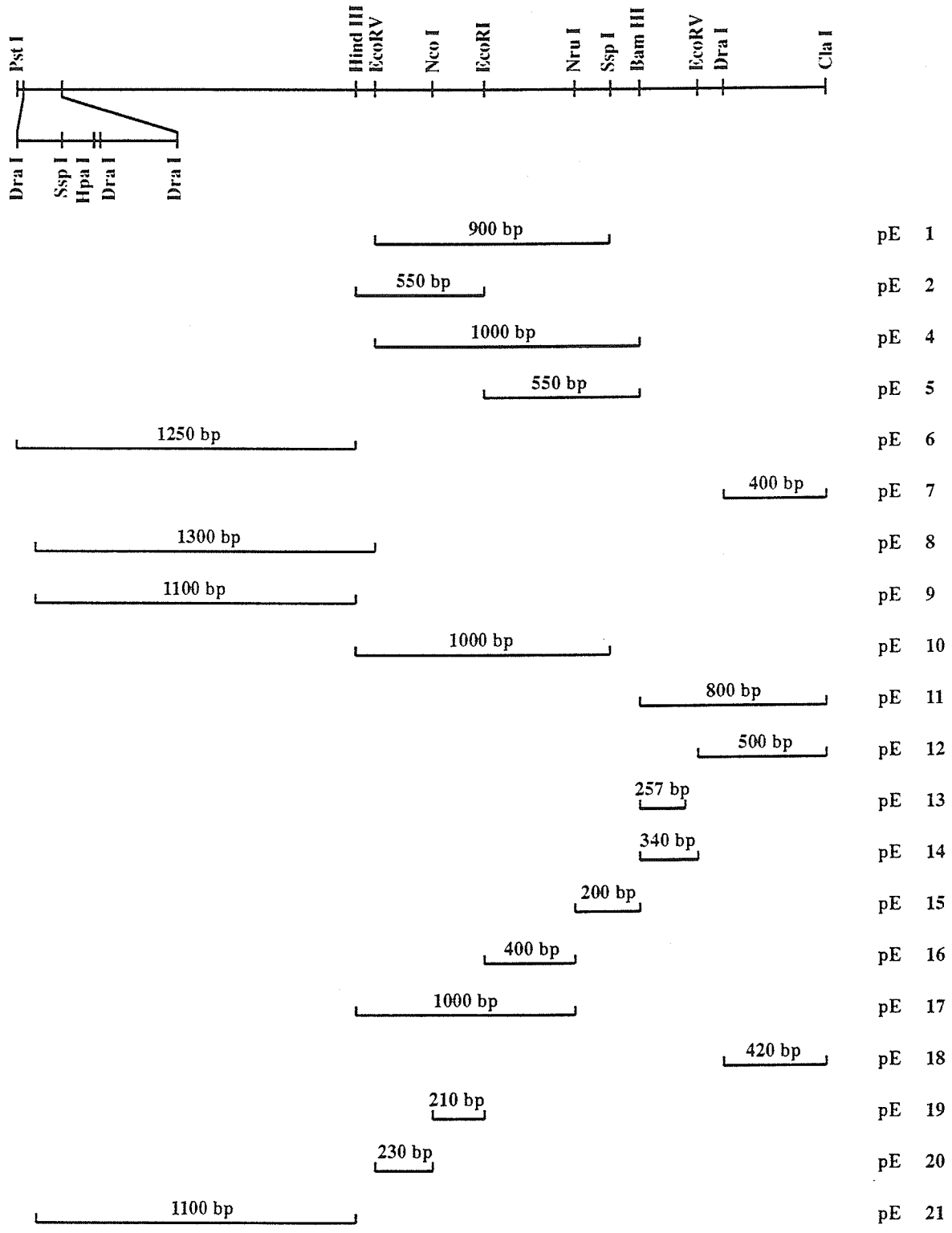

Pst I
Hind III
EcoRV
Nco I
EcoRI
Nru I
Ssp I
Bam HI
EcoRV
Dra I
Cla I
Dra I
Ssp I
Hpa I
Dra I
Dra I
900 bp
pE 1
550 bp
pE 2
1000 bp
pE 4
550 bp
pE 5
1250 bp
pE 6
400 bp
pE 7
1300 bp
pE 8
1100 bp
pE 9
1000 bp
pE 10
800 bp
pE 11
500 bp
pE 12
257 bp
pE 13
340 bp
pE 14
200 bp
pE 15
400 bp
pE 16
1000 bp
pE 17
420 bp
pE 18
210 bp
pE 19
230 bp
pE 20
1100 bp
pE 21

Table 4.2 Subclones of pAMkatE72

pEKS+	pEKS-	pESK+	pESK-		
+	+	+		1	*Eco*RV-*Ssp*I
+	+	+		2	*Eco*RI-*Hin*dIII
+	+	+	+	4	*Eco*RV-*Bam*HI
+	+	+		5	*Eco*RI-*Bam*HI
+	+		+	6	*Pst*I-*Hin*dIII
+		+	+	7	*Dra*I-*Cla*I
+		+	+	8	*Ssp*I-*Eco*RV
+	+	+	+	9	*Ssp*I-*Hin*dIII
+		+	+	10	*Ssp*I-*Hin*dIII
+	+	+	+	11	*Bam*HI-*Cla*I
+	+		+	12	*Eco*RV-*Cla*I
+	+	+	*	13	*Eco*RV-*Bam*HI
	+	+	+	14	*Bam*HI-*Dra*I
+				15	*Nru*I-*Bam*HI
+	+	+		16	*Nru*I-*Eco*RI
	+	+	+	17	*Nru*I-*Hin*dIII
	+		+	18	*Eco*RV-*Ssp*I
				19	*Nco*I-*Eco*RI
				20	*Nco*I-*Eco*RV
				21	*Dra*I-*Hin*dIII

*not required
+subclone obtained
The subclones isolated were identified by the system pEKS+1 being the *Eco*RV-*Ssp*I fragment, #1, indicated in fig. 4.10 as 900 bp in size inserted into Bluescript M13KS+.

DISCUSSION

5.0 Discussion

The ability to study the independent induction of HPI or HPII was greatly aided by the construction of transposon insertion catalase mutants (Loewen *et al.* 1985). These mutants also allowed for mapping of *katG*, *katE*, and *katF* to the positions of 89.2, 37.8 and 59 min respectively on the *E. coli* chromosome (Loewen, 1984; Loewen and Triggs, 1984; Loewen *et al.*, 1985). The *katG* gene had been located on a plasmid in the Clarke and Carbon plasmid bank (Loewen *et al.*, 1983), but attempts to clone *katE* or *katF* directly from restriction enzyme digested genomic DNA had been unsuccessful. This is because, in the case of *katE* the restriction enzymes used, with the exception of *Sal*I, all lie within the functional region of the gene. The lack of success in cloning *katE* on *Sal*I fragments may have arisen for the same reason that the cloning of *katF* from genomic DNA was unsuccessful, that is, that the DNA fragments created with *Sal*I and some other enzymes did indeed carry the gene but the fragments were too large to be efficiently ligated into pAT153. The existence of the transposon mutants allowed for the development of the isolation procedure utilized (Mulvey *et al.*, 1988). The transposon could be identified in a UM120 (*katE*::Tn10) library using the plasmid pBT107 (Moyed *et al.*, 1983). This method isolated a region of DNA containing *katE*. The insertion of the transposon in very close proximity to a *Bam*HI site facilitated the localization of the gene on the 18 kb fragment isolated in λkatE6 (section 4.2). The transposon mutations also reduced the amount of time required to screen for *katE* positive clones. One transposon mutant, UM255 is catalase negative (*katG*, *katE*) and was used to screen clones which could complement

the *katE* mutation indicating successful cloning of the gene. Presence of *katE* in the clones was confirmed by isolation of plasmid DNA, retransformation and complementation of UM255 and high levels of HPII in extracts of transformed cells. The *katE* positive cultures appeared green, a reflection of the overproduction of HPII which is green in color.

Using pAMkatE6 to probe the genomic DNA of UM120 confirmed the presence of the transposon in *katE* in UM120 and even allowed a definition of its location in the gene.

At the start of this project the individual functions of *katF* and *katE* were unknown but it was known that both were required for HPII synthesis (Loewen, 1984; Loewen and Triggs, 1984). Maxicell analysis of proteins expressed from pAMkatE6 indicated that a 93,000 dalton protein was encoded by the plasmid. This was the same as the previously determined size of the HPII subunit. Analysis of proteins encoded by the *katF* clone, pMMkatF2, in maxicells (Fig. 4.4, Lane D) showed a 42,000 dalton protein which was much too small to be the HPII subunit. From these data the respective functions of *katE* and *katF* have now been determined as the structural gene for HPII and some form of positive regulator of *katE* activity.

Confirmation that pAMkatE6 was producing HPII was shown through visualization of HPI and HPII activities in pAMkatE6 transformed and untransformed strain extracts following separation on acrylamide gels stained for catalase activity. The results show the catalase activity in UM255[pAMkatE6] to be primarily due to HPII production. The plasmid did not complement UM202 (*katG*) by producing HPI. HPII was produced in equivalent quantities to HPI in transformed UM122 (*katF*) and this may be due to a

"leakiness" in the *katF* mutation or simply that the presence of the *katE* gene in such high copy number may override its reliance on *katF* for HPII production. The recently deduced function of *katF* as a sigma factor (Mulvey and Loewen, 1989) involving it in some type of preferential transcription as its role in positive regulation of *katE* may explain the production of HPII in UM122[pAMkatE6]. HPII may be produced in extremely low levels in the absence of *katF* function but in high copy number this relatively low expression of *katE* in UM122[pAMkatE6] is detectable. The results of the UM255[pAMkatE6] time course catalase assay (Fig. 4.7) indicate that HPII is overproduced when *katE* is present on a multicopy plasmid, at levels 30 fold greater than wild-type, but that the number of copies of the gene has no influence on the normal pattern of induction. HPII production begins to rise in midlog phase and reaches a maximum in stationary phase, usually peaking at 16 hrs incubation.

The DNA fragment contained on pAMkatE22 and pAMkatE24 included the region required for HPII production and various methods were used to locate *katE* more precisely. A 93,000 dalton protein would require approximately 2.5 kb of DNA and portions of the 4.0 kb insert were deleted using various restriction enzymes and BAL31 nuclease. The combination of deletions obtained led to the conclusion that the functional region of *katE* lies within the 2.6 kb between the *Dra*I sites at 12 bp and 2.8 kb in fig. 4.8.

Attempts made to isolate the *katE* promoter in the vector pKK232-8 were successful in isolating fragments with promoter activity but inconclusive in defining the *katE* promoter. The promoter is most likely located near the *Pst*I site at the 0 kb end of the insert (fig. 4.9) on

fragment B given recent sequence data indicating an open reading frame extending away from this end (P.C. Loewen, unpublished). The high level of promoter activity from fragment A is probably due to a promoter belonging to an adjacent gene. Isolation of the *katE* promoter on a small fragment will be useful in confirming the role of the Kat F protein as a novel sigma factor and must be continued.

The 3.1 kb fragment contained on pAMkatE72 was used to create small subclones for sequencing of *katE*. The procedure chosen for sequencing was the dideoxy chain termination method using single-stranded DNA from clones in Bluescript M13 phagemids. This protocol would enable sequencing of each fragment from both directions on each strand. Subclones obtained at the conclusion of this work are listed in table 4.2 and the sequencing of these clones is underway. The only region without suitable restriction sites for subcloning is the 1.1 kb region between the *Dra*I site (194 bp) and the *Hin*dIII site. Attempts were made to create subclones of the region using the Stratagene Exo-Mung protocol, a *Sau*IIIA deletion method (Lee and Lee, 1988) and four modifications of the *Sau*IIIA method, but to date no subclones of this region have been obtained.

Loewen *et al.* (1985) had proposed that the presence of two catalase genes, *katG* and *katE* may have resulted from a duplication of the *E. coli* chromosome during the evolution of the organism. The possibility that this is the case seems slight as the two genes seem to belong to at least two different regulons or systems. The *katG* gene has been shown to be under the positive control of the *oxyR* gene product (Christman *et al.*, 1985) and *katE* under positive control of the *katF* gene product (Mulvey *et al.*, 1988). The Kat F protein is believed to be a novel sigma

factor with homology to *rpoD*, the *E. coli* sigma subunit of RNA polymerase, and also shows homology to the heat shock regulatory protein encoded by *htpR*. These two genes have not been reported to be homologous to Oxy R protein, furthermore *katG* and *katE* do not cross-hybridize under stringent conditions (Triggs-Raine, unpublished). Determination of the protein sequence of *katE* from the DNA sequence and comparison to *katG* should definitely answer this question. As well, the two catalases are immunologically distinct and vary in most characteristics making such homology unlikely.

It appears that the *E. coli* catalases HPI and HPII have evolved in response to different pressures because of their distinct differences in structure, function, and regulation. The sequence analysis of *katE* will be a first step in identification and characterization of the catalytic domains and functional regions of HPII and this will allow a comparison of the functional, structural, and regulatory differences between HPI and HPII. In turn this may increase our understanding of the complex mechanisms involved in an organism's adaptation to stresses related to surviving in oxygenated environments.

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6.0 References

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