Cloning and Characterization of *katF*, a Gene Controlling a Novel Regulon in *Escherichia coli*

by

MICHAEL RICHARD MULVEY

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TO JOANNE

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ABSTRACT

1.0 ABSTRACT

The *Escherichia coli katE* and *katF* genes encoding catalase HPII were cloned in a multistep procedure involving the transposon Tn*10*. Two libraries were constructed from the strains UM120 (*katE*::Tn*10*) and UM122 (*katF*::Tn*10*). Sequences containing the Tn*10* were isolated by probing the library with the plasmid pBT107. λ clones from each library were mapped and found to contain the Tn*10* sequences along with adjacent DNA. Sequences adjacent to the Tn*10* were cloned into the vector pAT153 producing the plasmids pMM120 and pMM122. These two plasmids were used as probes to isolate *katE* and *katF* sequences from a wild type MP180 library. The *katF* gene was localized to a 1.5 kb *Nru*I-*Hin*cII fragment which complemented the *katF*::Tn*10* mutation in UM258 (*katG2*, *katF13*::Tn*10*).

Fragments of *katF* were subcloned into M13mp18/19 or pM13KS+/- and sequenced by dideoxy chain termination sequencing. The entire 1.5 kb *Nru*I-*Hin*cII fragment was sequenced in both directions and found to contain an open reading frame 1086 bp in length. Two potential Shine-Dalgarno sequences existed resulting in a 362 or 350 amino acid protein with predicted molecular weights of 41.5 and 40.1 kDa respectively. This was similar to the 44 kDa size of the protein determined by maxicell analysis. Preliminary primer extension analysis to determine the transcriptional start site of *katF* suggests the gene may be part the flagellar regulon. Comparison of the amino acid sequence of KatF to other known protein sequences revealed strong similarity to a group of proteins termed sigma factors.

The regulation of the *katE* and *katF* genes were studied by inserting the two promoters in front of a promoterless *lacZ* gene in

pRS415 producing the plasmids pRSkatE16 and pRSkatF5. In rich medium the expression of *katF* increased during log phase growth reaching a maximum as cells entered stationary phase whereas *katE* expression remained low throughout early log phase and increased rapidly just prior to stationary phase. In minimal medium expression from both promoters was fully induced throughout growth with the exception of *katE* expression in glucose minimal medium where expression was half the fully induced levels. Removal of all carbon from the medium prevented expression from both promoters but if glucose was present in the absence of nitrogen, expression occurred. Simple aromatic weak acids were capable of inducing expression from both promoters as was spent rich medium from stationary phase cells. Regardless of the medium, *katF* expression was always turned on prior to or coincidently with *katE* expression, and in the presence of benzoate and glucose *katF* was fully induced while *katE* was only partially induced. During prolonged aerobic incubation, cells lacking *katF* were killed off more rapidly than cells lacking either *katE* or *katG*.

List of Abbreviations

A	absorbance
amp	ampicillin
Ap^R	ampicillin resistant
ATP	adenosine 5′-triphosphate
bp	base pair
BRL	Bethesda Research Laboratory
BSA	bovine serum albumin
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
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dTTP	2′-deoxthymidine 5′-triphosphate
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl ß-D-thiogalactoside
kan	kanamycin
kan^R	kanamycin resistant
kb	kilobase pairs
MOPS	3-[N-Morpholino]propanesulfonic acid
MSH	2-mercaptoethanol
NEN	New England Nuclear

PFU	plaque forming units
PMSF	phenylmethylsulfonylfluoride
PPO	2,5-diphenyloxazole
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
Tc^R	tetracycline resistant
TEMED	N,N,N^1,N^1-tetramethylethylenediamine
tet	tetracycline
Tn	transposon
U	units
UV	ultraviolet
V	volts
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

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HISTORICAL

2.0 HISTORICAL

2.1 Life in Oxygen

2.1.1 Oxygen Toxicity

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, 1978).

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

The chemistry of oxygen dictates this stepwise reduction. Oxygen contains two unpaired electrons of parallel spin in the π^* orbitals which require two electrons of antiparallel spin to reduce them. The Pauli exclusion principle states that electrons occupying the same orbital must have equal and opposite spin (Dickerson *et al.*, 1979) making it impossible for two electrons of the same spin to be passed to oxygen simultaneously. Therefore, the complete reduction of oxygen to water requires the sequential addition of four electrons.

The reduction takes place during aerobic respiration via the electron transport chain. The passage of electrons through the electron transport chain facilitates the extrusion of protons from the cell. As the electrons pass from compounds of very negative E_o's to compounds with less negative E_o's a proton motive force is produced which can then be coupled to the formation of ATP, transport of various compounds, or used to drive the flagellar motor. The loss of single electrons during this process can lead to the formation of the toxic oxygen intermediates O_2^-, H_2O_2, and $OH^\cdot$. The most common points of loss are in the transfers leading to coenzyme Q and from coenzyme Q itself (Halliwell and Gutteridge, 1985). These partially reduced derivatives can cause

extensive damage to biological systems including nucleic acid strand scission (Brawn and Fridovich, 1981), lipid oxidation (Haliwell and Gutteridge, 1985), and oxidation of aromatic amino acids (Davies *et al.*, 1987).

The formation of O_2^- leads to the production of H_2O_2 through the action of the enzyme superoxide dismutase (SOD) (Fridovich, 1975). These two compounds, in the presence of widely available metal ions (Halliwell *et al.*, 1983; Fridovich, 1986), can react in the Haber-Weiss reaction, a specific example of the Fenton reaction, where O_2^- acts as the reductant for the metal (Winterbourn, 1983) to form the $OH^{\cdot}$.

$$(1) \quad O_2^- + Fe(III) \longrightarrow O_2 + Fe(II)$$

$$(2) \quad Fe(II) + H_2O_2 \longrightarrow Fe(III) + H_2O + OH^{\cdot}$$

$$(1) + (2) \quad O_2^- + H_2O_2 + H^+ \longrightarrow O_2 + H_2O + OH^{\cdot}$$

The hydroxyl radical is one of the most reactive molecules known and can react with every type of molecule found in living cells (Beauchamp and Fridovich, 1970; Haliwell and Gutteridge, 1985).

2.1.2 Damage Produced by Toxic Oxygen Species

All biological molecules can react with at least one of the toxic oxygen species. These reactions have been reviewed by Halliwell and Gutteridge (1985). The hydroxyl radical and singlet oxygen can abstract protons from membrane lipids forming H_2O and a new lipid radical which can react with other lipid molecules in a chain reaction. The reactions can cause an overall loss in membrane fluidity, inactivation of membrane-bound proteins, and an increase in membrane permeability.

Cellular proteins are another target for toxic oxygen species. Methionine, tryptophan, histidine, and cysteine can all become oxidized resulting in loss of protein function.

There is a large accumulation of evidence demonstrating that DNA is a target of toxic oxygen species. The superoxide anion is mutagenic, causing both base pair substitutions and frameshift mutations (Gregory *et al.*, 1973; Moody and Hassan, 1982). The quinone plumbagin, which diverts electrons from the electron transport chain to oxygen, is also mutagenic (Farr *et al.*, 1985). Hydrogen peroxide has been shown to cause DNA strand scission (Ananthaswamy and Eisenstark, 1977; Hagensee and Moses, 1986), inhibit the transforming activity, and decrease the melting temperature of DNA (Yamamoto, 1969). Ionizing radiation and aerobic respiration lead to the formation of $OH^{\cdot}$ which can cause ss and ds DNA breaks (Breimer and Lindahl, 1985).

2.2 Protection From Toxic Oxygen Species

E. coli has evolved two modes of protection from toxic oxygen species, predamage and postdamage defence mechanisms (Yonei *et al.*, 1987). The predamage defence relies on the ability of certain enzymes, such as superoxide dismutase, catalase, and peroxidase to remove the toxic oxygen species before they harm cellular components. The postdamage defence mechanism repairs damage resulting from toxic oxygen species.

2.2.1 The Predamage Defence Mechanism

The enzymes traditionally included in the predamage defence mechanism include superoxide dismutase (SOD), catalase, and peroxidase. The reactions catalysed by these enzymes are shown below.

SOD	$O_2^- + O_{2\cdot} + 2H^+$	$H_2O_2 + O_2$	
Catalase	$H_2O_2 + H_2O_2$	$2H_2O + O_2$	
Peroxidase	$H_2O_2 + RH_2$	$2H_2O + R$	(Fridovich,1978)

These enzymes protect the cell by removing H_2O_2 and O_2^- before they can react with cellular components or themselves to produce more reactive oxygen species.

E. coli contains two superoxide dismutase isoenzymes, one containing manganese (MnSOD) and the other containing iron (FeSOD) (Fridovich, 1975). MnSOD is induced when cultures are shifted from anaerobic to aerobic conditions whereas FeSOD is constitutive with respect to oxygen (Hassan and Fridovich, 1977). The presence of SOD in nearly all aerobes confirms the role of SOD as a major enzyme involved in oxidative stress. However, the fact that *E. coli* mutants that lack SOD can survive aerobically suggests the enzyme is not essential for growth in oxygen (Schellhorn and Hassan, 1988a).

E. coli contains two different catalases HPI (Claiborne and Fridovich, 1979) and HPII (Claiborne *et al.*, 1979). The ability of these mutants to grow normally at standard oxygen concentrations (Loewen, 1984) shows that catalase is not essential for aerobic growth. The regulation of these two catalases will be discussed in detail below.

Schellhorn and Hassan (1988a) have constructed an *E. coli* strain completely deficient in both catalase and superoxide dismutase. Superoxide dismutase was found to be more important than catalase in preventing oxygen dependent growth inhibition and mutagenesis. However they also reported that both types of enzymes were required for effective defence against O_2^- and H_2O_2.

2.2.2 The Postdamage Defence Mechanism

There are a number of enzymes which have been implicated in postdamage repair of DNA damaged by oxidation. The *polA* (DNA

polymerases I; Hagensee and Moses, 1986), *polC* (DNA polymerase III; Hagensee *et al.*, 1987), *recA* (Carlsson and Carpenter, 1980), and *xthA* (exonuclease III; Demple *et al.*, 1983) mutants are more sensitive to H_2O_2 than wild type cells. The order, in terms of decreasing sensitivity to H_2O_2, is *recA*, *polC*, *xthA*, and *polA* (Hagensee and Moses, 1989). The repair of hydrogen peroxide damaged DNA seems to involve two possible pathways. The first pathway would require exonuclease III, DNA polymerase III, and DNA polymerase I, while the other would be DNA polymerase I dependent. The RecA protein seems to have little or no direct function in either repair pathway (Hagensee and Moses, 1989).

2.3 Catalase

2.3.1 Structure and Function

The catalase enzyme (H_2O_2:H_2O_2 oxidoreductase EC1.11.1.6) was one of the first proteins isolated, characterized and crystallized (Herbert and Pinsent, 1948). Some catalases have an associated peroxidase activity (donor:H_2O_2 oxidoreductase EC1.11.1.7). The dismutation of H_2O_2 to H_2O by catalase is a two step process. In the first step H_2O_2 reacts with the heme group of the catalase to form compound I. A second H_2O_2 molecule can then react with compound I to form water and oxygen (Fita and Rossmann, 1985).

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

$$H_2O_2 + \text{O=Fe-E} \qquad H_2O + O_2 + \text{Fe-E}$$

Catalase enzymes isolated from various sources share common features. Most contain four identical subunits that form a tetramer of MW between 225 000-270 000. Each subunit contains one heme (usually a protoheme IX group) which acts as an independent unit. Typical

catalases have a broad pH optimum and are sensitive to inhibition by 3-amino-1,2,4-triazole (AT) (Nadler *et al.*, 1986).

A number of catalases have been crystallized and studied using X-ray diffraction. The spatial organization of a non-heme hexameric catalase from *Thermus thermophilus* (Vainshtein *et al.*, 1985), three tetrameric catalases from *Penicillium vitale* (Vainshtein *et al.*, 1986), from beef liver (Murthy *et al.*, 1981), and from *Micrococcus lysodeikticus* (Yusifov *et al.*, 1989) and the HPII catalase of *E. coli* (Tormo *et al.*, 1990) are known.

2.3.2 Catalase HPI and HPII from *Escherichia coli*

The two catalases HPI and HPII have been purified and characterized (Claiborne and Fridovich, 1979; Claiborne *et al.*, 1979; Loewen and Switala, 1986).

HPI is a bifunctional catalase and broad spectrum peroxidase (Claiborne, 1978) that is composed of four identical 84 000 dalton subunits which combined to form a 337 000 dalton tetramer containing two protoheme IX groups (Claiborne and Fridovich, 1979). Purified HPI electrophoresed through native gels run in Tris-glycine buffer separates into two isoenzyme forms labelled HPI-A and HPI-B (Loewen *et al.*, 1985a; Meir and Yagil, 1985). The significance of the two forms is unknown. HPI has an optimum catalase activity at pH 7.5, an optimum peroxidase activity at pH 6.5 and a Km (at pH 7.5) of 3.9 mM H_2O_2 (Claiborne and Fridovich 1979). Some of the properties of HPI make it unusual to the typical catalase. It is resistant to inhibition by AT, is heat labile at 70°C (Meir and Yagil, 1985), is larger than most tetrameric catalases, and it has an associated broad spectrum peroxidase activity.

These differences have led Nadler *et al.* (1986) to proposed the formation of a new group of catalases containing *E. coli* HPI, *Rhodopseudomonas capsulata* catalase (Nadler *et al.*, 1986), *Mycobacterium avium* type-T catalase (Mayer and Kalkinbam, 1986), and *Comamonas compransoris* catalase (Nies and Shlegel, 1982) all of which share common features.

The gene affecting HPI synthesis has been mapped to 89.2 minutes on the *E. coli* chromosome and has been designated *katG* (Loewen *et al.*, 1985b). It has been cloned (Triggs-Raine and Loewen, 1987) and sequenced (Triggs-Raine *et al*, 1988). Comparison of the amino acid sequence to other catalases did not reveal any homology, but the amino acid sequence of the bifunctional catalase peroxidase from *Bacillus stearothermophilus* showed 48% homology to HPI (Loprasert *et al*, 1989).

HPI levels increase during logarithmic growth and decline as the culture enters stationary phase (Loewen *et al.*, 1985a). The levels were also inducible by ascorbate or H_2O_2 (Loewen *et al.*, 1985a; Richter and Loewen, 1982). HPI has been identified as one of 30 proteins induced during H_2O_2 adaptation and is positively regulated by the *oxyR* protein (Christman *et al.*, 1985). The *oxyR* regulon will be discussed further in section 2.6.7.

The HPII catalase also shows a variety of atypical properties. It is a monofunctional catalase composed of six identical 93 000 dalton subunits with one heme group per subunit having a combined molecular weight of 532 000 (Loewen and Switala, 1986). The prosthetic group is an unusual green chromophore that is similar to the family of *d*-type hemes with a proposed structure of 12-hydroxy-13-*cis*-spirolactone-2,7,12,18-tetramethyl-3,8-divinyl-17-propionylporphyrin (Chiu *et al.*,

1989). HPII is heat stable at 70°C and unlike HPI is sensitive to inhibition by AT. It is resistant to 0.1% SDS or 7M urea (Loewen and Switala, 1986; Meir and Yagil, 1985). The enzyme has catalase activity over a broad pH range (pH 4-11) and two peaks of optimum activity, one at pH 6.8 and the other at pH 10.5 with corresponding Km values of 18.2 mM and 10 mM (Meir and Yagil, 1985).

HPII does not cross react with antisera prepared against HPI (Loewen *et al.*, 1985b) nor did HPI cross react with antisera prepared against HPII (Claiborne *et al.*, 1979). Different patterns were observed by cyanogen bromide cleavage of HPI and HPII (Claiborne *et al.*, 1979). Recently the *katE* gene has been cloned (Mulvey *et al.*, 1988) and sequenced (I. von Ossowski, personal communication). The amino acid sequence of HPII shows homology to rat liver catalase, bovine liver catalase, human kidney catalase *S. cerevisiae* type T catalase, and *Candida tropicalis* catalase (I. von Ossowski, unpublished data). This is very surprising since HPII shares very few properties with the typical catalases. The HPII protein has been crystallized and examined using X-ray diffraction to 3.2 angstroms (Tormo *et al.*, 1990). It will be interesting to see how the structure of the hexamer differs from that of the other typical tetrameric catalases.

HPII is positively regulated by the *katF* gene product (Mulvey *et al*, 1988). The regulation of HPII will be discussed in section 2.4.2.

2.4 Genes Regulated by the KatF protein

The *katF* gene maps at 59 minutes on the *E. coli* chromosome (Loewen and Triggs, 1984). It has recently been shown to positively control two genes in *E. coli* (Mulvey *et al.*, 1988; Schellhorn and Hassan, 1988b; Sak

et al., 1989), *katE* and *xthA*, mapping at 37.8 and 38.2 minutes on the *E. coli* chromosome respectively. It has been suggested that they may form an operon (Sak *et al.*, 1989). In this next section I will be discussing the various characteristics and functions of these two genes.

2.4.1 The *xthA* gene

The *xthA* gene has been sequenced and codes for a protein with a predicted molecular weight of 30,921 (Saporito *et al.*, 1988). The protein, named exonuclease III, is the major apurinic/apyrimidinic (AP) endonuclease in *E. coli* accounting for greater than 85% of the total activity in the cell. In addition to this activity, the protein has a number of other distinct enzyme activities including; i) an exonuclease activity specific for dsDNA in the 3′ to 5′ direction; ii) an urea endonuclease activity that hydrolyzesincises the phosphodiester bond 5′ to a urea residue in DNA; iii) a 3′ phosphatase activity and possibly related functions that remove 3′terminal deoxyribose-5′-phosphate as well as phosphoglycolate esters to activate the 3′ terminus for DNA polymerase I; and iv) an RNase H activity which hydrolyses only the RNA part of a hybrid duplex exonucleolytically in the 3′ to 5′ direction. Mutations in the *xthA* gene result in significantly enhanced sensitivity to near-ultraviolet radiation (NUV) and H_2O_2-mediated inactivation (Demple *et al.*, 1983 and Eisenstark and Perrot, 1987). The exonuclease III protein has recently been implicated, along with DNA polymerase I and III, in a pathway that repairs DNA damaged by oxidative stress (Hagensee and Moses, 1989)

Two genes are required for the production of HPII in *E. coli* (Loewen, 1984; Loewen and Triggs, 1984). *katE* encodes a 93 000 dalton protein, the same size as the structural subunit of HPII. Unlike HPI, HPII is not inducible by ascorbate or H_2O_2 (Loewen *et al*, 1985a) nor is it positively regulated by the *oxyR* regulon (Schellhorn and Hassan, 1988b). HPII levels increase dramatically as cells enter stationary phase (Hassan and Fridovich, 1978; Loewen *et al.*, 1985a; Schellhorn and Hassan, 1988b) and during logarithmic growth on TCA cycle intermediates (Loewen *et al.*, 1985a). Meir and Yagil (1990) have reported that mutants in the electron transport chain failed to induce HPII in the stationary phase, whereas mutants in the glycolytic pathway were normal confirming the suggestion by Hassan and Fridovich (1978), but contradicting the findings of Loewen *et al.* (1985a). *katE* was expressed under anaerobic conditions at levels that were approximately one-fourth of those found in aerobically grown cells but followed the same pattern of induction increasing as cells entered stationary phase (Schellhorn and Hassan, 1988b). This contradicted the findings of Meir and Yagil (1990) who reported a shift to anaerobic conditions increased HPII levels by three- to four-fold. These authors also reported HPII levels were two- to six-fold lower in *fnr*$^-$ mutants, a protein that positively regulates a group of genes induced under anaerobic conditions.

2.5 Catabolite Repression and Catalase Synthesis

Catabolite repression has been suggested to be involved in catalase expression in yeast (Sulebele and Rege, 1968), *Bacteriodes fragilis* (Gregory *et al.*, 1977), and *E. coli* (Hassan and Fridovich, 1978; Yoshpe-Purer *et al*, 1977; and Meir and Yagil, 1990). However

Richter and Loewen (1982) found that glucose added to the media did not inhibit catalase levels in *E. coli*. Furthermore, they found that addition of cAMP to the media had no effect on catalase levels adding further support to the idea that catabolite repression is not involved in the regulation of the catalases.

Recently, Meir and Yagil (1990) have reported that the addition of glucose to cultures of *E. coli* strongly inhibited the synthesis of the catalases as the cells entered stationary phase. They also found that the addition of cAMP did not alleviate the repression by glucose. However, when catalase levels in *crp* mutants were assessed it was found that the levels were quite low. They suggested that HPII is under the control of catabolite repression in a manner that is independent of cAMP. This group has also identified a gene (labelled *gle-12*) that maps at 61.5 minutes on the *E. coli* chromosome which alleviates the repression of HPII levels by glucose by approximately 50%.

2.6 Regulons and Stimulons

E. coli has evolved mechanisms that permit rapid growth under favorable conditions and adapt for survival when conditions are unfavorable. Very few proteins are constitutive and most are produced only when required. The ability of *E. coli* to adjust protein synthesis to its environment was first illustrated in two-dimensional O'Farrell gels (Pederson *et al.*, 1978). These adjustments require large changes in gene expression. A group of unlinked genes and/or operons that are controlled by a single regulatory protein is defined as a regulon (Maas, 1964). The regulatory protein can be an inducer or a repressor.

The regulation of regulons is complex. Many of the operons can

The regulation of regulons is complex. Many of the operons can belong to more than one regulon and some can be induced by a number of different environmental changes. Also, a single environmental change may induce several regulons. Therefore the term stimulon has been introduced to accommodate the induction of several regulons to a particular environmental stimulus (VanBogelen *et al.*, 1987).

In recent years, the area of the identification and study of regulons has been increasing rapidly. During the course of my research, it has become clear that the *katF* gene product is similar to a group of regulatory proteins termed sigma factors (Mulvey and Loewen, 1989). Also, the *katF* gene has been shown to control two genes that may be individually transcribed (Sak *et al.*, 1989). This suggests that KatF is a regulatory protein involved in an unknown regulon. In this section I will be discussing the transcriptional process and the control a number of different regulons in *E. coli*.

2.6.1 Transcription

Transcription is a complex process involving a number of different proteins and in some cases small inducing molecules. In *E. coli*, the enzyme responsible for transcription is RNA polymerase. It is composed of four subunits with a stoichiometry of $\beta\beta'\alpha_2$. This core enzyme can nonspecifically bind to DNA. When the core enzyme interacts with a group of proteins called σ factors, the holoenzyme ($\beta\beta'\alpha_2\sigma$) is formed which accurately initiates transcription from specific DNA sequences called promoters. Following transcription initiation, the σ factor is released and the core enzyme elongates the RNA chain. Transcription is terminated by the interaction of RNA polymerase with a termination

structure or specific termination factors.

2.6.2 Sigma Factors

A sigma factor is a protein which has a number of different activities that include (a) core polymerase binding, (b) activation of promoter recognition, and may include, (c) DNA melting, and (d) inhibition of nonspecific transcription. All sigma factors known, by definition, have at least the first two of these activities (Helmann and Chamberlin, 1988). The point of contact between the sigma factor and the core enzyme is thought to be conserved among sigma factors. Once bound to the core, the sigma factors confer the ability to recognize specific DNA promoter sequences. The sigma factors are thought to make direct contact with the DNA during the recognition process.

A number of reviews have compared various sigma factor primary structures. The most recent (Helmann and Chamberlin, 1988), has aligned thirteen of the sigma proteins into four conserved regions (regions 1 through 4). All sigma factors share 20 to 30% identical amino acids and all are acidic with an excess negative charge at pH 7.0.

Region 1 is found in three major sigma factors of *E. coli* (σ^{70}), *B. subtilis* (σ^{42}), and *M. xanthus* (σ^{80}). The function of region 1 is unknown.

Region 2 spans approximately 70 amino acids and is subdivided into four subregions labelled 2.1-2.4. Some of the most highly conserved sequences occur in region 2. Subregion 2.2 is composed of 15 highly conserved amino acids. This region is thought to form the heart of the core binding region. Subregion 2.3 is 18 residues in length and is extremely rich in aromatic amino acids. It has been suggested that this

region binds to single-stranded DNA during formation of the open complex (Helmann and Chamberlin, 1988). The sequence in this region is similar to the ribonucleoprotein consensus sequence which is responsible for RNA binding. Subregion 2.1 may also play a role in this process. It contains a number of highly conserved aromatic residues. These residues may directly facilitate DNA melting through intercalation of the aromatic side chain into the nontranscribed template DNA strand. The predicted secondary structure of subregion 2.4 is α-helical. Mutational analysis of this region suggests it may be involved in -10 recognition.

Region 3 contains 45 amino acids and is weakly conserved among sigma factors. Many sigma factors are predicted to form α-helicies in this region but do not form the DNA binding (helix-turn-helix) motif. The function of this region is probably structural.

Region 4 is divided into two subregions 4.1 and 4.2. Subregion 4.2 is composed of a block of 28 amino acids which form a helix-turn-helix motif. Mutational evidence has implicated this region in -35 recognition. The *E. coli* σ^{70}, *B. subtilis* σ^{42}, and *M. xanthus* σ^{80} 4.2 regions are very similar and all recognize the same -35 promoter sequence.

2.6.3 The pH Regulon

The proton electrochemical gradient (or proton-motive force) $\Delta\tilde{\mu}_{H+}$ is comprised of two components, $\Delta\psi$ and ΔpH. The $\Delta\psi$ represents the electrical potential of the membrane whereas the ΔpH is the difference between the cytoplasmic pH (pH_i) and the pH of the bacterial environment (pH_o). The $\Delta\tilde{\mu}_{H+}$ is related to the ΔpH and $\Delta\psi$ in the formula below:

$$\Delta\tilde{\mu}_{H+} / F = \Delta\psi - Z\ \Delta pH$$

where R is the gas constant, T is the temperature in K, and F is the Faraday constant; Z = 2.3 RT/F.

The regulation of cytoplasmic pH is essential for every living cell. *E. coli* maintains a constant pH_i of between pH 7.7 to 8.0 over a broad pH_o range of pH 6.0 to 8.6 (Padan and Schuldiner, 1986). This constant pH level is maintained through the action of the primary proton pumps. When inhibitors are used to block respiration and the H^+/ATPase, protons rapidly equilibrate across the membrane.

When the pH_o is lower than pH 7.8, the ΔpH is basic inside the cell. It ranges from a value of 2 at a pH of 6 to 0 at pH 7.8. The $\Delta\psi$ changes in a compensatory fashion with ΔpH. It increases from 80 mV at pH 6.0 to its highest value of 160 mV at pH 7.8. At pH_o above pH 7.8 the ΔpH actually reverses and becomes acidic inside whereas the $\Delta\psi$ remains constant (Padan and Schuldiner, 1986).

Through the various changes of the $\Delta\psi$ and ΔpH, the $\Delta\tilde{\mu}_{H+}$ remains constant over the pH range of 6-8.5 (Zilberstein *et al.*, 1984). Therefore, over this pH range, pH homeostasis (pH_i) as well as the bioenergetic homeostasis ($\Delta\tilde{\mu}_{H+}$) is achieved in the cell.

The concentration of protons in the cell plays a fundamental role in the physical and chemical reactions of the cell. Most proteins and other biologically important compounds have narrow pH ranges of optimum activity and stability. Most of these pH optima fall around neutrality. It is therefore extremely important for the cell to maintain a constant pH.

Recently, there has been some interest in a number of proteins that have been found to be induced by changes in either pH_i or pH_o

(reviewed in Padan and Schuldiner, 1987). An alkaline shift in the pH_o induces the heat shock response in *E. coli* (Taglicht *et al.*, 1987). The synthesis of the H^+-ATPase in *S. faecalis* has been shown to be activated upon acidification of the cytoplasm (Kobayashi *et al.*, 1986). Induction of the SOS response in *E. coli* is brought on by alkalinization of pH_i (Schuldiner *et al.*, 1986). Two new regulons, one that is sensitive to internal acidification and one that is induced by external acidification, have been demonstrated through the use of Mu d-directed *lacZ* fusions in *E. coli* (Solnczewski *et al.*, 1987).

HPII levels remain low in logarithmic growth but increase tenfold during growth into stationary phase (Loewen *et al.*, 1985a). In this same study it was also found that growth in minimal media with any carbon source derived from the TCA cycle caused a five- to ten-fold induction of HPII. Preliminary results by Schellhorn and Hassan (1988) have indicated that nonmetabolic weak acids can act as inducers of a *katE::lacZ* fusion. These acids are thought to lower the internal pH of the cell in a pH-independent fashion. In the same study *katE* expression was induced by suspending noninduced, exponentially growing cells in early stationary phase media that was buffered to pH 7.0.

2.6.4 The Heat Shock Regulon

When cells are shifted from 30°C to 42°C there is an induction of a number of unlinked genes encoding at least 17 heat shock proteins in *E. coli*. Some of the proteins are structurally or functionally analogous to the heat shock proteins of yeast, *Drosophila spp.*, and human cells (Neidhardt and VanBogelen, 1987). A gene required for the induction of these genes has been mapped to 75 min on the *E. coli*

chromosome and is named *rpoH* (formerly *htpR*; Neidhardt and VanBogelen, 1981). The *rpoH* gene encodes a protein with a M_r of 32,000 (Neidhardt *et al.*, 1983). The predicted amino acid sequence of *rpoH* resembles the predicted amino acid sequence of *rpoD*, the major sigma factor in *E. coli* (Landick *et al.*, 1984). The protein is now termed σ^{32} or σ^{H}.

The regulation of the *rpoH* gene is complex. During heat shock σ^{32} increases 20 fold within a minute of the temperature shift. After 20 to 30 minutes at the elevated temperature the level declines to about 4 times the value at low temperatures (Straus *et al.*, 1987). The *rpoH* gene is transcribed from three promoters named P1, P3 and P4. Only the P3 promoter functions following a shift to 50°C (Erickson *et al.*, 1987). The P1 and P4 promoters are recognized by RNA polymerase with σ^{70} and are not transcribed at 50°C. This is probably due to the fact that σ^{70} activity is reduced at high temperatures (Skelly *et al.*, 1987).

Recently the factors required for transcription at *rpoH* P3 have been purified. The P3 promoter is recognized by a polymerase containing a sigma subunit of M_r 24,000. This new sigma factor has been named σ^{E} (Erickson and Gross, 1990). The σ^{E} protein also directs the transcription of *htrA* (*degP*) (Lipinska *et al.*, 1988) that is required for growth and survival at high temperatures (Lipinska *et al.*, 1989).

The induction of the heat shock response (via σ^{32}) is not sufficient to induce thermotolerance (VanBolgelen, 1987) and it has been suggested that the new σ^{E} may play a role in thermotolerance (Erickson and Gross, 1990).

Exposure of mid-log cultures to heat shock has been shown to induce MnSOD (Privalle and Fridovich, 1987). It is not known whether the induction is dependent on σ^{32} or σ^{E}. Heat shock may produce

elevated levels of H_2O_2 or O_2^- in the cell possibly by disrupting the electron transport assembly within the plasma membrane. The O_2^- may then induce MnSOD (Privalle and Fridovich, 1987).

2.6.5 The Nitrogen Regulon

The nitrogen regulon is positively controlled by the protein σ^{54} (encoded by *ntrA* also known as *glnF* and *rpoN*). It was initially identified as a positive regulator of the gene encoding glutamine synthetase (Garcia *et al.*, 1977). It was later found to control the genes involved in nitrogen assimilation (such as the amino acid transport components and degradative enzymes [Magasanik, 1982]). The gene is also required for transcription of the nitrogen fixation (*nif*) genes in *Klebsiella pneumonia* (Gussin *et al.*, 1986), and *Azotobacter* (Santero *et al.*, 1986). In *E. coli* σ^{54} controls the selenopeptide of formate dehydrogenase F (*fdhF*) and hydrogenase isoenzyme 3 (*hyd3*) both of which are components of formate hydrogen lyase.

The σ^{54} consensus sequence is different from other sigma factors in a number of ways. Firstly, it does not have a -10 and a -35 consensus binding sequence. Instead, the σ^{54} promoters are characterized by a conserved GC doublet that lies 11-14 bp upstream from the transcriptional start site. Exactly 10 bp upstream from the GC doublet lies a conserved GG doublet (Kustu *et al.*, 1989). Secondly, this sigma factor requires an activator protein called NTRC, also known as NRI (encoded by *ntrC*, also known as *glnG*) (Ninfa *et al.*, 1987). NTRC catalyses the isomerization of the closed complexes between σ^{54} holoenzyme and the *gln* promoter to transcriptionally productive open complexes in which the DNA strands are locally denatured in the region

of the transcriptional start site (Popham *et al.*, 1989). The reaction requires ATP. The third difference is that σ^{54} shows little amino acid sequence homology to other sigma factors (Merric and Gibbins, 1985). Instead of sharing homologous regions with other sigma factors, σ^{54} has a glutamine rich region at its amino terminus similar to the mammalian transcription factor Sp1. The region is required for NTRC-dependent isomerization (Kustu *et al.*, 1989).

The amount of σ^{54} does not vary much under different cellular conditions. Regulation of the various genes is controlled by the amount of activator protein. For the NTRC activator, the protein is synthesized in an inactive form and is positively regulated by phosphorylation and negatively regulated by dephosphorylation. The NTRC protein is phosphorylated by NTRB, also known as NRII (encoded by *ntrB* also known as *glnL*) (Nixon *et al.*, 1986).

2.6.6 The Starvation Regulon

During the first four to five hours of starvation for carbon substrates (glucose or succinate) approximately thirty proteins are induced in *E. coli* (Groat *et al.*, 1986). Similar findings have also been demonstrated in *S. typhimurium* (Spector *et al.*, 1986). The starvation induced proteins have been shown to be involved in *E. coli* starvation survival (Reeve *et al.*, 1985) enabling it to survive starvation for several weeks (Reeve *et al.*, 1984).

The regulation of the starvation proteins is complex and probably involves a number of different regulons. Some of the proteins are synthesized transiently during starvation whereas others have a broader peak of synthesis, similar to the *Bacillus spp.* sporulation in which

proteins require the cAMP-CRP complex for induction during starvation, while the other one-third do not. All of the cAMP-dependent proteins were induced only by carbon source starvation whereas the cAMP-independent proteins were induced regardless of whether the starvation was due to carbon, nitrogen, or phosphorus limitation (Schultz *et al.*, 1988).

It has been suggested that the cAMP-induced proteins (designated cst) are probably not involved in conferring resistance to starvation. These proteins are more likely to be involved in preparing the cell for escape from starvation since many cAMP-controlled proteins mediate transport and catabolism of carbon substrates. The induction of these proteins would enlarge the range of substrates that the cell can utilize without a lag thus increasing its chances of escape (Schultz *et al.*, 1988).

Spector *et al.* (1988) used the technique of Mu d-directed *lac* operon fusions to identify 8 loci in *S. typhimurium* which exhibited increased transcription when starved for two or more of the following nutrients: nicotinate, phosphate, ammonium, glucose, or sulfate. The loci were designated *stiA* to *stiH* for starvation inducible. The *stiC* and *stiD* loci are linked at approximately 30 min. The *stiC*, *stiE*, *stiG*, and *stiH* loci mapped at approximately 77, 43, 88, and 56 min, respectively, on the *S. typhimurium* linkage map. Two of the loci (*stiC* and *stiD*) significantly decreased cell viability during prolonged periods of nicotinate starvation.

2.6.7 The Oxidative Stress Regulon

When *S. typhimurium* or *E. coli* cells are pretreated with low doses of hydrogen peroxide they become resistant to lethal doses of hydrogen peroxide (Demple and Halbrook, 1983; and Christman *et al.*, 1985). This response was sensitive to chloramphenicol demonstrating the requirement for protein synthesis. These cells were also resistant to other chemical oxidants such as N-ethlymaleimide and heating at 50°C. Increased resistance to hydrogen peroxide was accompanied by the induction of at least 30 proteins as seen on two-dimensional gels. Twelve of these proteins reached maximal synthesis 10 minutes after the treatment and the remaining 18 reached maximal synthesis 10-20 minutes from the time of hydrogen peroxide addition. The OxyR protein was found to be the positive regulator of at least nine of the thirty proteins induced by hydrogen peroxide. Three have been identified as catalase HPI (*katG*; 88 min on the *E. coli* chromosome), a novel alkylhydroperoxide reductase (*ahp*; 13.8 min) (Jacobson *et al.*, 1989) and glutathione reductase (*gsh*; 13.8 min) (Christman *et al.*, 1985).

The OxyR protein has recently been shown to protect sequences upstream from the *ahpC* and *katG* promoters (Tartaglia *et al.*, 1989). Both of the OxyR footprints of *katG* and *ahpC* extended into the -35 σ^{70} consensus binding sequence in the promoter. It was suggested that the OxyR protein may stimulate the promoters by making direct contact with the RNA polymerase at this site. There were no statistically significant similarities between the two protected regions suggesting that the secondary structure of the promoter region may be more important than the actual sequence in OxyR binding.

The *oxyR* gene has recently been sequenced (Christman *et al.*,

1989). It codes for a 305 amino acid protein with a molecular weight of 34.4 kDa. The protein shows significant homology to a new family of regulatory proteins that includes the *Rhizobium meliloti* NodD and *E. coli* LysR proteins.

The molecular mechanism by which the OxyR protein is activated by oxidative stress is beginning to emerge. Christman *et al.*(1989) has shown that a single missense mutation resulting in the change from an alanine to a valine at position 234 causes the constitutive *oxyR2* mutant. The fact that the constitutive mutant is due to a missense mutation and not due to overproduction of the *oxyR* mRNA suggests that the OxyR protein itself may be modified in order to induce the regulon under stress conditions. It was suggested that one or more of the six cysteine resides found in OxyR may be involved in an "activation by oxidation" mechanism for the induction of the *oxyR* regulon. The oxidation of a cysteine may result in conformational change in the protein that enables it to activate transcription of a specific promoter.

2.6.8 The Flagellar Regulon.

The bacterial flagellum consists of the external filament, a short curved segment called the hook, and a complex structure of rings and rods called the basal body (Macnab, 1987). There are at least forty genes in the flagellar regulon; thirty three *fla* (*flb*) genes, two motility genes (*mot*) and a single *hag* gene coding for flagellin. In addition to these genes there are numerous regulatory genes as well as genes involved in chemotaxis. The *flbB/flaI* operon positively controls all of the operons in the regulon and is itself positively controlled by

cAMP/CRP. The *flaI* gene is thought to code for a transcription factor termed σ^F (Arnosti and Chamberlin, 1989). This sigma factor recognizes the same -10 and -35 promoter sequences as σ^{28}, which is also responsible for the flagellar and chemotaxis genes of *B. subtilis*. The function of the second gene in the operon, the *flbB* gene is unknown. The remaining flagellar genes can be grouped into four blocks, the hook and basal body operons, the HAP (hook accessory proteins) operon, the flagellin operon, and the operon for motility and chemotaxis (Komeda, 1982). The *flaU* gene, a structural protein in the hook assembly, also acts as a repressor of the three remaining blocks of genes and operons. When the *flaU* gene product is incorporated into the hook assembly, the repression is alleviated. The three blocks of operons also require the product of the *flaD* gene, a positive activator protein. The *hag* gene, which codes for the flagellin protein, is controlled by another positive regulatory protein, the product of the *flaZ* gene.

2.7 *katF* and Its Role in Near-Ultraviolet Radiation Protection (NUV)

The action of NUV (290-400 nm) on *E. coli* cells is complex and has been extensively reviewed (Eisenstark, 1989 and Eisenstark, 1987). The DNA can be altered directly via direct photon action on DNA or indirectly by numerous routes including; 1) photodynamically by striking an absorbing chromophore that transfers energy to adjacent DNA; 2) via photooxidation which generates toxic oxygen species (O_2^-, H_2O_2, $OH^\cdot$, or singlet oxygen); 3) via destruction of critical enzymes that assist in DNA repair or protection; 4) via destruction of thiolated tRNA and 2-thiouracil photosensitization (Eisenstark, 1989). Most of the DNA damage is probably due to oxygen in an excited state. NUV killing was

found to be oxygen dependent and irradiation of tryptophan was found to produce H_2O_2 (McCormic *et al.*, 1976). H_2O_2 may also be a photoproduct of cysteine irradiation (Greenberg and Demple, 1986). H_2O_2 treatment at high doses has been found to cleave DNA and at low concentrations with low concentrations of ferric chloride supercoiled ϕX174 DNA was nicked (Ananthaswamy and Eisenstark, 1977). NUV may also convert H_2O_2 to O_2^- which in turn may produce $OH^\cdot$ through the Fenton reaction. These radicals can in turn act to produce DNA lesions (Hartman *et al.*, 1979, and Imlay and Linn, 1988).

A number of mutants have been constructed that are sensitive to both NUV and H_2O_2. Cells which lack the enzyme exonuclease III (*xthA* gene) are very sensitive to NUV and H_2O_2 but only slightly sensitive to alkylating agents which produce a large number of AP sites. This suggests that the DNA lesion produced by NUV and H_2O_2 is not an AP site but some other damage on which exonuclease III can act (Kow and Wallace, 1985). Strains with a mutation in DNA polymerase I (*polA* gene) which are deficient in the 5′ to 3′ exonuclease (but not in the 3′ to 5′) activity are sensitive to both NUV and H_2O_2 (Ananthaswamy and Eisenstark, 1977). During the repair process, all four bases are removed from the DNA backbone (Breimer and Lindahl, 1985).

The DNA lesion produced by NUV and H_2O_2 may be DNA-protein cross-links. The cross-links have been demonstrated in NUV irradiated phage (Hartman *et al.*, 1979; and Casas-Finet *et al.*, 1984) but not in *E. coli*. As mentioned above, single-strand breaks may also occur. The breaks produce a 3′-end blocking group that may either be a phosphoglycoaldehyde ester (Demple *et al.*, 1986) or a urea moiety (Kow and Wallace, 1985). These breaks may be activated by exonuclease III to

allow synthesis by DNA polymerase I (Demple *et al.* 1986). Endonuclease IV may also be involved in the process by initiating repair of ruptured 3'-deoxyribose. DNA polymerase III (*polC* gene) may either be required for a small (but undetectable) amount of synthesis or may provide a terminus modification function for subsequent DNA polymerase I synthesis since *polC* mutants are also sensitive to NUV (Hagensee and Moses, 1986).

Are the catalase genes involved in NUV protection? Bovine catalase added to the recovery media reduces the lethal effects of NUV (Kramer and Ames, 1987). The *oxyR* regulon is induced by both H_2O_2 and by NUV but the proteins synthesized in response to each stress are not identical (Kramer *et al.*, 1988). Mutants that lack the catalase HPI (*katG*) are not particularly sensitive to NUV (Eisenstark and Perrot, 1987) so it seems likely that genes, other than *katG*, induced by OxyR play a more important role in NUV recovery. When HPI is overproduced in the cell the strain is actually more sensitive to NUV (Eisenstark and Perrot, 1987). The HPI in this case may act as a photosensitizer when present in excess. This is very similar to the findings of Yallaly and Eisenstark (1990) who have found that the lack of Dam methylase resulted in the cells being sensitive to NUV and H_2O_2, but overproduction of the Dam methylase resulted in an increase in sensitivity rather than resistance.

Sammartano *et al.* (1986) has shown that *katF* mutants, but not *katE* mutants, are sensitive to NUV killing. Since *katE* has been shown to be the structural gene for HPII in *E. coli* (Mulvey *et al.*, 1988) it seems unlikely that HPII plays an important role in NUV defence. The *katF* gene resembles a group of regulatory genes termed sigma factors (Mulvey and Loewen, 1989). Besides *katE*, the KatF protein also is a positive

regulator of the *xthA* gene (Sak *et al.*, 1989) which has been shown to play an important role in repair of NUV lesions (see above). The *katF* and *xthA* mutants showed similar survival patterns (Sak *et al.*, 1989) suggesting that the *xthA* gene may be the only important gene in the *katF* regulon involved in NUV protection.

recA mutants are also highly sensitive to NUV when cells are in logarithmic phase but not when they are in stationary phase (Peak *et al.*, 1983; Tuveson *et al.*, 1983). The *recA* gene is induced only by far-UV light (FUV), not by NUV (Turner and Eisenstark, 1984). However, the reason other SOS genes are not induced may be that there is a transient cessation of growth and protein synthesis caused by NUV (Caldeira de Araujo and Favre, 1986). The RecA protein has a dual function: it has a recombinase activity and it acts as a protease to turn on genes involved in the SOS response. It is possible that the recombinase activity of the protein may be more important for NUV recovery (Turner and Eisenstark, 1984).

To further understand the mechanism of H_2O_2 protection in *E. coli*, the cloning and characterization of the *katE* and *katF* genes is the subject of the study presented in this thesis.

MATERIALS AND METHODS

3. Materials and Methods

3.1 Bacterial Strains, Bacteriophage, and Plasmids

Bacterial strains used in this study are listed in Table 1 along with their genotype and source. Table 2 lists the bacteriophage and Table 3 lists the plasmids used. Both tables list the important characteristics and the source.

3.2 Bacterial Growth and Storage Conditions

Unless otherwise stated, all cultures were grown at 37°C with aeration. Cultures were started in 10 ml of LB broth supplemented with the required antibiotics with a loop full of a glycerol stock. The culture was incubated overnight then stored at 4°C for up to a month. Log-phase cultures were prepared by subculturing 0.1 ml of the 4°C stock into 10 ml of LB broth and shaking for 2 to 4 hrs.

Cell densities were determined by measuring the absorbance at 600 nm or using a Klett-Summerson colorimeter equipped with a blue filter.

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.

3.3 Media and Solutions

LB Medium (Miller, 1972)

10.0 g tryptone (Difco)
5.0 g yeast extract (Difco)
5.0 g NaCl
per liter of distilled H_2O
for agar plates 10 g of agar (Difco) was added
for bacteriophage growth the media was supplemented with 0.2% maltose and 10 mM $MgCl_2$.

Table 1. List of Bacterial Strains

Strain	Genotype	Source	
MP180	*thi-1* Hfr H	Pearson (1972)	
HB101	*recA ramC pro gal rpsL leu hsdM TnsdR endI lacY*	Boyer & Roulland-Dussoix (1969)	
JM101	*supE thi* Δ(lac-proAB) [F′ *traD36 proA*$^{+}$ *pro B*$^{+}$]	Yanisch-Perron *et al.* (1985)	
NM522	*supE thi* Δ(*lac-proAB*) *hsd5* [F′ *proAb lacI*^{q} *lacZ* ΔM13]	Mead *et al. (1985)*	
Q359	P2 lysogen *hsdR hsdM*$^{+}$	Elledge and Walker (1985)	
UM120	as MP180 but *katE*::Tn*10*	Loewen *et al.* (1985)	
UM122	as MP180 but *katF*::Tn*10*	Loewen *et al.* (1985)	
UM202	as MP180 but *katG17*::Tn*10*	Loewen *et al.* (1985)	
UM255	*pro leu rpsL hsdM hsdR endI lacY katG2 katE12*::Tn*10 recA*	Mulvey *et al.* (1988)	
UM258	*pro leu rpsL hsdM hsdR endI lacY katG2 katF13*::Tn*10*	Mulvey *et al.* (1988)	
UM259	as UM255 but tet^{S}	UM255 Maloy	Tc^{S}
UM261	as UM258 but tet^{S}	UM258 Maloy	Tc^{S}
UM315	as NM522 but *katF*::Tn*10*	P1(UM122)xNM522	Tc^{R}

Table 2 Bacteriophage

Bacteriophage	Source
λEMBL3	Frischauf *et al.* (1983)
λkatE::Tn*10*	19 kb fragment from UM120 containing Tn*10* in λEMBL3
λkatE6	16.5 kb fragment from MP180 in λEMBL3 complementary to pMM120
λkatF::Tn*10*	19.4 kb fragment from UM122 containing Tn*10* in λEMBL3
λkatF3	17.2 kb fragment from MP180 in λEMBL3 complementary to pMM122

Table 3 Plasmids

Plasmid	Characteristics	Source
pBT107	Tc^R	Moyed *et al.* (1983)
pAT153	Ap^R Tc^R	Twigg & Sherratt (1980)
pMM120	Ap^R	6.2 kb *Bam*HI fragment from λ*katE*::Tn*10* in
pMM122	Ap^R	5.3 kb *Hin*dIII fragment from λ*katF*::Tn*10* in pAT153
pMMkatF1	Ap^R	10.1 kb *Bam*HI fragment from λ*katF3* in pAT153
pMMkatF2	Ap^R	4.2 kb *Cla*I fragment from pMMkatF1 in pAT153
pMMkatF3	Ap^R	4.2 kb *Cla*I fragment from pMMkatF1 in pAT153
pM13-F1	Ap^R	4.2 kb *Cla*I fragment from pMMkatF3 in KS M13-
pM13-F2	Ap^R	2.3 kb *Kpn*I fragment from pMMkatF3 in KS M13-
pM13-F4	Ap^R	1.8 kb *Kpn*I/*Dra*I fragment from pMMkatF3 in KS M13- *Kpn*I/*Eco*RV
pM13-F5	Ap^R	2.0 kb *Nru*I fragment from pMMkatF3 in KS M13- *Eco*RV
pM13-F6	Ap^R	1.7 kb *Apa*I/*Hin*cII (partial) from pM13-F2 in KS M13- *Apa*I/*Eco*RV

pGP1-2	kan^R	Tabor and Richardson (1985)
pT7-5	Ap^R	Tabor and Richardson (1985)
pT7F2	Ap^R	4.2 kb *Hin*dIII/*Eco*RI fragment from pMMkatF2 in pT7-5
pT7F3	Ap^R	4.2 kb *Hin*dIII/*Eco*RI fragment from pMMkatF3 in pT7-5
pRS415	Ap^R	Simons *et al.* (1987)
pRSE16	Ap^R	1.4 kb *Sma*I/*Eco*RV fragment from pAMkatE72 into *Sma*I of pRS415
pRSF5	Ap^R	600 bp *Eco*RI/*Dra*I fragment from pM13-F2 in pRS415 *Sma*I/*Eco*RI
pAMkatE72	Ap^R	3.1 kb *Pst*I/*Cla*I fragment containing *katE* in Bluescript KSM13+

Ampicillin and kanamycin was added to 100 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.

R-Top Agar (Miller, 1972)

10 g	tryptone
1.0 g	yeast extract
8.0 g	NaCl
8.0 g	Agar

per liter of distilled H_2O, supplemented after autoclaving with

2.0 mM	$CaCl_2$
16.6 mM	glucose

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

per liter of distilled H_2O
for agar plates add 10 g agar
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	$FeSO_4.H_{20}$
2.9 g	H_3BO_3
1.2 g	$CoSO_4.7H_2O$
0.1 g	$CuSO_4.5H_2O$
2.5 g	$Na_2MoO_4.2H_2O$
2.1 g	$ZnSO_4.7H_2O$
90 mg	$MnCl_2.4H_2O$
5.0 ml	conc. H_2SO_4

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

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

M9 medium supplemented with 1% casamino acids and after autoclaving supplemented with 0.3 μM vitamin B1 and 16.6 mM glucose.

Hershey's Salts (Worcel and Burgi, 1974)

5.4 g	$NaCl$
3.0 g	KCl
1.1 g	NH_4Cl
15 mg	$CaCl_2.2H_2O$
20 mg	$MgCl_2.6H_2O$
0.02 mg	$FeCl_3.6H_2O$
87 mg	KH_2PO_4
28 mg	Na_2SO_4
12.1 g	Trizma base

pH to 7.4 with HCl
per liter of distilled H_2O

Hershey's Medium (Worcel and Burgi, 1974)

Hershey's salts supplemented after autoclaving with 3 mM vitamin B1, 22.2 mM glucose, and 0.16 mM of required amino acids.

Maloy Medium (Maloy and Nunn, 1981)

5.0 g	tryptone
5.0 g	yeast extract
10.0 g	NaCl
50 mg	chlorotetracycline hydrochloride

after autoclaving add:

20 ml	3.5 M $NaH_2PO_4.H_2O$
6.0 ml	10 mM fusaric acid
5.0 ml	20 mM $ZnCl_2$

SM Buffer (Miller, 1972)

0.05 M	Tris-HCl pH 7.5
0.01 M	$MgSO_4$
0.1%	gelatin

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

10 mM	Tris-HCl pH 8.0
1.0 mM	EDTA pH 8.0

Phenol

phenol was distilled and buffered with

0.1 M	NaCl
0.1 M	Tris-HCl pH 7.6
1.0 mM	EDTA

3.4 P1 Transductions

A P1 lysate was prepared by growing the appropriate strain to mid-log phase in 10 ml of LB broth. The culture was centrifuged at 10,000 rpm for 5 min and the pellet was taken up in 2.5 ml of SM, 0.05 ml of 1.0 M $CaCl_2$, and 0.1 ml of 1.0 M $MgSO_4$. A 0.1 ml aliquot of the culture was mixed with 20 μl of $P1_{(vir)}$ and incubated at 37°C for 30 min. Three ml of molten R-top was added and the lysate was poured onto fresh LB plates. The plates were incubated inverted overnight. The next day the R-top was scraped off the plates and mixed with 2.0 ml of LB and 0.5 ml of chloroform in a 10 ml Corex centrifuge tube. The lysate was vortexed for 1 min and left at room temperature for 1 hr. The debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C and the supernatant was collected as the P1 lysate.

The strain to be transduced was grown to mid-log phase in 10 ml of LB broth, then centrifuged and resuspended as above. A 0.1 ml aliquot of cells was mixed with 0.1 ml of undiluted, 10^{-1}, and 10^{-2} dilutions of the lysate prepared above. After 45 min at 37°C, 0.2 ml of 1.0 M sodium citrate was added along with 3.0 ml of molten R-top and the mixture was plated on the appropriate selective medium. After 24-48 hrs the colonies were picked and assayed for the presence or absence of the particular genetic marker.

3.5 Genomic DNA Isolation

Media

500 ml of LB supplemented with the appropriate antibiotics if necessary in 2.0 l fluted flask. A fresh overnight culture of *E.coli* was used as an inoculum.

Solutions

Saline-EDTA
0.15 M NaCl
0.10 M EDTA pH 8.0

Saline-EDTA-Lysozyme
same as Saline-EDTA but contains 2.0 mg of lysozyme per gram of cells.

Tris-SDS
0.1 M Tris-HCl pH 7.6
1.0% SDS (w/v)
0.1 M NaCl

Sodium Citrate (10X)
0.150 M NaCl
0.015 M trisodium citrate (pH 7.0)

A fresh 5.0 ml overnight culture was used to inoculate a 2 l fluted flask containing 500 ml of LB media along with the appropriate antibiotics if necessary. The flask was then incubated at 37°C with vigourous shaking overnight. The next day the cells were collected by centrifugation for 10 min at 5000 rpm at 4°C. The cells were washed once by resuspension in Saline-EDTA, then centrifuged as above. The pellet was then resuspended in Saline-EDTA-Lysozyme to a concentration of 1 g of cells per ml. The suspension was incubated at 37°C for 30 min, then frozen at -60°C. To the frozen cells, 10X the volume of a Tris-SDS solution was added and the cells were resuspended as the mixture thawed. The freeze thaw cycles were repeated two more times before an equal volume of TE saturated phenol was added. The emulsion was shaken for 20 min at 4°C, then separated in 150 ml Corex centrifuge bottles at 5000 rpm for 10 min at 4°C. The aqueous phase was removed and 2 volumes of 95% ethanol were added. The precipitate was collected by centrifugation in 150 ml Corex centrifuge bottles and the pellet was dissolved in 20 ml of sodium citrate. The RNA was removed by the addition of 1.0 ml of a 1.0 mg per ml solution of RNase

A (Sigma), and incubated for 30 min at 37°C. The DNA was precipitated by the addition of 300 ml of 3.0 M sodium acetate (pH 6.0) and one volume of room temperature 95% ethanol. The mixture was incubated at room temperature for 10 min and the DNA was collected by centrifugation in 30 ml Corex centrifuge tubes at 8000 rpm at 4°C for 10 min. The procedure was repeated until a clear supernatant was obtained. The final pellet was dissolved in TE buffer to an approximate concentration of 1.0 mg per ml.

3.6 Construction of Genomic Libraries

3.6.1 Preparation of 15-20 Kb Fragments

The preparation of 15-20 Kb fragments is a slightly modified version of Maniatis *et al.* (1982).

A reaction mixture was prepared containing 10 μg of genomic DNA, 15 μl of 10X *Sau* 3A buffer, and distilled water to a final volume of 150 μl. Thirty μl of the mixture was dispensed into tube 1 and 15 μl were dispensed into the tubes labelled 2 thru 9. Once all the tubes were cooled on ice, 4 units of *Sau* 3A were added to tube one then mixed. The concentration of the enzyme in tube one was 2 units per μg of DNA. Fifteen μl of the reaction mixture from tube one were transferred to tube two. The enzyme concentration was diluted two fold to 1 unit per μg of DNA. This two fold serial dilution was continued through to tube eight, tube nine was left as a control containing no enzyme. All the tubes were incubated at 37°C for one hour. The reactions were stopped by the addition of 3 μl of gel loading dye and then the samples were electrophoresed through a 1.0% agarose gel along with λ*Hin*dIII DNA standards at approximately 100 V. After 3 to 4 hrs, the gel was stained in a 0.5 μg

per ml ethidium bromide solution and a photograph was taken. The concentration of enzyme (units per μg of DNA) found to give the maximum fluorescence in the 15-20 kb range was chosen for use in a large-scale genomic DNA digest.

3.6.2 Large Scale Genomic DNA Digest

With the concentration of enzyme that yields maximum 15-20 kb fragments ascertained, a large scale digest was set-up using 100 μg of DNA. This digest was identical to the small scale digest but 100 fold larger. After one hr at 37°C, a 15 μl sample was loaded onto a 1.0% agarose gel and electrophoresed at 100 V for 3 hrs. This step was performed to verify a partial digest in the range of 15-20 kb. While the sample was electrophoresed, the remainder of the digest was extracted with phenol, phenol:chloroform, and finally chloroform. One tenth the volume of 3 M sodium acetate (pH 4.8) was added to the aqueous phase along with two volumes of ice cold 95% ethanol. The DNA was allowed to precipitate for 20 min at -20°C, then centrifuged at 8000 rpm for 10 min at 4°C. The pellet was dried under vacuum, then resuspended in 150 μl of TE buffer. After a 10 min incubation at 65°C, 75 μl of the solution was loaded onto a 10-40% continuous sucrose gradient and centrifuged in a SW50.1 rotor at 30,000 rpm for 18 hrs at 15°C. After the centrifugation, approximately 100 μl samples were collected from the bottom of the tube. Every second fraction was electrophoresed along with λ*Hin*dIII DNA markers on a 1.0% agarose gel at 100 V for 3-4 hrs. The gel was stained with ethidium bromide and a photograph was taken. Fractions containing fragments between 15-20 kb were pooled, diluted two fold with distilled water, and precipitated with ice cold 95% ethanol. The pellet was dried under vacuum

and dissolved in 50 μl of TE buffer. The DNA concentration was estimated by comparison to λ*Hin*dIII DNA on a 0.7% agarose gel.

3.6.3 Ligation of 15-20 Kb Fragments into λEMBL3

10X Ligation Buffer

0.02 M Tris-HCl pH 7.6
0.01 M $MgCl_2$

Approximately 0.5 μg of 15-20 kb fragments were mixed with 1.0 μg of λEMBL3 arms (BRL) along with 0.5 μl of 10x ligation buffer, 0.5 μl of 6 mM ATP, 0.5 μl 0.1 M DTT, 1 unit T4 DNA ligase (Boehinger Mannheim), and sterile water to 5.0 μl. The ligation was carried out at 5°C for 15 hrs. After the ligation reaction, 2.0 μl of the mixture was added to 3.0 μl of gel loading buffer and electrophoresed through a 1.0% agarose gel along with 15-20 kb fragments and λ*Hin*dIII DNA as standards. After 3 hrs at 100 V, the gel was stained with a 0.5 μg per ml solution of ethidium bromide and examined under ultraviolet light to determine if the ligation had taken place. If the DNA appeared to ligate, the remaining 3.0 μl of ligation mixture was packaged using an *in vitro* packaging kit (Boehinger Mannheim).

3.6.4 Packaging λEMBL 3

The procedure used for the packaging reaction was previously described as per Boehringer Mannheim instructions contained in the kit. Briefly, a pipet tip containing sonic extract was placed in an Eppendorf tube containing freeze thaw lysate and both were allowed to thaw on ice. While the extracts were being thawed, 3 μl of the ligation mixture was added. After complete thawing, the mixture was carefully homogenized and

then centrifuged in an Eppendorf centrifuge for 10 sec. The mixture was incubated at room temperature for one hr and then diluted with 0.5 ml of SM buffer. Finally, 10 μl of chloroform was added and the dilution was stored at 4°C.

3.7 Titration and Amplification of the Library

3.7.1 Preparation of Cells for Infection

A 10 ml LB broth supplemented with maltose to 2% was inoculated with 0.1 ml of a fresh overnight culture of Q359. This strain selects for recombinant phage via the spi selection (Elledge and Walker, 1985). The culture was incubated with shaking at 37°C. When the culture reached approximately 50 Klett units, it was centrifuged in a 10 ml Corex centrifuge tube at 5,000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in 5.0 ml of a sterile 0.1 M $MgSO_4$ solution and stored on ice for no more than 2 hours before use.

3.7.2 Titration of Lambda

The titration of λ phage has been previously described by Maniatis *et al.* (1982). All dilutions were performed in SM buffer. Ten fold dilutions were carried out using 1.0 μl of the λ phage and 9.0 μl of SM. One μl of each dilution was used to infect 100 μl of Q359 as prepared above. The phage and cells were mixed and then placed in a 37°C water bath for 20 min without shaking to allow for infection. During the incubation, 3 ml of melted R-top was dispensed into prewarmed sterile tubes then placed in a heating block set at 45°C. After the incubation, 3 ml of R-top was added to each of the phage-cell mixtures and then each was mixed and

immediately poured and spread evenly onto LB plates. After allowing 5 min for the R-top to solidify, the plates were inverted and incubated over night at 37°C. The next day, plates containing between 30 and 300 plaques were counted and the titer determined.

3.7.3 Amplification of the Library

After the titer of the library had been determined, an amplification step was then used to increase the titer. This has been previously described by Frischauf *et al.*, (1983). Infections were carried out using an appropriate amount of phage to yield confluent lysis on 5 freshly poured LB plates. After incubating the plates overnight at 37°C, the R-top was scraped off of the LB plates using a sterile spatula or glass spreading rod and the R-Top was put into a 30 ml Corex centrifuge tube. One ml of SM was added to each plate, then swirled, and finally removed using a sterile Pasteur pipette into the tube containing the R-top. Approximately 5 drops of chloroform were added per plate and the tube was vortexed for one minute. After half an hour at room temperature, the R-top was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was decanted and 3 drops of chloroform was added before the lysate was stored at 4°C.

3.8 Large Scale Lambda Phage DNA Isolation

A rapid biochemical procedure has been developed by Klaslow (Klaslow, 1986) for the isolation of λ DNA from liquid lysates. Approximately 250-500 μg of purified λ DNA can be isolated from a 100 ml liquid lysate.

Liquid lysates were prepared as per Maniatis *et al.* (1982).

Any remaining cells in the 100 ml liquid lysate prepared above were lysed and the genomic DNA and RNA were digested by the addition of chloroform to 2%, DNase I and RNase A to 1.0 μg per ml, and solid NaCl to a final concentration of 1 M. The mixture was incubated at 37°C with shaking for 30 min. The cell debris was removed by centrifugation at 5000 rpm for 10 minutes at 4°C. Solid polyethylene glycol 6000 was added to the supernatant to 10% (w/v) and the phage were precipitated overnight at 4°C. The next day the phage were pelleted at 7000 rpm for 20 min at 4°C. The pellet was resuspended in 3 ml of SM and any remaining traces of bacterial genomic DNA and RNA were removed by the addition of DNase I and RNase A to 5.0 μg per ml and 100 μg per ml respectively. After a 30 min incubation at 37°C, the phage were lysed by the addition of SDS to 0.5%, 0.5 M EDTA (pH 8.0) to 20 mM, and Proteinase K to 100 μg per ml. The mixture was heated to 68°C for 30 min. The protein was removed by phenol, phenol:chloroform, and chloroform extraction. The phage DNA was precipitated by the addition of 0.5 volumes of 5 M ammonium acetate and 2 volumes of ice cold 95% ethanol. After a 15 min incubation on ice, the DNA was pelleted by a 15 min centrifugation at 10,000 rpm at 4°C. The pellet was dissolved in 1.6 ml of sterile water. Any remaining traces of RNA were removed by preferentially precipitating the phage DNA by the addition of 0.4 ml of 4 M NaCl and 2.0 ml of 13% polyethylene glycol 6000 (w/v). After a one hour incubation on ice, the DNA was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C. The pellet was washed once with 95% ethanol, dried under vacuum, and finally dissolved in 0.5 ml of TE buffer.

3.9 DNA Cloning

3.9.1 Restriction Enzyme Digests

Restriction enzymes used were purchased from either Boehringer Mannheim, Pharmacia, or BRL. Unless otherwise stated, restriction reactions were carried out in 10 or 20 μl volumes using 1 to 10 U of restriction enzyme and the recommended buffer. When digests were carried out with more than one enzyme, the buffer that gave the highest activity for both enzymes was chosen. All reactions were carried out for a minimum of 1.5 hrs at 37°C, except for the enzymes *Sma*I and *Taq*I which were carried out at 30°C and 65°C respectively.

3.9.2 Agarose Gel Electrophresis of DNA

3.9.2.1 Preparation of the Gel

10X TBE
108 g Trizma Base
55 g Boric acid
8.5 g EDTA
per liter of distilled H_2O

10X TAE
48.4 g Trizma Base
11.4 ml glacial acetic acid
3.7 g EDTA
per liter of distilled H_2O

Tracking Dye
200 mM EDTA
35% glycerol
25 mg Bromophenol Blue
in 10 ml of distilled H_2O

The 0.7% to 1.2% agarose gels were prepared by boiling the appropriate amount of agarose (Sigma type II: medium EEO) in 1X TBE or 1X TAE until the agarose was dissolved. The TAE buffer was used when a DNA

fragment was to be Genecleaned, the TBE has a higher buffering capacity and can be reused for more gel runs. When the agarose had cooled to approximately 55°C, the gel was poured and a well maker was placed into the molten agarose. After the agarose solidified, the well maker was removed and the gel was placed in a Bio-Rad DNA Sub Cell apparatus containing either 1X TBE or 1X TAE.

Tracking dye was added to DNA samples to a final concentration of 10% and samples were pipetted into the submerged wells. The gel was electrophoresed for either 2-4 hrs. at 90 V or overnight at 15 V.

3.9.2.2 Pulse Field Gel Electrophoresis

Pulse field gel electrophoresis was used for sizing large fragments of DNA (15-40 kb) on agarose gels. This procedure was used in mapping the λ clones. A 0.7% agarose gel was prepared as above and samples were run through at 90 V. When the tracking dye was three quarters of the way into the gel it was stained and a photograph was taken to facilitate the sizing of small DNA fragments. The gel was then placed into the pulsing apparatus which was a normal DNA Sub Cell that allowed the buffer to be circulated and cooled via a parastolic pump. The buffer used was 0.5X TBE. The pulsing was controlled by a computer program which was set to allow maximum separation between 20-40 kb. After pulsing the gel for approximately 2 hrs, a photograph was taken and the large fragments were sized.

3.9.2.3 Visualization, Photographing and Sizing of DNA

The agarose gel was placed into 1X TBE or 1X TAE containing 0.5 mg/ml of ethidium bromide for 20 min. The gel was then photographed on a

transilluminator with a Polaroid MP4 Land Camera equipped with a Kodak 22A Wratten filter and Polaroid Type 667 film. Typical exposure times ranged from 3 to 5 sec depending on the intensity of the DNA bands.

The sizes of unknown DNA fragments were determined by measuring the distance from the origin and then comparing them with known DNA size markers. The markers used were either λ DNA digested with *Hin*dIII or BRL 1 kb Ladder. The distance the standard markers migrated from the origin was plotted as a function of the ln of the size (bp) to obtain a standard curve.

3.9.3 Isolation of DNA from Agarose Gels.

Isolation of DNA from agarose gels was achieved using the Geneclean procedure from BIO 101 Inc.. The DNA band selected for purification was excised from an ethidium bromide-stained agarose gel run using 1X TAE buffer. The gel slice was weighed and placed in an Eppendorf tube. Sodium iodine was added to the gel slice to give a concentration close to 4 M. The tube was then heated to 55°C for 5 min or until the agarose slice was completely dissolved. Five μl of glassmilk was added, the solution was mixed, and then placed on ice for 15 min. The glassmilk was pelleted by a 5 sec spin in a microfuge. The pellet was washed three times with 0.5 ml of NEW Wash (supplied with the kit). After the third wash the glassmilk pellet was resuspended in 5 μl of sterile distilled water. The DNA was eluted from the glass beads by a 55°C incubation for 3 min. The beads were again pelleted by a 30 sec spin and the supernatant was removed and stored at 4°C until used.

3.9.4 Ligations

Typical ligation reactions contained approximately 0.5 μg of the appropriate digested vector DNA and 0.5 to 1.0 μg of insert DNA. Both the vector and the insert DNA had the restriction enzymes inactivated either at 65°C for 30 min or by phenol extraction followed by ethanol precipitation. BRL ligation buffer was added to a final concentration of 1X and 2 U of T4 DNA ligase (BRL) were added. Final ligation volumes were 15 μl unless otherwise specified. Typical ligations were carried out at room temperature for 3 to 4 hrs.

3.9.5 Transformations

Transformations of *E. coli* were preformed as per Chung *et al.* (1989) with slight modifications. A 10 ml LB broth culture of the strain to be transformed was grown to mid-log phase. The cells were transferred to a 10 ml Corex centrifuge tube and spun at 5,000 rpm for 10 min at 4°C. The cell pellet was gently resuspended in 0.5 ml of ice cold 0.1 M $CaCl_2$. A 0.1 ml aliquot was mixed with the DNA to be transformed and placed on ice for 15 min. The mixture was heat shocked at 42°C for 90 sec and then diluted with 0.4 ml of LB broth. After 1 hr at 37°C the cells were plated onto 3 LB plates with the appropriate antibiotics and incubated inverted overnight.

3.9.6 Recombinant Selection

3.9.6.1 pAT153 Selection

The plasmid pAT153 is 3.6 kb in size and contains two genes that confer resistance to ampicillin and tetracycline. *Bam*HI and *Cla*I fragments containing the *katF* gene were cloned into the unique *Bam*HI and

ClaI sites in the Tc^R gene. Transformed cells were plated onto LB amp allowing both recombinants and nonrecombinants to grow. Colonies were then picked onto LB amp and tet plates. Cells which did not grow on LB tet presumably contained inserts and DNA was isolated and characterized from these cells. To identify the inserts containing the *katF* gene, the plasmid was transformed into UM258. This strain is tet resistant and could not be used for the initial screening. If the *katF* gene was encoded on the plasmid it would complement the *katF* mutation in the genome and revert the strain from a catalase negative to a catalase positive phenotype.

3.9.6.2 M13 mp18/19 Recombinants

The M13mp18/19 vector system is used primarily for subcloning small fragments of DNA for dideoxy sequence analysis. The vectors secrete single stranded DNA which is used as templates in the sequencing process. The vector also contains a multiple cloning site located in the ß-galactosidase gene. When the phage infect a lac^- host, nonrecombinant phage produce blue colored plaques while recombinant phage produce colorless plaques when plated on X-gal IPTG plates.

3.9.6.3 Bluescript M13 Recombinants

The Bluescript vector is very similar to the M13mp18/19 vector system. The vector contains a multiple cloning region in the ß-galactosidase gene allowing for color selection of recombinants. The difference between the two systems is that the Bluescript exists as a plasmid conferring Ap^R to its host. The plasmid also contains M13 sequences allowing the plasmid, or phagemid as it is sometimes called, to produce

single stranded DNA when infected with a helper phage. This enables the isolation of single-stranded template for dideoxy sequencing of the inserted fragment.

3.9.6.4 pT7-5 Recombinants

The pT7-5 plasmid contains a small multiple cloning site in front of a T7 polymerase promoter as well as an Ap^R gene. Transformed cells were plated on LB amp plates allowing both non-recombinants and recombinants to grow. Inserts were identified by plasmid isolation and restriction enzyme mapping.

3.9.6.5 pRS415 Recombinants

The plasmid pRS415 contains a multiple cloning site in front of a promoterless ß-galactosidase gene as well as an Ap^R gene. Transformants were plated on LB amp and inserts were identified by plasmid isolation and DNA restriction enzyme mapping.

3.10 Plasmid DNA Isolation

3.10.1 Rapid Lysates

STE Buffer

100 mM NaCl
20 mM Tris-HCl pH 7.5
10 mM EDTA

Isolation of plasmid DNA from overnight bacterial colonies was performed as described in the Bluescript Manual. Most of a 1.0 cm colony streak was picked with a sterile toothpick and resuspended in 30 μl of STE

buffer. After the colony was resuspended, 20 μl of TE saturated phenol was added and the mixture was vortexed for 30 sec. The mixture was centrifuged for 3 min and 20 μl of the aqueous phase was removed and mixed directly with 4 μl of tracking dye on Parafilm. Approximately 20 μl of this mixture was dry loaded onto a 1.0% agarose gel. Once the samples were run into the gel, 1X TBE buffer was added to completely submerge the gel and the electrophoresis was allowed to continue for about 1 hr at 100 V. The gel was stained in ethidium bromide and examined under UV light.

3.10.2 Alkaline DNA Extraction

Lysozme Solution
50 mM Glucose
10 mM EDTA
25 mM Tris-HCl pH 8.0
25 mg/ml lysozme

Alkaline-SDS
10 % SDS (w/v)
0.2 M NaOH

High Salt
3.0 M potassium acetate
1.2 M formate

Acetate-MOPS
0.05 M MOPS
0.1 M sodium acetate pH 8.0

This plasmid preparation (Birmboim, 1983) was initially used to isolate plasmid DNA suitable for restriction enzyme digests. The pellet from 0.5 ml of an overnight culture grown in LB with the appropriate antibiotics was resuspended in 0.15 ml of lysozyme solution. After 5.0 min on ice, 0.3 ml of alkaline-SDS was added and the suspension was gently mixed and placed on ice for 5.0 min. A 0.225 ml volume of high salt was added and the solution was mixed gently and placed on ice. After 15 min

the cellular debris was removed by centrifugation in a microfuge for 10 min at 4°C. The supernatant was removed and added to a tube containing 0.9 ml of ice cold 95% ethanol and was placed at -20°C for 15 min. The precipitate was pelleted by a 10 min centrifugation at 4°C. The pellet was drained and dissolved in 0.15 ml of Acetate-MOPS and reprecipitated with 0.3 ml of ice cold 95% ethanol. The DNA was pelleted by a 10 min centrifugation at 4°C and the pellet was vacuum dried and resuspended in 50 μl of sterile water. The solution was stored at -20°C.

3.10.3 Rapid Plasmid Preps.

Lysozyme Solution
this solution is identical to the lysozyme solution above.

NaOH/SDS
0.2 M NaOH
1.0 % SDS

This rapid plasmid preparation was previously described by Morelle (1989) and was used to replace the alkaline extraction procedure above. The pellet from a 1.5 ml overnight LB culture grown with the appropriate selection pressure was resuspended in 200 μl of lysozyme solution. After a 5.0 min incubation at room temperature, the tube was placed on ice and 400 μl of NaOH-SDS was added. The solution was gently mixed and left on ice. After 5 min, 300 μl of 7.5 M ammonium acetate (pH 7.8) was added and the solution was gently inverted several times. After 10 min on ice, the debris was removed by a 15 min centrifugation at 4°C. The supernatant was transferred to a clean tube and the centrifugation was repeated. The DNA was precipitated by removing the supernatant into a tube containing 0.5 ml of 25°C isopropanol. After 10 min at room temperature the DNA

was pelleted by centrifugation at 4°C in a microfuge for 15 min. The pellet was washed twice with 70% ethanol and dried under vacuum. The dried pellet was resuspended in 25 μl of sterile water and stored at -20°C.

3.10.4 Large Scale Plasmid DNA Isolation.

The method of Birnbiom and Doly (1979) was employed to isolate plasmid DNA in quantities of up to 1 mg.

Lysis Buffer
25 mM Tris-HCl pH 7.6
10 mM EDTA
50 mM glucose
2.0 mg per ml Lysozyme

NaOH-SDS
0.2 M NaOH
1.0% SDS (w/v)

Tris-Acetate-EDTA-SDS (TAES)
40 mM Tris-HCl pH 7.6
0.1% SDS (w/v)
1.0 mM EDTA
0.1 M Sodium Acetate

Five ml of a fresh 10 ml overnight culture was used to inoculate a 2 l fluted flask containing 500 ml of LB broth supplemented with the appropriate antibiotics. The culture was shaken vigorously at 37°C for approximately 4-6 hours before a 0.5 ml solution of a freshly prepared 170 mg per ml chloramphenicol solution (dissolved in 95% ethanol) was added to every 500 ml culture. The incubation was continued overnight (Clewell, 1972).

The next day, the cells were pelleted in plastic bottles at 5000 rpm for 10 minutes at 4°C. The pellet was resuspended in 40 ml of Lysis Buffer and the suspension was incubated on ice for 30 minutes. After the incubation, 80 ml of a freshly prepared NaOH-SDS solution was added and

mixed gently. After 5 minutes on ice, 60 ml of a 3.0 M sodium acetate solution (pH 4.8) was added and the mixture was gently inverted before being placed on ice for one hour. The lysed cell mixture was then centrifuged in plastic 30 ml tubes at 12,000 rpm for 10 minutes at 4°C to remove the cell debris. Two volumes of ice cold 95% ethanol were added to the supernatant. After 20 minutes at -20°C, the precipitate was pelleted at 5,000 rpm for 10 minutes at 4°C in 150 ml Corex centrifuge bottles. The pellet was dissolved in 40 ml of TAES and then the solution was extracted with 40 ml of a phenol:chloroform mixture (1:1). The phases were separated by centrifugation at 5,000 rpm for 10 minutes at 4°C. The aqueous phase was saved while the phenol:chloroform was re-extracted with 40 ml of TAES then centrifuged as above. The two aqueous phases were combined and 2 volumes of ice cold 95% ethanol were added. After 20 minutes at -20°C, the precipitate was collected by centrifugation at 5,000 rpm 4°C for 10 minutes. The pellet was dissolved in 10 ml of sterile distilled water. Four ml of 4 M sodium acetate (pH 8.0) was added followed by the addition of 25 ml of ice cold 95% ethanol. Following centrifugation at 5,000 rpm for 10 minutes at 4°C, the pellet was resuspended and precipitated as above. The pellet from the second precipitation was dissolved in 4.0 ml of distilled sterile water. To remove any RNA, 0.2 ml of a DNase free, RNase A solution (2.0 mg per ml) was added and incubated at 37°C for 30 minutes. The plasmid DNA was preferentially precipitated by the addition of 75 μl of 4 M sodium acetate (pH 6.0) plus one volume of room temperature 95% ethanol. After 10 minutes at room temperature, the plasmid DNA was pelleted at 10,000 rpm at 4° C for 10 minutes. The pellet was dissolved in 4 ml of distilled sterile water and the above procedure was repeated until a clear supernatant was

obtained. The final pellet was dissolved in TE buffer to a final concentration of approximately 1.0 mg per ml.

3.11 DNA/DNA Hybridizations

3.11.2 Southern Blots

Denaturing Solution
1.5 M NaCl
0.5 M NaOH

Neutralizing Solution
1.0 M Tris-HCl pH 8.0
1.5 M NaCl

20X SSC
3.0 M NaCl
3.0 M sodium citrate

Southern blots were prepared as per Maniatis *et al.* (1982). DNA samples to be analyzed were electrophoresed on agarose gels. The gels were stained and photographed with a ruler along the side so as to visualize the actual size of the gel in the photograph. The excess agarose was removed and the gel was placed in several volumes of denaturing solution and left at room temperature for 1 hr with gentle agitation. The denaturing solution was removed and the gel was rinsed with distilled H_2O before the addition of several volumes of neutralizing solution. The neutralization was allowed to continue for 1 hr at room temperature. A piece of Whatman 3MM paper was placed on top of a glass plate so that each end of the paper was submerged in a tray of 10X SSC allowing it to act as a wick. The gel was placed on top of the filter paper and surrounded with Saran Wrap to prevent the buffer from moving around the gel. A nitrocellulose filter cut to the same dimensions as the

gel was soaked in 2X SSC and placed on top of the gel. Two pieces of filter paper were placed on top of the filter. At each stage of the layering, any air bubbles were removed by smoothing the bubbles out with a glass rod. A 3-5 inch stack of paper towels was placed on top of the filter paper to draw the buffer up through the gel and filter. A weight was placed on top of the stack and the transfer was allowed to continue for 16 hrs. The filter was removed and soaked in 6X SSC for 5 min before it was air dried and baked at 80°C for 2 hrs.

Blots prepared with nylon filters were set up the same way except the DNA was crosslinked by UV light for 2 min on a transilluminator instead of by baking.

3.11.2 Plaque Blots

20X SSPE

3.6 M NaCl
200 mM NaH_2PO_4 pH 7.4
20 mM EDTA

The transfer of phage particles to nitrocellulose was achieved by the method of Maniatis *et al.* (1982). The phage were grown on LB plates as per section 3.7.2.

The plates containing the plaques to be blotted were placed at 4°C for at least 1 hr to allow the top agar to harden. A nitrocellulose filter was labelled and carefully placed on to the surface to avoid the formation of any air bubbles. The filter and plate were marked in 3 locations for identification purposes. After 60 sec the filter was peeled off the top agar and placed on top of denaturing solution saturated Whatmann 3MM paper with the plaque side up. This prevents any smearing of the plaques. The filter was then transferred to Whatmann 3MM saturated

neutralizing solution. After 60 sec the blots were transferred to a solution of 2X SSPE for 5 min before being placed on Whatman 3MM paper to air dry at room temperature. The blots were wrapped in foil and then baked for 2 hrs at 80°C. The blots were stored under vacuum until used.

3.11.3 Nick Translations

5X Nick Translation Buffer
0.1 mM dCTP (Pharmacia)
0.1 mM dGTP (Pharmacia)
0.1 mM dTTP (Pharmacia)
0.05 M $MgCl_2$
0.5 mM DTT (BMC)
0.25 M Tris-HCl pH 7.6
250 μg/ml BSA (BMC)
prepared using sterile HPLC H_2O

DNaseI
0.1 μg/ml DNaseI in 1X nick translation buffer containing 50% glycerol

Sephadex G-50 (Sigma)
mixed with several volumes TE buffer and allowed to expand overnight at 4°C

The nick translation procedure was a slight modification from Maniatis *et al.* (1982). The reaction mixture was set up as follows:

10 μl 5X nick translation buffer
1 μg DNA
5 μl α^{32}P-dATP (1 mCi/ml)
1 μl DNA polymerase I (7300 μ/ml)
sterile HPLC H_2O to 50 μl

Immediately after the DNaseI was added the solution was mixed and incubated at 16°C for 1 hr. The unincorporated nucleotides were separated from the labelled DNA by loading the mixture onto a Sephadex G-50 column. The peaks were localized using a Geiger counter and the faster moving peak was collected. A 1 μl aliquot was counted in a liquid scintillation counter and the DNA was stored at -20°C until used.

3.11.4 Hybridization

10X SSC
1.5 M NaCl
1.5 M sodium citrate

50X Denhardt's Solution
5.0 g Ficoll
5.0 g polyvinylpyrrolidine
5.0 g BSA

Salmon Sperm DNA
stock solution (1.0 mg/ml) prepared by passage through a 20 gauge needle. The solution was boiled for 5 min before being stored at 20°C

Prehybridization Solution
6x SSC
0.5% SDS
5X Denhardt's Solution
100 μg/ml Salmon Sperm DNA

Hybridization Solution
Prehybridization solution plus 0.01 M EDTA.

The nitrocellulose filter to be hybridized was immersed in 6X SSC for 2 min before being placed in a bag with approximately 0.2 ml/cm^2 of prehybridization solution. If nylon filters were used the initial step was omitted and the filters were placed directly into a bag containing prehybridization solution. The prehybridization took place for 2-4 hrs at 65°C with gentle agitation. The solution was replaced with an equal volume of hybridization solution preheated to 65°C. Approximately 1x10^6-1X10^7 cpm of DNA probe, boiled for 5 min, was added to a bag and sealed. This bag was placed into a second bag to avoid leakage and the hybridization was allowed to continue overnight at 65°C. The next day the hybridization solution was removed and the filter was washed with 2X SSC/0.5% SDS for 5 min with gentle agitation. The filter was then transferred to a second container containing 2X SSC/0.1% SDS and the washing was continued for another 15 min before being transferred to a solution of 0.1X SSC/0.5% SDS

for 2 hrs at 68°C. Nitrocellulose filters were removed, dried and wrapped in Saran Wrap. Nylon filters were removed and wrapped wet in Saran Wrap to prevent the probe from irreversibly attaching to the filter.

The filters were exposed to Kodak X-Omatic-AR X-ray film using a Picker Source One cassette containing one or two Cronex Quanta III intensifying screens. The film was exposed between 6 and 72 hrs before being developed. Developing was achieved by placing the exposed film in developer for 3 min, rinsing with water, and finally placing it in fixer for 3 min. The film was rinsed in water and dried before being analyzed.

3.12 Maxicell Determination of Plasmid Encoded Proteins

The KatF protein size was determined by maxicell analysis as described by Sancar *et al.* (1979).

Cells containing the appropriate plasmid were grown to mid-log phase in K medium with the appropriate antibiotic selection. The cells were transferred to a petri dish and irradiated (lid off) with a Mineralight 115 V UV lamp at a distance of approximately 36 cm for 30 sec at room temperature. At this fluence rate, UM255 harbouring the plasmid pMMkatF2 was 95% killed. After shaking the irradiated cells in a sterile 125 ml flask at 37°C for 1 hr, cycloserine was added to 100 μg per ml and the mixture was shaken overnight at 37°C. The cells were collected by centrifugation and washed twice in Hershey's salts. The pellet was resuspended in 5.0 ml of Hershey's medium after which 20 μCi of [^{14}C]amino acids (NEN) was added. The plasmid encoded proteins were allowed to label at 37°C for 1.5 hrs before being collected by centrifugation. The cells were washed once in SM buffer before being resuspended in 50 μl of sample buffer and stored at -20°C.

A 25 μl sample was thawed and mixed with tracking dye (0.25% bromophenol blue) before being boiled for 3 min and loaded onto an 8% SDS-polyacrylamide gel. The samples were electrophoresed, stained, and destained as described in section 3.14.2.

The gel was then treated as per Boulnois and Timmis (1984) before exposure to X-ray film. The fixer was replaced with 2 changes of DMSO over a period of 1 hr before a solution of 22% (w/v) PPO in DMSO was added. After 1 hr the solution was replaced with water. The water was changed several times over the course of 1 hr before the gel was dried and exposed to X-ray film.

3.13 Dideoxy Sequence Anlysis

3.13.1 M13 RF DNA Prep

A 1 ml inoculum of an overnight JM101 culture was used to start a 100 ml broth culture of LB. The culture was incubated with shaking at 37°C for 1.5 hrs. A 5.0 ml aliquot was used to start a 500 ml broth of LB. This culture was incubated at 37°C for 1.5 hrs with shaking before a 1 ml inoculum of M13 phage was added. The phage inoculum was prepared by inoculating a 1.5 ml broth culture of JM101 with a single blue plaque and then growing the mixture overnight. The cells in the 500 ml flask were harvested after 6 hrs and washed once in SM buffer. The replicative form (RF) DNA was isolated using the large scale plasmid preparation in section 3.10.4.

3.13.2 Cloning using M13 Vectors

The restriction enzyme digests, ligations and transformations were

carried out as described in section 2.9. The transformations were heat shocked for 15 min at 37°C instead of 90 sec at 45°C. Immediately after the heat shock, 50 μl of a 100 mM solution of IPTG, 50 μl of a 2% X-gal solution, and 0.2 ml of a log phase culture of JM101 were mixed with the transformation mixture and 2.5 ml of R-top and the mixture was plated on LB plates. The plates were incubated overnight at 37°C inverted.

3.13.3 Construction of *Sau*3A Deletion Mutants

Approximately 2 μg of plasmid DNA was linearized by *Bam*HI digestion in a reaction volume of 20 μl. The digest was carried out at 37°C for 2 hrs. A 2 μl sample was electrophoresed and examined to ensure complete digestion. The volume was brought up to 50 μl by the addition of 5 μl of 10X *Sau*3A and 27 μl of sterile water. A 9 μl aliquot was added to 5 tubes labelled 1 through 5, the remaining 5 μl was used as a control. *Sau*3A was added to each tube to yield a *Sau*3A concentration in tubes 1 to 5 of 0.35, 0.175, 0.088, 0.044, and 0.022 units per μl respectively. The DNA was digested for 5 min after which all 5 tubes and the control were immediately placed at 65°C for 20 min to inactivate the enzyme. A 3 μl sample from each tube was electrophoresed on a 0.7% agarose gel. The amount of enzyme that gave a partial digest was selected for further study.

An equal volume of 1.5 M NaCl and 20% PEG were added to the tube and the DNA was allowed to precipitate on ice for at least 1.5 hrs. The DNA was pelleted by centrifugation in a microfuge for 15 min at 4°C. The pellet was washed twice with 70% ethanol and dried under vacuum. The DNA was dissolved in 10 μl of sterile water after which 3 μl of 5X ligation buffer and 2 units of T4 DNA ligase were added. After 3 hrs at room

temperature, the ligation was heat inactivated at 65°C for 10 min. The ligated DNA was then subjected to digestion with a restriction enzyme that linearized any DNA that was not deleted. The digest was carried out by the addition of 3 μl of 10X restriction enzyme buffer and 12 μl of sterile water giving a final volume of 30 μl. The reaction was carried out at 37°C for 2 hrs. The DNA was transformed into JM101 and plated onto LB amp. The next day the colonies were picked onto LB and 24 hrs later the transformants were screened using the rapid lysate procedure.

3.13.4 Single Stranded DNA Isolation

Single stranded DNA isolations were performed as per Schreier and Cortese (1979).

PEG Solution

20% (w/v) polyethylene glycol 6000 (Fisher)

2.5 ml NaCl

A clear plaque was picked with a sterile toothpick and used to inoculate a 50 ml tube containing 1.5 ml of a log phase JM101 growing in LB. The tube was vigorously shaken for 6-8 hrs at 37°C. The cells were pelleted in an Eppendorf tube and the supernatant was transferred to a new tube containing 0.2 ml of ice cold PEG solution. The phage were allowed to precipitate for at least 15 min on ice before being pelleted in a microfuge for 10 min. The supernatant was removed by aspiration and any remaining traces of PEG were removed by a second spin for 2 min. The pellet was resuspended in TEN_{250} and extracted consecutively with 50 μl of phenol, phenol:chloroform (1:1) and chloroform. After the 3 extractions, the DNA was precipitated using 250 μl of ethanol. After 15 min at -20°C the ssDNA was pelleted in a microfuge for 25 min. The pellet was dried

and resuspended in 15 μl of TE buffer and stored at -20°C.

3.13.5 Preparation of Sequencing Gels

10X TBE
108 g Trizma base (Sigma)
55 g boric acid
9.3 g EDTA

40% Acrylamide
38 g acrylamide (Bio-Rad)
2 g N,N'-methylene bisacrylamide (Sigma)
The solution was filtered and stored at room temperature in the dark.

Sialyzing Solution
2% dichloromethylsilane (Aldrich)
(v/v) in carbon tetrachloride

Gel Mixture (for 1 gel)
21 g Urea (BRL-ultra pure)
5.0 ml 10X TBE
7.5 ml 40% acrylamide
distilled water to 50 ml

Two 38.5 x 20 cm plates were washed with an SOS pad and rinsed with distilled water. One plate was sialyzed 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 50 μl of TEMED (BRL-ultra pure) was added along with 25 mg of ammonium persulfate (Fisher). The mixture was poured between the two glass plates using a 50 ml syringe. A sharks tooth comb (flat side in) was placed into the glass plates to form a trough.

3.13.6 Electrophoresis of Sequencing Samples

The sharks tooth comb 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 for 15 min before samples were loaded. The gel was run in 1X TBE at a constant voltage of 1200 V. The first 100 bp of sequence was obtained by ending electrophoresis after the bromophenol blue had reached the bottom of the gel, approximately 1.5 hrs. Another 100 bp could be read by allowing the xylene cyanol to reach the bottom of the gel (about 3 hrs). To obtain more sequence the gels were run for longer periods of time.

3.13.7 Sequencing and T-Tracking DNA

Klenow Buffer (10X)
100 mM Tris-HCl pH 8.0
50 mM $MgCl_2$

Klenow Dilution Buffer
10 mM Tris-HCl pH 8.0

Dideoxy Working Solutions
0.1 mM ddATP (Pharmacia)
0.1 mM ddCTP "
0.3 mM ddGTP "
0.5 mM ddTTP "
working solutions were prepared from 10 mM stocks and stored at -60°C.

Deoxynucleotide Stocks
10 mM stocks were stored at -60°C and were purchased from Pharmacia.

Deoxynucleotide Working Solutions
Each nucleotide was diluted from the 10 mM stocks to 0.5 mM. The nucleotide mixtures were prepared as follows:

Mix	0.5 mM dCTP	0.5 mM diazoGTP	0.5 mM dTTP	Tris pH8
A°	20 µl	20 µl	20 µl	5 µl
C°	1 µl	20 µl	20 µl	5 µl
G°	20 µl	1 µl	20 µl	5 µl
T°	20 µl	20 µl	1 µl	5 µl

M13 primer

17 bp universal primer (Regional DNA Synthesis Laboratory, Calgary Alberta) was dissolved in HPLC water to a concentration of 2 ng/μl.

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 (NEN) 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.

3.13.7.1 T-Tracking (10 clones)

Primer Mix

4 μl primer
6 μl 10X Klenow buffer
12 μl HPLC water

T-reaction mix

8 μl ddTTP
8 μl dTTP
4 μl [α^{32}-P]dATP (diluted)
1 μl Klenow (Pharmacia)

The T-tracking procedure was used to screen potential clones for inserts. The annealing reaction used 2 μl of ssDNA template and 2 μl of primer mix. The mixture was placed at 65°C for 5 min and then left to anneal at room temperature for 15 min. Immediately after the Klenow was added to the T-reaction mix, 2 μl was added to each tube of primed templates. After a 15 min incubation at 50°C, 2μl of formamide dye was added to each tube and the tubes were boiled for 3 min before being loaded onto the gel.

3.13.7.2 Sequencing Reactions

The sequencing procedure used 7 μl of single stranded template. The DNA was mixed with 1.5 μl of 10X Klenow buffer and 2 μl of primer. The samples were heated to 65°C for 5 min before being placed at room temperature for 15 min. Tubes labelled A,C,G,T containing 1 μl of the appropriate ddNTP, 1 μl of the corresponding dNTP solution, and 0.5 μl of [α^{32}P]dATP were set-up for each clone to be sequenced. Each of the 4 reaction tubes received 2 μl of the primer template mixture. Two μl of diluted Klenow (1 μl of Klenow in 24 μl of Klenow dilution buffer) were added to each tube and the tubes were incubated at 50°C for 15 min. Four μl of formamide dye was added to each tube and boiled for 3 min before being loaded onto the prerun sequencing gel.

3.13.8 Fixing, Drying, and Autoradiography of the Gel

Once the samples had run the desired distance the gel was removed from the apparatus and the plates were separated. The gel and nonsialyzed glass plate were transferred to a fixing bath (5% methanol/5% acetic acid) and left to soak for 20 min. The gel was removed from the fixing solution and blotted dry with Kim Wipes. The gel was transferred to Whatman 3MM paper and dried using a Bio-Rad Model 483 Slab Drier under vacuum for 1 hr at 80°C. The gel was exposed to Kodak X-Omatic AR X-ray film overnight at room temperature. The X-ray film was developed as described in section 3.11.4.

3.14 Kat F Protein Isolation

3.14.1 Protein Labelling Using the T7 Polymerase System

3.14.1.1 Protein Labelling

A fragment containing the *katF* gene was cloned in front of the T7 polymerase promoter in the pT7-5 vector. This plasmid along with the plasmid pGP1-2 was transformed into NM522 and selected on LB amp kan plates at 30°C. The cells were grown to an A_{590} of 0.5 at 30°C. A 0.2 ml aliquot of cells was centrifuged in a microfuge and washed with 5 ml of M9. The pellet was resuspended in 1 ml of M9 media and grown at 30°C with shaking for an additional hour. The temperature was shifted to 42°C for 15 min and then rifampicin was added (20 mg/ml stock) to a final concentration of 200 μg/ml. After 10 min at 42°C, 20 μCi of ^{14}C-labelled mixed amino acids (NEN) were added and the temperature was shifted down to 30°C for 25 min. The cells were harvested in a microfuge and resuspended in 50 μl of cracking buffer. The samples were boiled for 3 min and then loaded on a 8% SDS-polyacrylamide gel. Before exposure to X-ray film the gel was treated with PPO as described in section 3.12.

3.14.1.2 Maximizing Protein Production from the T7 Vector System

Cells harbouring both the pT7F2 and pGP1-2 plasmids were grown in 10 ml of LB amp kan broth at 30°C overnight. This culture was used to inoculate 2 l fluted flasks containing LB amp kan (1% inoculum). The flasks were shaken at 30°C until the A_{590} was approximately 1.5. An equal volume of 50°C LB was added to each flask giving a final temperature of

approximately 42°C. The temperature was kept at 42°C for 25 min before being shifted down to 37°C for 2 hrs. The cells were harvested in 250 ml centrifuge bottles at 5000 rpm for 10 min at 4°C. The cell pellet was weighed and stored at -60°C until enough cells were collected for a protein isolation attempt.

3.14.2 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Running Buffer
0.1% SDS
25 mM Tris
0.2 M glycine

Sample Buffer
2% SDS
0.2 M MSH
8% glycerol
68 mM Tris (pH 6.8)

Staining Solution
0.25% Coomassie Brilliant Blue
10% Acetic Acid
40% Methanol

Destaining Solution
7% Acetic Acid
15% Methanol

Gels for SDS-PAGE were prepared and electrophoresed in a Bio-Rad Protean Dual Slab Cell apparatus as described by Laemmli (1970).

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 15x15 cm vertical gel plates. Distilled H_2O (approximately 3 ml) was layered onto the mixture and the gel was allowed to polymerize for 30 min.

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 of distilled H_2O. This mixture was also 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.

3.14.3 Column Preparations

3.14.3.1 Preparation of the Heparin-Sepharose Column.

Coupling Buffer
0.1 M $NaHCO_3$ pH 8.3
0.5 M NaCl

High pH Buffer
0.1 M NaOAc pH 4.0
0.5 M NaCl

KCl Buffer
0.01 M Tris-HCl pH 8.0
0.01 M $MgCl_2$
1.0 mM EDTA pH 8.0
0.3 mM DTT
7.5 % glycerol (vol/vol)
0.1 M KCl
0.05 mg/ml PMSF

CNBr-activated Sepharose 4B was purchased from Pharmacia. Six grams

of CNBr-activated Sepharose 4B were reswelled in 50 ml of 1.0 mM HCl for 15 min at room temperature and then washed on a sintered glass funnel with 1 l of the same solution. The Sepharose was washed with 30 ml of coupling buffer and immediately mixed with 60 ml of a 4 mg/ml heparin solution in coupling buffer. The heparin was coupled to the Sepharose for 2 hrs at room temperature in a 150 ml glass Corex centrifuge bottle with gentle mixing. Any remaining active groups were blocked by transferring the gel to a 60 ml solution of 0.2 M glycine (pH 8.0) for 2 hrs at room temperature. Excess uncoupled ligand was removed by washing alternately with coupling buffer (high pH buffer) and low pH buffer solutions five times. The Sepharose was resuspended 0.1 M KCl buffer and then packed in a 20 ml glass syringe.

3.14.3.2 Preparation of the DNA Cellulose Column.

Tris/EDTA
10 mM Tris-HCl pH 7.4
1.0 mM EDTA

DNA cellulose was prepared as per Alberts and Herrick (1971). A 2 mg/ml solution of Type III salmon sperm DNA (Sigma) was prepared in Tris/EDTA. Clean dry cellulose was added with stirring (1.0 g cellulose per 3.0 ml of DNA) until the cellulose was thoroughly mixed. The mixture was spread evenly on a baking dish and left at 50°C for 3 days. The dry cellulose was scraped off the dish and ground into a fine powder with a mortar and pestle. Any remaining water was removed by overnight lyophilization. The next day the powder was suspended in 20 volumes of

Tris-EDTA and left at 4°C overnight. The cellulose was washed twice with Tris-EDTA, packed into a column, and finally equilibrated with TGED + 0.15 M NaCl (see section 3.14.4). The amount of DNA bound to the cellulose was determined by DNA released from an aliquot heated at 100°C for 20 min in Tris-EDTA. There was typically 1.0 mg of DNA released from 1.0 ml of packed column.

3.14.4 RNA Polymerase Isolation

3.14.4.1 Growth and Induction of Cells

A 100 ml preculture of NM522 harbouring the plasmids pT7F2 and pGP1-2 was grown at 30°C overnight in LB amp kan. This culture was used to inoculate 12-2 l fluted flasks containing 500 ml of LB amp kan. The flasks were shaken at 30°C to an OD_{600} of 1.5. At this time an equal volume of 50°C LB was added to each flask and the cultures were incubated at 42°C with shaking. After 25 min the temperature was shifted down to 37°C for 2 hrs with shaking. The cells were pelleted at 5000 rpm for 10 min before being weighed and stored at -70°C. Typically, 30 to 35 g of cells were harvested.

3.14.4.2 Transcriptional Assays

Transcription buffer
- 40 mM Tris-HCl pH 8.0
- 20 mM $MgCl_2$
- 1 mM EDTA
- 50 mM NaCl

Nucleotide Mix
80 μM ATP
8 mM CTP
8 mM GTP
8 mM UTP
6 Ci/ml ^{3}H-ATP

A typical transcriptional reaction mixture contained 0.4 ml of transcription buffer, 0.05 ml of nucleotide mix, 0.02 ml of a RNA polymerase fraction and 10 μl of pAMkatE72 DNA (1.0 mg/ml). The mixture was incubated at 37°C for 10 min. The RNA was precipitated by the addition of 1.0 ml of 10% ice cold trichloroacetic acid (TCA) and then bound to a glass fiber filter via vacuum filtration. The filter was washed with 6 ml of ice cold 5% TCA and placed in 3 ml of scintillant before being counted.

3.14.4.3 RNA Polymerase Isolation

CB Buffer
10 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
1.0 mM EDTA (pH 8.0)
0.9 mM MSH
50 μg/ml PMSF

Sonication Buffer
CB buffer with 6.25 mg/ml lysozyme, 0.25 M Tris-HCl (pH 8.0), and 300 μg/ml pf PMSF

Low Salt Elution Buffer
CB buffer with 8.8% PEG and 2 M NaCl

High Salt Elution Buffer
CB buffer with 8.8% PEG and 5 M NaCl

Biogel Buffer

0.1 M Tris-HCl (pH 8.0)
0.1 M $MgCl_2$
0.5 M NaCl
1.0 mM EDTA (pH 8.0)
10% Glycerol
17 mM MSH
50 μg/ml PMSF

No Salt Buffer

10 mM Tris-HCl (pH 8.0)
10 mM $MgCl_2$
1.0 mM EDTA (pH 8.0)
7.5 % glycerol
0.3 μM DTT
50 μg/ml PMSF
containing either 0.1 M or 1.0 M KCl where specified

Unless otherwise specified, all centrifugations were carried out at 4°C. Approximately 30 g of induced cells were thawed on ice and then resuspended in 50 ml of sonication buffer. The cells were sonicated in 20 ml batches using the intermediate probe 4x 30 sec. The debris was removed by centrifugation in 50 ml plastic centrifuge tubes at 17,000 rpm for 15 min. The volume of the supernatant was measured and for every 42 ml of crude extract, 5.8 ml of 20% dextran (in CB buffer) and 19.3 ml of 30% PEG (in CB buffer) were added. The mixture was stirred on ice for 20 min before being centrifuged at 12,000 rpm for 15 min. The pellet was resuspended in 32 ml of low salt elution buffer and was stirred on ice for 20 min. The precipitate was pelleted by centrifugation at 12,000 rpm for 15 min and then resuspended in 32 ml of high salt elution buffer. The RNA polymerase was eluted from the pellet for 35 min with gentle stirring. The remaining protein was removed by centrifugation at 15,000 rpm for 15 min. The supernatant was diluted with an equal volume of CB buffer and

then 16.5 g of ammonium sulfate was added for every 100 ml of diluted supernatant. The mixture was stirred on ice for 5 min after the ammonium sulfate had dissolved. The solution was centrifuged at 8,000 rpm for 20 min in 30 ml Corex centrifuge tubes. At this point two phases exist, the upper phase containing PEG and the lower aqueous phase containing the RNA polymerase. A needle was used to draw the aqueous phase into a syringe leaving the PEG layer behind. The volume of the aqueous layer was measured and 1.5 volumes of 63% ammonium sulfate solution (dissolved in CB buffer) and 23.6 g of solid ammonium sulfate per 100 ml of undiluted aqueous layer were added and the mixture was stirred at room temperature for 15 min. The precipitate was pelleted in 50 ml plastic centrifuge tubes at 17,000 rpm for 15 min. The pellet was resuspended in 8 ml of BioGel buffer and then loaded onto a BioGel A 1.5 M column (100 cm x 2.7 cm) that had been washed with at least 200 ml of BioGel buffer. The column had a flow rate of about 50 ml/hr. Approximately one hundred 5.5 ml fractions were collected overnight at 7°C. The next day fractions were assayed for RNA polymerase activity and the OD_{600} was measured. Fractions containing peak RNA polymerase activity were pooled (usually 10-12 fractions) and concentrated over an ultrafiltration membrane at a pressure no greater than 40 psi of nitrogen with very gentle stirring. When the volume was reduced to approximately 10 ml, 20 ml of no salt buffer was added and the concentration was repeated. This process was repeated until the conductivity of the flow through equalled that of 0.1 M KCl buffer (usually two dilutions). The sample was then loaded onto a 20 ml Heparin-

Sepharose column that had been previously equilibrated with 0.1 M KCl buffer. The column was washed with 200 ml of 0.1 M KCl buffer or until the OD_{600} of the flow through equalled that of 0.1 M KCl buffer. The RNA polymerase was eluted off of the column using a 300 ml 0.1-1.0 KCl continuous gradient. Approximately 55 fractions of 5.5 ml each were collected. The fractions were assayed for RNA polymerase activity and OD_{600}. The polymerase eluted at the second OD_{600} peak.

3.15 Measurement of DNA and Protein Concentrations

The concentration of DNA was determined spectrophotometrically by determining the absorbance at 260 nm. One absorbance unit is equal to approximately 50 μg of DNA.

DNA concentrations were also estimated by agarose gel electrophoresis. Unknown DNA samples were run next to known concentrations of DNA and the intensities were compared after staining with ethidium bromide.

Protein concentrations were determined spectrophotometrically by the ratio of the A_{280} to the A_{260}. The ratio was used to determine F from a table of known values where:

$$F = \frac{2.303}{A_{280}} \times \frac{\text{per cent protein}}{100}$$

F is then used in the formula below to give the protein concentration in mg/ml (Layne, 1957).

$$[\text{protein mg/ml}] = F \times 1/d \times A_{280}$$

where d is the cuvette width in cm.

3.16 Measurement of Catalase Activity

3.16.1 Colony Assay

A qualitative method was used to determine catalase activity in colonies on plates. A drop of 30% H_2O_2 was applied to the edge of a colony. Cells containing catalase decomposed the H_2O_2 evolving oxygen which appeared in the form of bubbles.

3.16.2 Quantitative Determination of Catalase

Catalase activity was determined in a Gilson oxygraph equipped with a Clark electrode (Rorth and Jensen 1967). Assays were carried out with a H_2O_2 concentration of 60 mM. One unit of catalase is defined as the amount of enzyme that degrades 1 μM of H_2O_2 per min at 37°C.

3.16.3 Visualization of Catalase on Polyacrylamide Gels

Extracts were electrophoresed on native polyacrylamide gels (Davis, 1964) and were stained for catalase by the method of Clare *et al.* (1984). Extracts were prepared from 250 ml broth cultures. The cells from mid-log or stationary phase cultures were pelleted and resuspended in 1.0 ml of SM. The cells were broken open by three 30 sec rounds of sonication. The debris was removed by centrifugation at 10,000 rpm in 10 ml Corex centrifuge tubes at 4°C for 10 min. Appropriate volumes of sample containing 1 to 2 units of catalase were electrophoresed on a 9.5% polyacrylamide gel prepared in 67 mM Tris-HCl pH 8.1 and electrophoresed

at 25 mA until the blue dye reached the bottom. The running buffer used was Tris-Glycine (10 mM Tris-HCl and 70 mM glycine).

The gel was soaked in 50 mM potassium phosphate pH 7.0 containing 0.4 mg/ml of diaminobenzidine hydrochloride and washed with H_2O. A solution containing 0.05 mg/ml horseradish peroxidase in H_2O was added and the gel soaked for 10 min. The gel was then rinsed with distilled water and soaked for up to 2 hrs in 20 mM H_2O_2 in 50 mM potassium phosphate buffer pH 7.6. The bands containing catalase activity appeared as white bands on a brown background.

3.17 ß-Galactosidase Determinations

Z Buffer

0.06 M $Na_{2HPO}4.7H_2O$
0.04 M $NaH_2PO_4.H_2O$
0.01 M KCl
1.0 mM $MgSO_4.7H_2O$
0.05 M MSH
pH adjusted to pH 7.0

ß-galactosidase assays were performed as per Maniatis *et al.* (1982). The cell density was determined by measuring A_{600}. A sample of cells was mixed with Z buffer so that the final volume was 1.0 ml. A couple of drops of chloroform and 0.1% SDS were added and the mixture was vortexed for 10 sec and then placed at 28°C to lyse the cells. After 5 min, 0.25 ml of ONPG (4 mg/ml) was added and the time was recorded. When the color change seemed adequate, 0.5 ml of 1.0 M $CaCO_3$ was added to stop the reaction and the time was again recorded. The tube was placed on ice. The A_{420} and A_{550} were recorded and the ß-galactosidase activity was then

determined by the formula below:

$$\text{ß-galactosidase (Miller Units)} = \frac{A_{420}-1.75(A_{550})}{A_{600} \times t \times v}$$

where t = time in min

v = volume of culture used (ml)

3.18 Mapping of the Transcriptional Start Site

3.18.1 Isolation of RNA

NM522 harbouring the plasmid pM13-F2 was grown overnight in 50 ml of LB broth with shaking. The cells were pelleted in 30 ml Corex centrifuge tubes at 5000 rpm for 10 minutes and then resuspended in 2.0 ml of 30 mM Tris-HCl (pH 7.6)/5 mM EDTA. The suspension was extracted with equal volumes of phenol; phenol:chloroform; and finally chloroform. The nucleic acids were precipitated with the addition of 0.2 ml sodium acetate (pH 4.8) and 4 ml of 95% ethanol. After 20 minutes at -20 °C the DNA and RNA were pelleted at 5000 rpm at 4°C for 10 minutes. The pellet was dried and resuspended in 1.5 ml of 0.1 M sodium acetate (pH 5)/5 mM $MgSO_4$. For every 100 μg of DNA present, 40 U of DNase were added along with Proteinase K to a concentration of 50 μg/ml. The mixture was incubated at 37°C for 2 hours. The DNase and Proteinase K were removed by a phenol:chloroform and chloroform extraction. The RNA was precipitated by the addition of 2 volumes of 95% ethanol and stored in ethanol until required.

3.18.2 5′ End Labelling of DNA

Reaction mixture

50 mM	Tris-HCl pH 7.5
10 mM	$MgCl_2$
5 mM	DTT
50 pmol	dephosphorylated DNA, 5′ ends
15 μCi	[γ-^{32}P]-dATP
50 μg/ml	BSA
20 U	T4 polynucleotide kinase

The end labelling reaction was carried out in a 30 μl volume and was performed as per Tabor (1989). The reaction mixture was incubated at 37°C for 60 minutes. The reaction was stopped by adding 1 μl of 0.5 M EDTA (pH 8.0) followed by an extraction with phenol/chloroform. The unincorporated nucleotides were separated from the labelled oligonucleotide by the addition of 3 μl of 3 M sodium acetate (pH 4.8) and two volumes of ice cold 95% ethanol. The labelled oligonucleotide was pelleted in a microfuge for 25 minutes after 15 minutes at -20 °C. The pellet was resuspended in 30 μl of TE buffer and the ethanol precipitations were repeated two more times. The pellet was air dried at room temperature, resuspended in 50 μl of TE and store at -20°C.

3.18.3 Primer Extension Analysis

Hybridization buffer

1 M NaCl
0.16 M HEPES, pH 7.5
0.33 mM EDTA

10x RT

0.5 M Tris-HCl (pH 8)
0.5 M KCl
50 mM $MgCl_2$
50 mM DTT
0.5 mg/ml BSA

RT mix

3.5 μl 4 mM dNTPs
2.5 μl 10x RT buffer
1.25 μl RNAse inhibitor (Pharmacia)
18 μl H_2O

Primer extension analysis was performed as per Kingston (1989). Approximately 50 μg of RNA was mixed with $5x10^4$ counts of labelled oligonucleotide. The salt concentration was adjusted to 0.3 M with sodium acetate and 2.5 volumes of 100% ethanol were added. After 20 minutes at -20 °C, the RNA and primer were pelleted in a microfuge for 25 minutes at 4 °C. The pellet was resuspended in 30 μg of hybridization buffer and the mixture was incubated overnight at 30 °C. The next day, 170 μl of 0.3 M sodium acetate and 500 μl of ethanol were added to the hybridization mixture and the mixture was pelleted as above. The pellet was washed with 75% ethanol/25% 0.1 M sodium acetate (pH 5.2) and dried.

The pellet was resuspended in 25 μl RT mix and 80 U of AMV reverse transcriptase (Pharmacia) were added. The reaction was incubated at 42°C for 90 minutes. The RNA was digested by the addition of 1 μl of 0.5 M EDTA and 5 μl of RNase A (Sigma) (1 mg/ml) and the incubation was continued at 37 °C for 2 hours. The RNase was removed by the addition of 100 μl of 2.5 M ammonium acetate and extraction with 125 μl phenol/chloroform. The DNA in the aqueous phase was precipitated by the

addition of 300 μl of 100% ethanol. The pellet was washed three times with 70% ethanol and air dried before being resuspended in 3 μl TE buffer and 4 μl formamide dye mix. Three μl of the sample was boiled for 3 minutes before being analyzed on a sequencing gel along side the same DNA sequence produced from the primer.

RESULTS

4.0 Results

4.1 Construction of Genomic Libraries

The cloning vector λEMBL3 was used to construct three *E. coli* genomic libraries. This vector can harbour inserts between 9 and 23 kb (Frischauf *et al* 1983). It contains two polylinker sequences (*Sal*I, *Bam*HI, and *Eco*RI) between the λ 19.4 and 9.4 kb arms and the nonessential stuffer region (Figure 1). λEMBL3 (purchased from BRL) was predigested with *Bam*HI and *Eco*RI and then precipitated with isopropanol. The above procedure does not precipitate the 9 bp *Bam*HI/*Eco*RI polylinker fragment. The absence of this small fragment prevents the religation of the arms and the stuffer fragment which would produce nonrecombinant phage.

The strains UM120 and UM122 carry Tn*10* in or near the *katE* and *katF* genes respectively. This insertion causes loss of the HPII catalase activity. These insertional mutations were used to map both the *katE* and *katF* genes (Loewen, 1984; Loewen and Triggs, 1984). Partial *Sau*3A genomic DNA digests in the range of 15-20 kb were isolated from UM120, UM122, and MP180. These fragments were ligated into the *Bam*HI site of the λEMBL3 arms and then *in vitro* packaged, producing three libraries. The titre of each library was determined and is shown in Table 4.

4.2 Probing the UM120 and UM122 Libraries

The plasmid pBT107 contains the Tet^R gene from the Tn*10* (Figure 2; Moyed *et al*, 1983). This plasmid was used to isolate DNA segments from the UM120 and UM122 libraries that contained *katE*::Tn*10* and *katF*::Tn*10*

Figure 1. Restriction map of λEMBL3. The thick black lines represent the essential 19.4 kb left arm and the 9.4 kb right arm. Important restriction enzymes are abbreviated as follows; *Bam*HI, B; *Eco*RI, E; *Hin*dIII, H; *Sal*I, S.

λ EMBL3

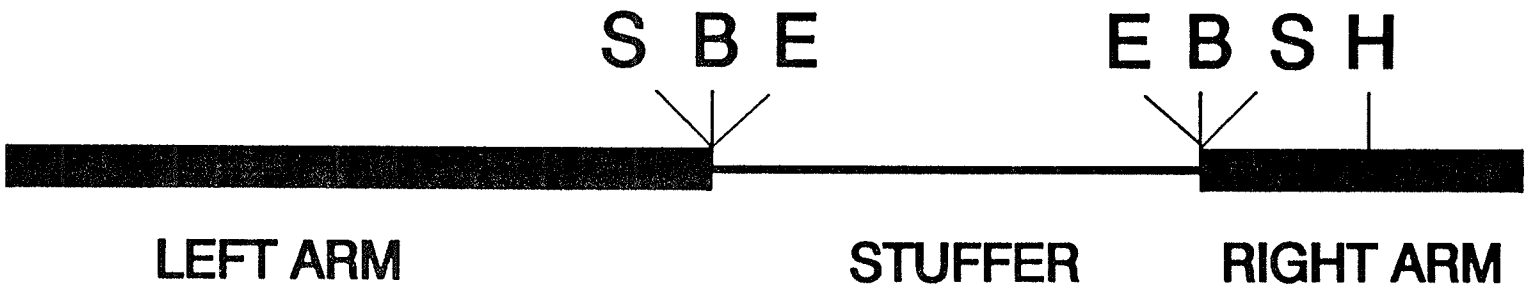


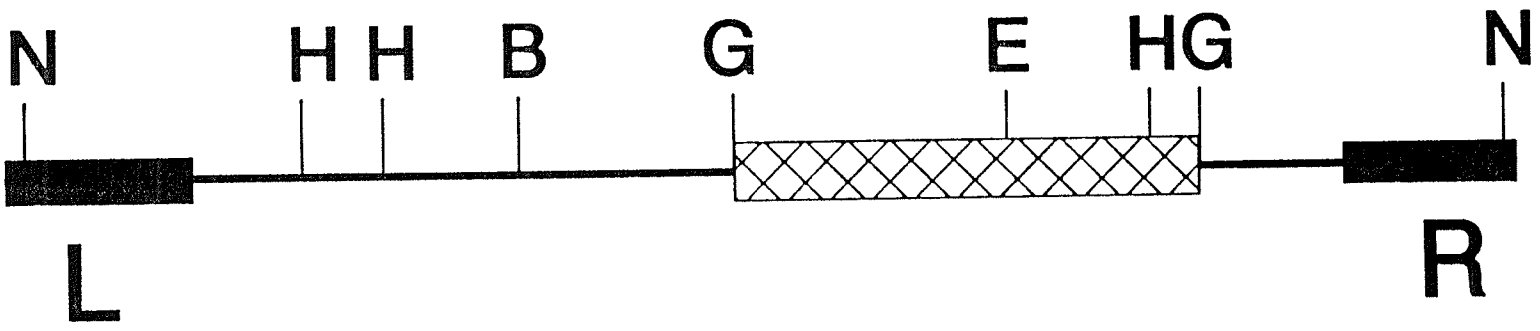
TABLE 4

Titration of the Genomic Libraries

STRAIN	TITRE (PFU/ml)	TOTAL PFU
UM120	3.0×10^5	1.5×10^5
UM122	7.6×10^4	3.8×10^4
MP180	1.05×10^5	5.25×10^4

Figure 2. Restriction map of the transposon Tn*10*. The thicker lines represent the left and right inverted repeat segments. The cross hatched region indicates the *Bgl*II fragment containing the Tet^R gene in the plasmid pBT107. New abbreviations for restriction sites are as follows: *Bgl*II, G; *Nru*I, N.

TN10



sequences respectively. Before the libraries were probed it was necessary to calculate the number of plaques (N) required to represent the entire *E. coli* genome. The formula is shown below (Maniatis *et al* 1982):

$$N = \frac{\ln (1.0 - P)}{\ln (1.0 - f)}$$

where: P is the desired probability

f is the fractional portion of the genome in a single recombinant

therefore, to represent an *E. coli* genome using 18 kb fragments with a probability of 99%:

$$N = \frac{\ln (1.0 - 0.99)}{\ln [1.0 - (1.8 \times 10^4 / 3.0 \times 10^6)]}$$

$$N = 765$$

A total of at least 765 plaques must be screened to represent the entire *E. coli* genome using 18 kb fragments.

Two plates, containing approximately 500 plaques per plate, were made for each of the UM120 and UM122 libraries. The plaques were transferred to nitrocellulose filters and probed with approximately 5×10^6 cpm/filter of nick translated pBT107 under stringent conditions (65°C and 6X SSC). After autoradiography, the film was placed under the plate and the markings were lined up. The positive plaques were picked and then replated with less than 100 plaques per plate. These plates were again blotted and probed with pBT107. Strong positive plaques that were well isolated were picked and stored. This procedure was repeated for each positive plaque isolated to insure purity.

Four positive plaques labelled λ120::Tn*10* 1-4 were isolated from

the UM120 library and six positive plaques labelled λ122::Tn*10* 1-6 were isolated from the UM122 library.

4.3 λ120::Tn*10* Mapping and Isolation of a *katE* Probe

Four restriction enzymes were selected to map the λ clones; *Bam*HI, *Eco*RI, *Hin*dIII, and *Sal*I. *Bam*HI and *Eco*RI were selected because there are no sites for these enzymes in the λ arms and only single sites in the Tn*10* (Figures 1 and 2). The enzyme *Sal*I was selected because it cuts at the polylinker sequence in λEMBL3 (Figure 1) facilitating the excision of the insert. There are no *Sal*I sites in the Tn*10*. *Hin*dIII cuts three times in the Tn*10* and once in the 9.4 kb λEMBL3 arm. These four enzymes were selected for two main reasons. Firstly, the EMBL3 clones are large (40-50 kb) and it was necessary to select enzymes which did not give a large number of fragments making mapping difficult. Secondly, it was necessary to use enzymes which cut in the Tn*10* enabling the mapping of the Tn*10* within the insert. The fragment sizes generated from the digestion of Tn*10* with the enzymes above are shown in Table 5.

Since the λ DNA molecule is linear, the number of restriction sites in a single digest is one less than the number of fragments generated. Restriction enzyme mapping of these clones was achieved by comparing double digests with single digests. The fragments from single digests which disappeared in double digests had to be cut by the second enzyme. The new fragments created by the double digests were identified by the addition of the two new fragments, the sum of which would equal the original size of the fragment restricted with the first enzyme. In

TABLE 5

Restriction Endonuclease Fragments Obtained by Digestion of Tn*10*

Restriction Endonuclease	Fragment Sizes (kb)
*Hin*dIII	4.7 0.5
*Hin*dIIIx*Bam*HI	3.85 0.85 0.5
*Hin*dIIIx*Eco*RI	3.85 0.868 0.5

some digests fragments having the same size were generated. In these cases the DNA double band was identified by its higher intensity as compared with adjacent single bands. All DNA mapping in this study was performed using these rules.

Two clones, λkatE4::Tn*10* and λkatE6::Tn*10*, were mapped using the four enzymes listed above. The fragment sizes generated from the digestion of λkatE4::Tn*10* and λkatE6::Tn*10* are listed in Tables 6 and 7 respectively. λkatE4::Tn*10* contained an insert size of 20.8 kb whereas λkatE6::Tn*10* harboured an insert of 18.7 kb. The two maps are shown in Figure 3. Both clones contained the entire Tn*10* along with adjacent DNA sequences upstream and downstream from it. The restriction of λkatE4::Tn*10* with *Bam*HI resulted in a 5.9 kb fragment that was directly adjacent to the Tn*10*. This fragment was cloned into the *Bam*HI site of the high copy number plasmid pAT153 (3570 bp). Transformants were picked onto LB amp and LB tet plates. Clones which did not grow on the LB tet plates were picked and plasmid DNA was isolated. A plasmid which produced two bands (5.9 and 3.6 kb) when digested with *Bam*HI was stored and named pMM120.

4.4 λkatF::Tn*10* Mapping and Cloning of a *katF* Probe

The same mapping strategy used in the λkatE::Tn*10* clones was employed in the mapping of the λkatF::Tn*10* clones. Two clones labelled λkatF3::Tn*10* and λkatF5::Tn*10* were mapped. Both clones contained inserts with similar sizes, 19.4 and 20.1 respectively. The restriction fragment sizes are listed in Tables 8 and 9. The two overlapping clones are diagrammed in Figure 4. A 5.4 kb *Hin*dIII fragment containing a 2.27

TABLE 6

Restriction Endonuclease Fragments Obtained by Digestion of λkatE4::Tn*10*

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Bam*HI	23.0 16.1 6.6 5.9	51.6
*Eco*RI	25.0 20.0 3.6	48.6
*Hin*dIII	24.3 6.6 4.7 4.4 4.4 3.6 1.8	
	(0.5)*	50.3
*Sal*I	19.0 14.8 9.4 2.8	46.0
*Bam*HIx*Eco*RI	23.0 16.1 5.0 3.0 3.0	50.1
*Bam*HIx*Hin*dIII	23.0 6.6 4.4 4.4 3.8 2.4 2.2	
	1.8 1.3 (0.9) (0.5)	51.3
*Bam*HIx*Sal*I	19.0 9.4 6.6 5.9 4.7 2.8	48.4
*Eco*RIx*Hin*dIII	24.3 6.6 4.6 4.4 4.4 3.5 1.8	
	(0.5)	50.1
*Eco*RIx*Sal*I	19.0 10.0 9.4 6.0 3.6	48.0
*Hin*dIIIx*Sal*I	19.0 4.9 4. 4.4 3.6 3.1 2.9	
	2.5 1.8 1.5 1.3 (0.5)	50.2
	Average size	**49.5**

* numbers in parenthesis indicate predicted fragments

TABLE 7

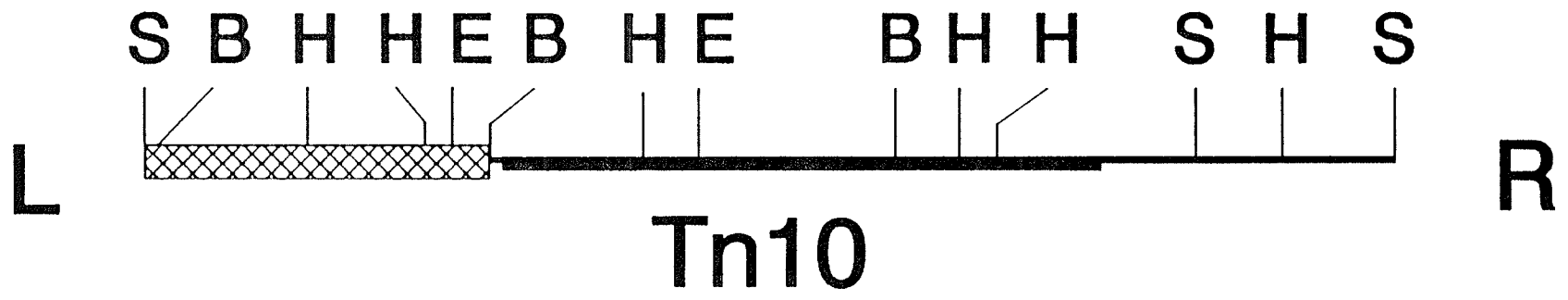
Restriction Endonuclease Fragments Obtained by Digestion of λkatE6::Tn*10*

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Bam*HI	25.0 14.0 6.6	45.6
*Eco*RI	25.0 17.0 3.6	45.6
*Hin*dIII	23.0 8.6 4.7 4.4 3.6 1.8	46.1
*Sal*I	19.4 17.6 9.4 2.4	48.4
*Bam*HIx*Eco*RI	25.0 14.0 3.0 3.0	48.0
*Bam*HIx*Hin*dIII	25.0 8.6 4.4 3.8 2.3 1.8 1.3	
	(0.8)* (0.5)*	46.5
*Bam*HIx*Sal*I	19.4 9.4 6.5 6.5 4.7 2.4	48.5
*Eco*RIx*Sal*I	19.0 9.4 8.0 6.0 3.6 2.4	48.4
*Sal*Ix*Hin*dIII	19.0 5.0 4.7 4.4 3.6 3.1 3.0	
	1.8 1.2	49.9
	Average size	47.4

* numbers in parenthesis indicate predicted fragments

Figure 3. Restriction enzyme maps of the λkatE::Tn*10* clones. The heavy dark line indicates the position of the transposon Tn*10*. L and R indicate the left and right arms of λEMBL3 respectively. The cross hatched region in λkatE4::Tn*10* indicates the 5.9 Kb *Bam*HI fragment that was cloned into pAT153.

λkatE4::Tn10



λkatE6::Tn10

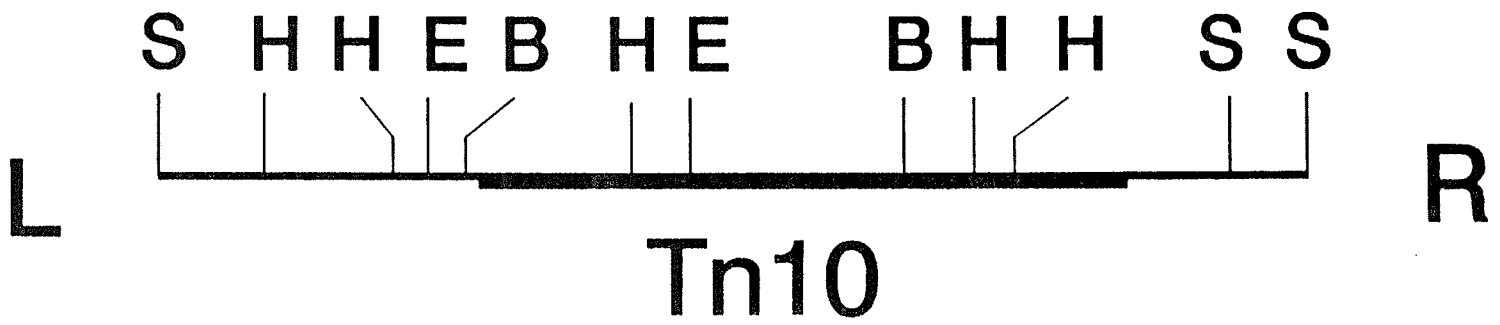


TABLE 8

Restriction Endonuclease Fragments Obtained by Digestion of λkatF3::Tn10

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Bam*HI	23.2 13.4 10.9	47.5
*Eco*RI	20.9 16.7 10.1	47.7
*Hin*dIII	23.1 8.5 5.6 4.8 4.4 0.44	46.8
*Sal*I	20.4 20.4 9.4	50.2
*Bam*HIx*Eco*RI	20.9 13.7 7.9 3.0 2.2	47.7
*Bam*HIx*Hin*dIII	23.1 8.5 5.6 4.4 4.0 1.5 0.5	47.6
*Bam*HIx*Sal*I	20.4 10.9 8.9 5.4 3.1	48.8
*Eco*RIx*Hin*dIII	20.9 8.6 5.4 4.4 3.6 3.55 1.0	
	0.72 (0.45)*	48.6
*Eco*RIx*Sal*I	19.4 10.1 9.4 7.5 1.5	47.9
*Hin*dIIIx*Sal*I	20.4 5.6 4.9 4.9 4.4 4.1 3.6	47.9
	Average size	**48.1**

* numbers in parenthesis indicate predicted fragments

TABLE 9

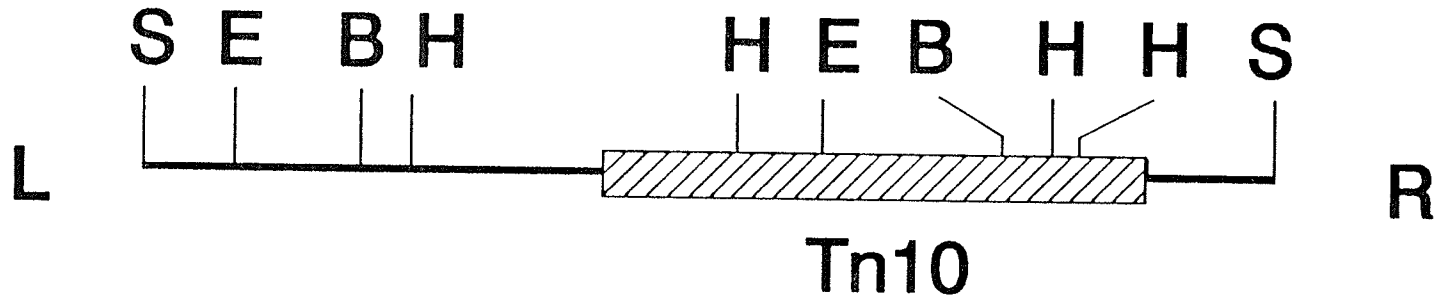
Restriction Endonuclease Fragments Obtained by Digestion of λkatF5::Tn10

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Bam*HI	30.4 10.2 9.4	50.0
*Eco*RI	32.9 17.2	50.1
*Hin*dIII	23.1 6.2 5.4 4.7 4.4 0.46 (0.38)*	44.6
*Sal*I	20.9 16.3 9.4 1.5	48.1
*Bam*HIx*Eco*RI	30.4 9.4 7.7 2.1	49.6
*Bam*HIx*Hin*dIII	30.4 5.7 4.9 4.4 3.8	49.2
*Bam*HIx*Sal*I	20.9 10.2 9.4 6.6 1.5	48.6
*Eco*RIx*Hin*dIII	23.2 6.2 5.7 4.9 4.4 3.6 1.0 0.72	49.7
*Eco*RIx*Sal*I	20.9 9.4 9.4 7.7 1.5	48.9
	Average size	**48.8**

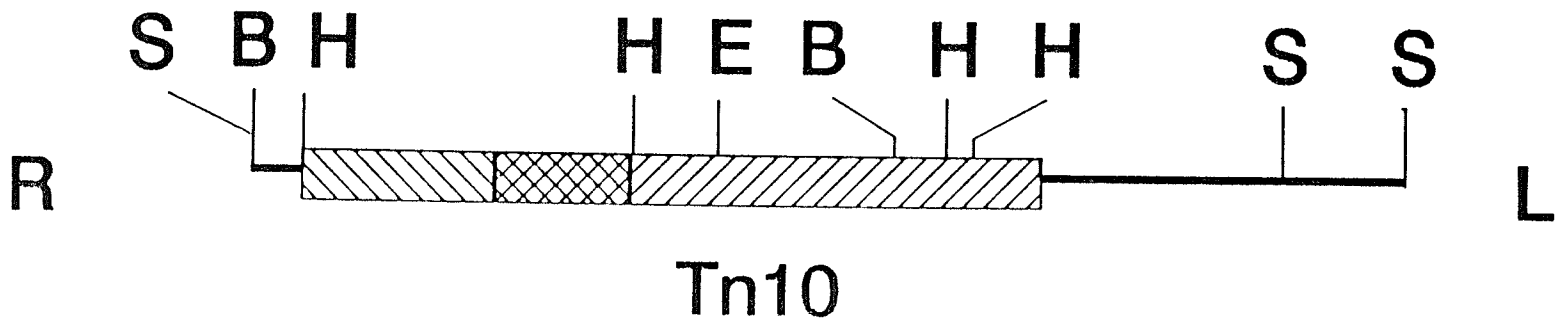
* numbers in parenthesis indicate predicted fragments

Figure 4. Restriction enzyme maps of the λkatF::Tn*10* clones. The fore slashed region indicates the position of the transposon Tn*10*. L and R indicate the left and right arms of λEMBL3 respectively. The back slashed region in λkatF5::Tn*10* indicates the 5.4 Kb *Hin*dIII fragment that was cloned into pAT153. The cross hatched area indicates the overlap of the *Hin*dIII fragment and the Tn*10*.

λ katF3::Tn10



λ katF5::Tn10



kb portion of Tn*10* and approximately 3.13 kb of adjacent DNA was cloned from λkatF5::Tn*10* into the *Hin*dIII site of pAT153. The resulting 9.0 kb clone was amp^R and tet^S. This clone was stored as pMM122.

4.5 Isolation of *katE* and *katF* λ Clones

The two clones, pMM120 and pMM122, were used as probes to isolate *katE* and *katF* containing λ clones from the MP180 (wild-type) library. The probing of the library and isolation of positive plaques were carried out as described in section 4.2. Approximately 1500 plaques were screened for each of the probes used. Six positive plaques were isolated from the probing of the MP180 library with pMM120. These clones were labelled λkatE1 to λkatE6. The probing of the library with pMM122 led to the isolation of 3 positive λ clones labelled λkatF1 to λkatF3.

4.6 Mapping of the λkatE and λkatF Clones

The restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, and *Sal*I were used to map the λkatE and λkatF clones isolated above. Two *katE* λ clones were mapped, λkatE4 and λkatE6. The sizes of the fragments are listed in Tables 10 and 11 respectively. λkatE4 contained a small, 13 kb insert whereas the insert size of λkatE6 was 17 kb. All of the λkatE4 sequence was contained in the λkatE6 sequence (Figure 5). These clones correlated with the *Escherichia coli* restriction map produced by Kohara *et al.* (1987). The *katE* gene was further localized to a 4.8 kb *Cla*I fragment by P. Sorby (Mulvey *et al.*, 1988).

A single *katF* clone, λkatF3, was mapped using the four restriction enzymes above. The fragment sizes are listed in Table 12. The clone

TABLE 10

Restriction Endonuclease Fragments Obtained by Digestion of λkatE4

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Bam*HI	30.0 11.8	41.8
*Eco*RI	24.5 7.0 10.4	41.9
*Hin*dIII	24.5 5.2 4.4 4.0 2.7 0.8	41.6
*Sal*I	19.4 9.4 9.4 3.2	41.4
*Bam*HIx*Eco*RI	24.5 10.4 7.0 0.7	42.6
*Bam*HIx*Hin*dIII	24.5 5.2 4.4 2.4 2.7 1.2 0.8	41.5
*Bam*HIx*Sal*I	19.4 9.4 9.4 1.8 1.4	41.4
*Eco*RIx*Hin*dIII	24.5 5.2 4.4 3.3 2.7 0.8 0.6	41.5
*Eco*RIx*Sal*I	19.4 9.4 5.0 5.0 2.1 1.1	42.0
*Hin*dIIIx*Sal*I	19.4 4.85 4.4 4.2 2.7 2.7 1.2 0.8 0.5	40.8
	Average size	**41.7**

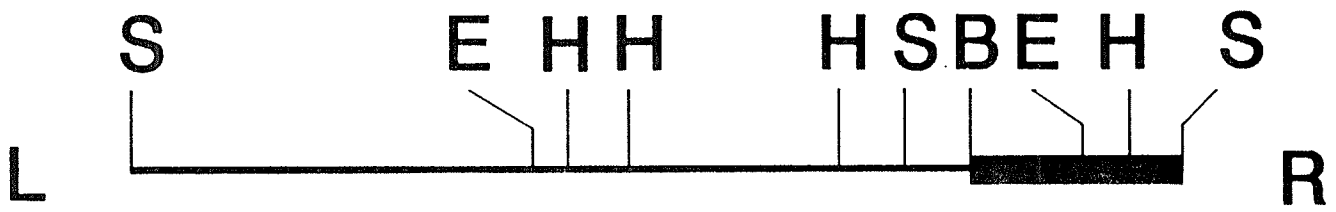
TABLE 11

Restriction Endonuclease Fragments Obtained by Digestion of λkatE6

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Bam*HI	30.0 15.5	45.5
*Eco*RI	24.5 15.5 6.6	46.6
*Hin*dIII	24.5 7.2 4.4 3.9 2.6 1.8 0.8	45.2
*Sal*I	19.4 9.4 9.4 7.7	45.9
*Bam*HI x *Eco*RI	24.5 15.5 6.6 0.7	47.3
*Bam*HI x *Hin*dIII	24.5 7.2 4.4 2.6 2.6 1.4 1.8	
	0.8	45.3
*Bam*HI x *Sal*I	19.4 9.4 9.4 7.7	45.9
*Eco*RI x *Hin*dIII	24.5 7.2 4.4 3.2 2.6 1.8 0.8	
	0.6	45.1
*Eco*RI x *Sal*I	19.4 9.4 5.0 5.0 3.8 2.0	44.6
*Hin*dIII x *Sal*I	19.4 5.0 4.7 4.4 3.0 2.9 2.6	
	1.8 1.3 0.8	45.9
	Average size	**45.7**

Figure 5. Restriction maps of the two λkatE clones isolated from the MP180 library. The dark line represents the portion of the clone that is homologous to pMM120.

λkatE4



λkatE6

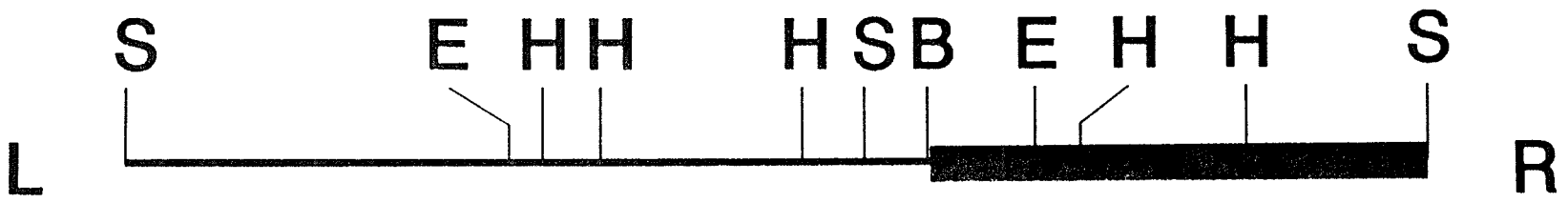


TABLE 12

Restriction Endonuclease Fragments Obtained by Digestion of λkatF3

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Bam*HI	22.3 13.0 10.2	45.5
*Eco*RI	35.9 10.3	46.2
*Hin*dIII	19.8 12.1 9.4 4.4	45.3
*Sal*I	19.4 11.5 9.4 5.8	46.1
*Bam*HIx*Hin*dIII	19.8 9.4 8.8 4.4 2.3 1.7	45.6
*Bam*HIx*Sal*I	19.4 9.4 8.4 3.9 2.9 2.0	46.0
*Eco*RIx*Hin*dIII	19.8 12.1 5.6 4.4 3.6	45.5
*Hin*dIIIx*Sal*I	19.4 6.5 4.9 4.6 4.4 4.2 1.5	45.5
*Eco*RIx*Sal*I	19.4 10.5 9.4 5.8 0.85	46.0
	Average size	**45.8**

contained an insert of 17.1 kb and is shown in Figure 6. This map is also in close agreement with the *E. coli* genomic map deduced by Kohara *et al.* (1987).

4.7 Subcloning of *katF*

The insertion of the transposon in the strain UM122 caused a KatF$^-$ phenotype (Loewen *et al.*, 1985). Therefore it was concluded that the transposon inserted into the *katF* gene. The insertion of the transposon in UM122 was close to the center of the 10.2 kb *Bam*HI fragment in λkatF3. This 10.2 kb fragment was Genecleaned from an agarose gel and cloned into the *Bam*HI site of pAT153. The insertion of a DNA fragment into the *Bam*HI site of pAT153 resulted in the inactivation of the Tet^R gene. DNA was isolated from Amp^R Tet^S transformants and restriction enzyme digests were performed using *Bam*HI. One of the plasmids harbouring the 10.9 kb *Bam*HI fragment was named pMMkatF1. It was not possible to directly determine if the *katF* gene was on this fragment because the strain used in the transformation (HB101) was wild-type with respect to catalase activity. Therefore, it was necessary to transform the catalase negative strain UM258 (*katG2*, *katF13*::Tn*10*) with pMMkatF1. Catalase activity was scored by applying a drop of 30% H_2O_2 directly onto one of the transformants. A positive result (the liberation of oxygen in the form of bubbles) was produced indicating the complementation of the *katF13*::Tn*10* mutation in UM258. This clone was then mapped using a number of other restriction enzymes (Table 13). A 4.1 kb *Cla*I fragment located in the center of the *Bam*HI fragment (Figure 7) was subcloned into the *Cla*I site of pAT153. The resulting Tet^S plasmid was labelled pMMkatF2. The same fragment in the reverse

Figure 6. Restriction map of λkatF3. The thick line represents the portion of λkatF3 that is homologous to pMM122.

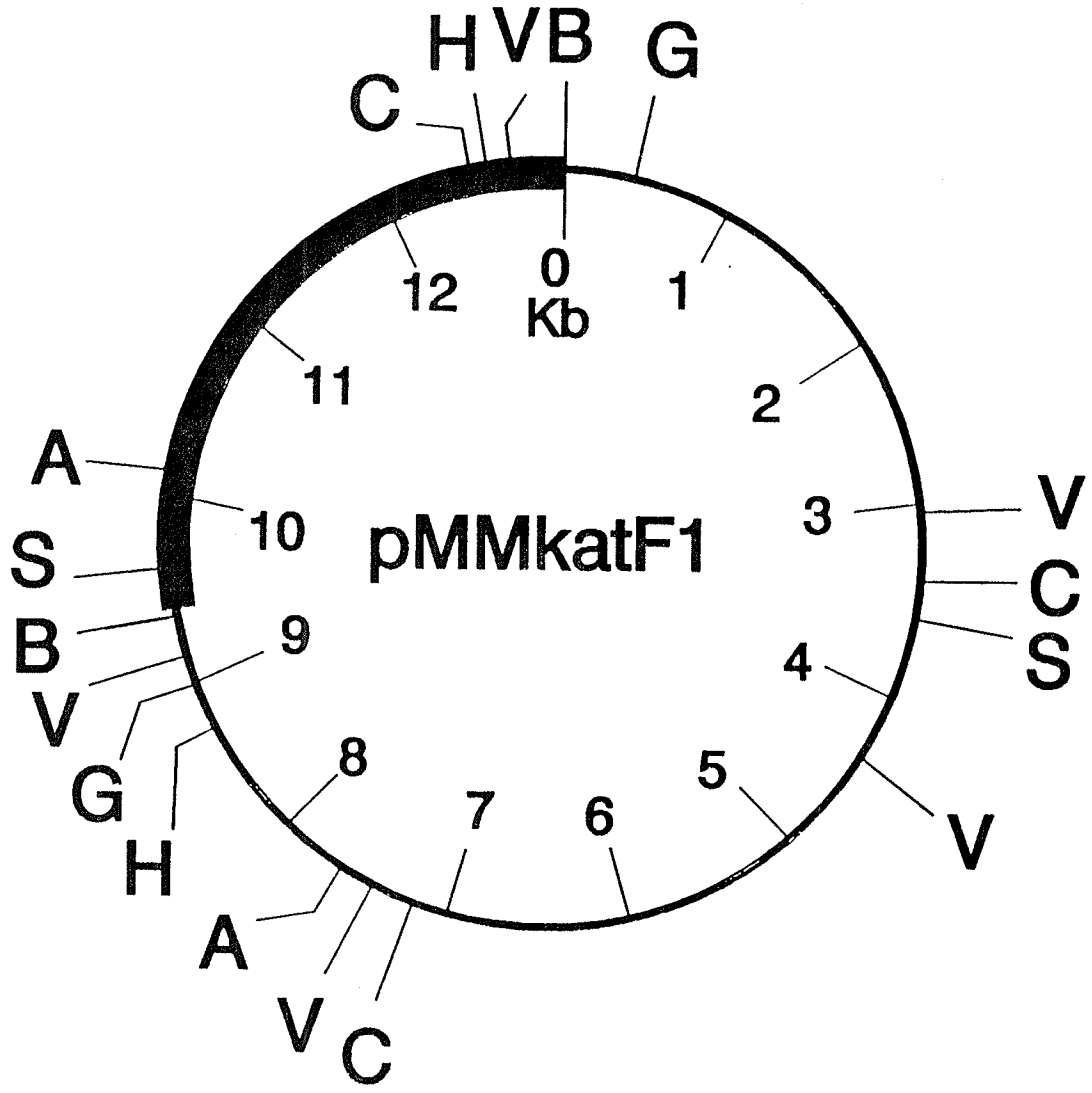
λ katF3

TABLE 13

Restriction Enzyme Fragment Sizes Obtained from the Digestion of pMMkatF1

Restriction Endonuclease	Fragment Sizes (kb)	Sum (kb)
*Ava*I	7.7 3.4	11.1
*Bam*HI	10.2 3.6	13.8
*Bgl*II	8.1 4.6	12.7
*Cla*I	6.3 4.2 2.8	13.3
*Eco*RV	3.7 2.8 2.4 2.3 1.3	13.0
*Ava*I x *Bgl*II	6.3 2.8 1.9 1.6	12.6
*Ava*I x *Cla*I	3.9 3.4 2.6 2.0	11.9
*Ava*I x *Eco*RV	2.6 2.3 2.1 2.0 1.4 1.2	11.6
*Bam*HI x *Cla*I	4.2 3.2 3.0 2.5	12.9
*Bam*HI x *Bgl*II	4.6 4.1 3.4	12.1
*Bam*HI x *Eco*RV	3.9 2.9 2.5 2.3 1.4	13.0
*Bgl*II x *Cla*I	4.1 3.6 2.3 1.9	11.9
*Bgl*II x *Eco*RV	3.6 2.7 2.2 2.0 1.3	11.8
*Cla*I x *Eco*RV	3.6 2.8 2.3 2.1 1.0	11.8
	Average size	**12.4**

Figure 7. Restriction map of the plasmid pMMkatF1. The thick line represents the vector pAT153, and the thin line represents the insert. New restriction enzyme abbreviations used are as follows: *Ava*I, A; *Cla*I, C; and *Eco*RV, V.


pMMkatF1
0
Kb
1
2
3
4
5
6
7
8
9
10
11
12
H
V
B
G
C
A
S
B
V
G
H
A
V
C
V
C
S
V

orientation was also isolated and named pMMkatF3. Both clones complemented the *katF* mutation in UM258. The fact that both orientations complemented the mutation confirmed that the entire gene, including the promoter, was located on this segment. The plasmid pMMkatF2 was used in a large number of experiments including maxicell analysis, catalase assays, and a source of DNA for sequence analysis. For these reasons, this clone was mapped using a large number of restriction enzymes (Table 14). The map of pMMkatF2 is shown in Figure 8.

4.8 HPII Production

The plasmid pMMkatF2 was transformed into MP180 (wild-type), UM120 (*katE*::Tn*10*), UM122 (*katF*::Tn*10*) and UM202 (*katG*::Tn*10*). Catalase levels were measured using the oxygraph in both mid-log and stationary phase cells. The levels are listed in Table 15. In MP180, catalase levels increased approximately two fold in mid-log phase and were only slightly higher in stationary phase. The strains UM120 and UM122 containing pMMkatF2 exhibited similar two to three fold increases in catalase activity in mid-log and stationary phase cultures. The plasmid pMMkatF1 had similar catalase levels to pMMkatF2 in the strain UM122. There was no increase in catalase levels in mid-log cells of UM202 and only a slight increase in stationary phase cells containing the plasmid.

In order to confirm that the plasmid pMMkatF2 complemented the *katF* mutation in UM258 and not the *katG* mutation, crude extracts of stationary phase cells with and without the plasmid were electrophoresed on non-denaturing polyacrylamide gels and then stained for catalase activity (Figure 9). In MP180 and MP180 [pMMkatF2] there was no

TABLE 14

Restriction Endonuclease Fragments Obtained by Digestion of pMMkatF2

Restriction Endonuclease	Fragment Sizes (Kb)	Sum (Kb)
*Cla*I	4.13 3.34	7.47
*Bst*EII	7.4	7.40
*Dra*I	2.9 2.47 1.79 0.717	7.88
*Eco*RV	4.45 2.80 0.52	7.70
*Hinc*II	2.44 2.0 1.29 1.07 0.94	7.74
*Nru*I	3.42 2.07 1.88	7.37
*Pvu*II	7.7	7.70
*Sal*I	4.0 3.4	7.40
*Cla*I x *Bst*EII	3.6 2.87 1.14	7.60
*Cla*I x *Dra*I	2.84 2.4 1.3 0.717 0.54	7.80
*Cla*I x *Eco*RV	3.25 2.8 1.03 0.42	7.50
*Cla*I x *Hinc*II	2.44 1.56 1.3 0.94 0.67 0.54	7.45
*Cla*I x *Nru*I	2.5 1.88 1.18 1.0 0.87	7.44
*Cla*I x *Pvu*II	3.42 2.8 1.27	7.49
*Cla*I x *Sal*I	3.42 2.8 0.63 0.63	7.48
*Bst*EII x *Eco*RV	4.5 2.08 0.854 0.49	7.90
*Dra*I x *Eco*RV	2.47 2.26 1.36 0.717 0.57 0.5	7.90
*Eco*RV x *Hinc*II	2.44 1.56 1.30 0.94 0.63 0.56	7.43
*Eco*RV x *Nru*I	3.8 1.95 0.85 0.73 0.5	7.83
*Eco*RV x *Pvu*II	4.45 2.77 0.52	7.74
*Eco*RV x *Sal*I	3.9 2.87 0.5 0.5	7.70

*Hin*cIIx*Nru*I	2.44 1.56 1.17 0.94 0.84 0.63	
	0.44	8.0
	Average size	**7.6**

Figure 8. Restriction map of the plasmid pMMkatF2. The thick line represents the vector pAT153. New restriction enzyme abbreviations introduced in this diagram are as follows: *Dra*I, D; *Hin*cII, Hc; *Kpn*I, K; and *Pvu*II, P.

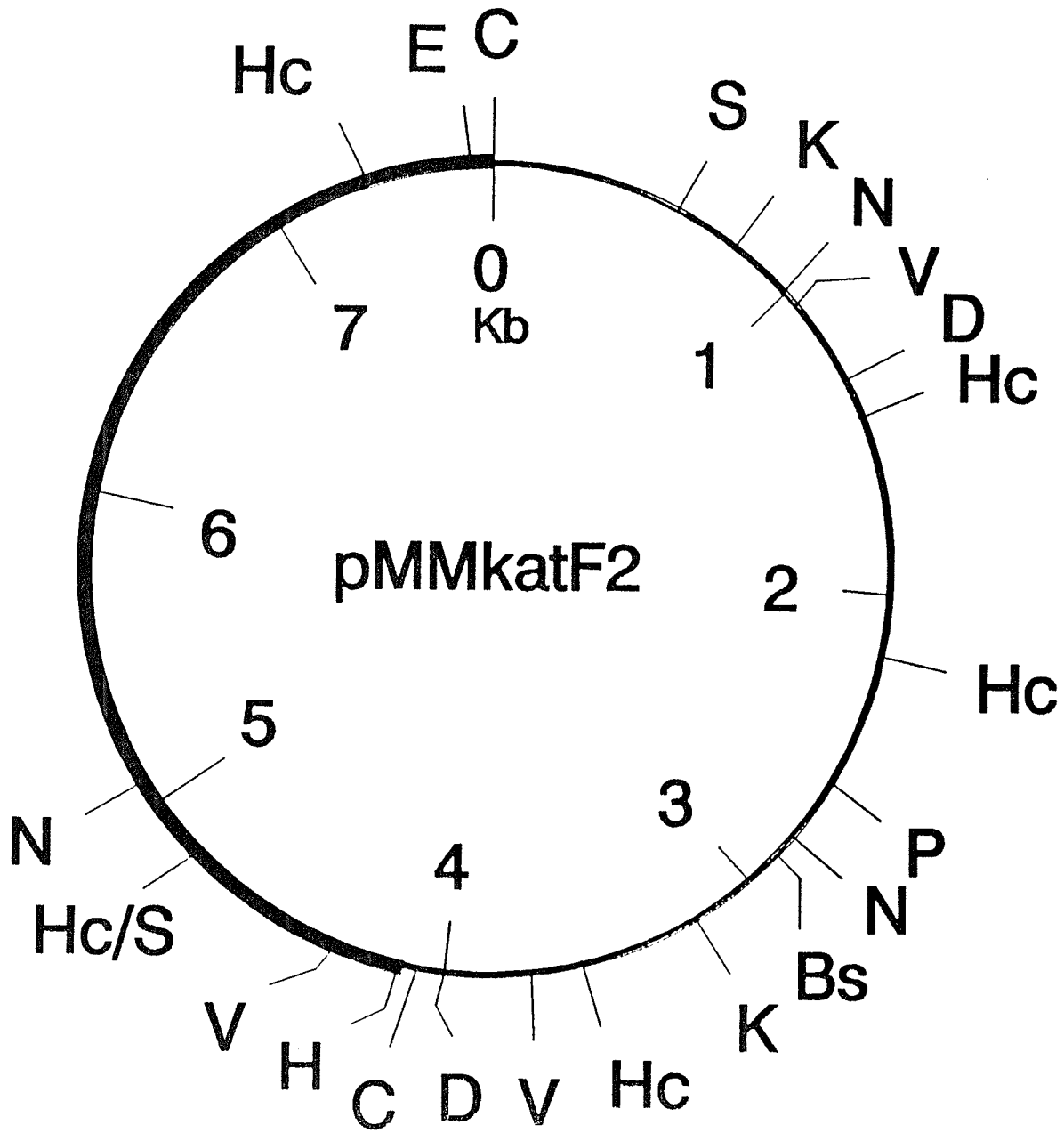

Hc
E
C
S
K
N
V
D
Hc
0
Kb
7
1
6
pMMkatF2
2
Hc
5
3
N
4
P
N
Hc/S
Bs
V
K
H
C
D
V
Hc

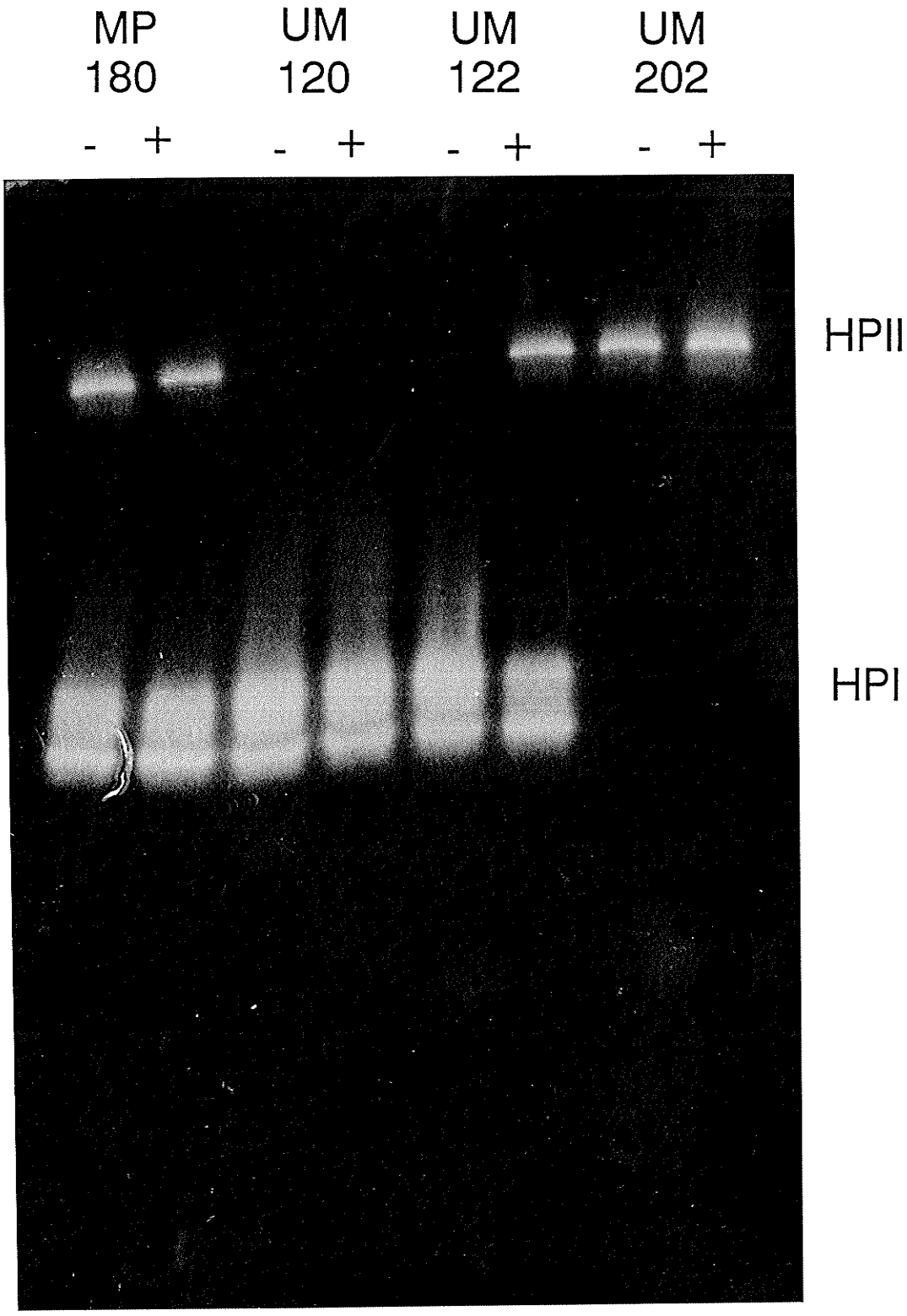
TABLE 15

Catalase Activity of pMMkatF1 and pMMkatF2 in Various Strains at Mid-log and Stationary Phase

Strain	Catalase Activity (u/mg dry cell wt)	
	Mid-log Phase	Stationary Phase
MP180 (wild-type)	5.7	58.8
MP180 [pMMkatF2]	14.8	78.2
UM120 (*katE*::Tn*10*]	8.8	17.3
UM120 [pMMkatF2]	15.7	41.3
UM122 (*katF*::Tn*10*]	4.3	17.8
UM122 [pMMkatF1]	ND[a]	49.4
UM122 [pMMkatF2]	18.4	60.3
UM202 (*katG*::Tn*10*)	2.0	33.7
UM202 (pMMkatF2]	1.4	54.1

[a] not determined

Figure 9. Visualization of catalase activities from crude extracts following electrophoresis on an 8.5% non-denaturing gel. Extracts of various strains are shown with (+) and without (-) pMMkatF2. The slower migrating band is HPII and the faster migrating HPI forms a doublet.


MP
180
UM
120
UM
122
UM
202
- + - + - + - +
HPII
HPI

apparent increase in HPII activity with the plasmid present. UM120 and UM120 [pMMkatF2] did not produce any visible HPII. This was expected because the *katF* gene should not complement the *katE* gene. There was no visible HPII produced from UM122 (*katF*::Tn*10*) but UM122 [pMMkatF2] produced a visible HPII band. In UM202 (*katG*::Tn*10*) there was no production of HPI and no visible increase in HPII levels with pMMkatF2 present.

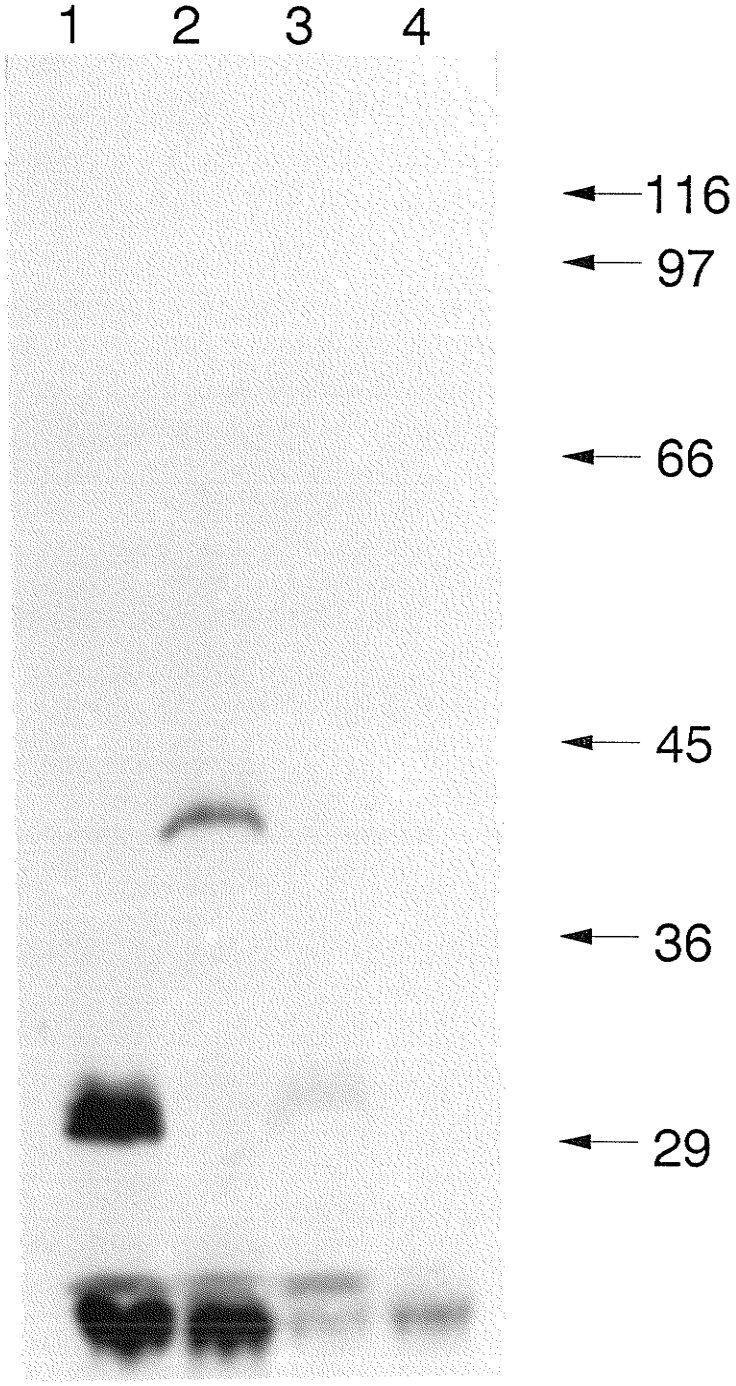
4.9 Maxicell Analysis of pMMkatF2

Proteins encoded by pMMkatF2 were preferentially labelled by the maxicell procedure descried by Sancar *et al.* (1979). The autoradiogram of the plasmid encoded proteins is shown in Figure 10. Cells with no plasmids present only produced a fast running band of cell debris. A double band of M_r 28 000 and 32 000 was produced from cells harbouring the plasmid pAT153. This plasmid encodes the amp^R gene which is responsible for the production of these two proteins. A second Tc^R gene found on the plasmid produces a protein of M_r 37 000. This protein was not visible in the autoradiographs. The plasmid which complemented the *katF* mutation in UM258, pMMkatF2, produced two protein bands. The first was very faint and corresponded to one of the amp^R proteins. The second band had an approximate M_r of 44 000 and did not correspond to any of the bands found in pAT153. This presumed KatF protein is much too small to be the 93 000 dalton HPII subunit.

4.10 Identification of the Chromosomal Location of the Tn*10* in UM122

The transposon Tn*10* was used to construct HPII deficient strains and map the *katF* gene to 59.0 minutes on the *E. coli* chromosome (Loewen

Figure 10. Maxicell analysis of plasmid encoded proteins. Lanes: 1, UM258 [pAT153]; 2, UM258 [pMMkatF2]; 3, MP180 [pAT153]; 4, UM258. The numbers along the side indicate the locations of the molecular weight standards ($x10^3$).


1
2
3
4
116
97
66
45
36
29

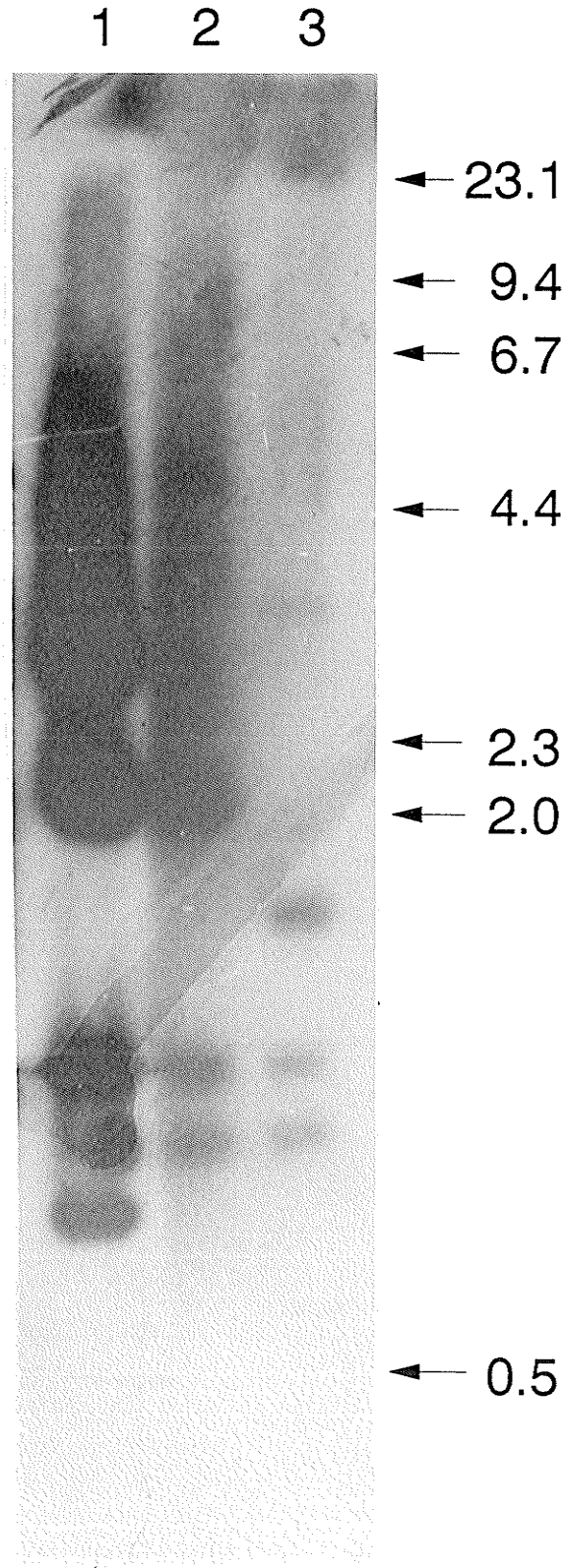
and Triggs, 1984). Southern blots of digested chromosomal DNA from MP180(wild-type) and UM122 (*katF*::Tn*10*) were probed with pMMkatF2 in order to verify that the *katF* gene had actually been cloned from the same chromosomal location as the Tn*10*. The autoradiograph obtained from the blot is shown in Figure 11. The first lane contains five bands (2.5, 1.9, 1.2, 1.0, and 0.87 kb) produced from the digestion of pMMkatF2 with *Cla*I and *Nru*I. Of the five bands in this lane, only three correspond to the insert DNA. The first band (1.2 kb) corresponded to the fragment starting from the *Cla*I site at the 0 position of pMMkatF2 and extending to the *Nru*I site, while the 1.9 kb band corresponded to the fragment between the two *Nru*I sites in the insert. The third 1.0 kb band was from the *Cla*I site (defining the other end of the insert) to the second *Nru*I in the insert.

The pattern obtained from the digest of MP180 DNA with *Cla*I and *Nru*I (Lane 2) is similar to pMMkatF2. The three bands corresponding to the insert DNA in pMMkatF2 hybridize in MP180. This result is expected because MP180 was used as a source of DNA to clone the *katF* gene.

The third lane containing the *Cla*I-*Nru*I digested UM122 (*katF*::Tn*10*) showed an altered hybridization pattern. The 1.9 kb *Nru*I fragment was replaced by a 1.6 kb fragment. *Nru*I cuts approximately 80 bp within either end of the Tn*10* (Way *et al.*, 1984) (Figure 2). The remainder of the original fragment, 0.3 kb plus the 80 bp of Tn*10*, was sufficiently small that it was lost from the bottom of the gel.

The change in the hybridization in UM122 (*katF*::Tn*10*) confirmed that this was the same gene found on the plasmid pMMkatF2. Furthermore, the restriction map of the clone λkatF3 is in close agreement with the *E. coli* genomic map deduced by Kohara *et al.* (1987).

Figure 11. Autoradiogram showing the change in hybridization pattern when genomic DNA with or without a Tn*10* insertion in *katF* was probed with α^{32}P-labelled pMMkatF2. Lanes: 1, pMMkatF2 digested with *Cla*I and *Nru*I; 2, MP180 genomic DNA digested with *Cla*I and *Nru*I; 3, UM122 (*katF*::Tn*10*) genomic DNA digested with *Cla*I and *Nru*I. The numbers along the side indicate the sizes in kb of the fragments obtained by digesting λ DNA with *Hin*dIII.


1
2
3
23.1
9.4
6.7
4.4
2.3
2.0
0.5

From the maps of the λkatF::Tn*10* clones and the data above it was also possible to predict that the transposon inserted into the 1.9 kb *Nru*I fragment approximately 400 bp from the *Nru*I site at the 3 Kb position of pMMkatF2 (Figure 12).

4.11 The Sequence Analysis of *katF*

4.11.1 Defining the Ends of the Gene

Before the *katF* gene could be sequenced it was necessary to further localize the gene on pMMkatF2. From maxicell analysis the size of the KatF protein was predicted to be 44 000 daltons. A minimum of 1.3 kb is required to encode a protein of this size.

The first subclone isolated from pMMkatF3 that retained the ability to complement the *katF* mutation was named pM13-F2. This clone was composed of a 2.3 kb *Kpn*I fragment from pMMkatF2 inserted into the *Kpn*I site of pM13KS(-) (Figure 13). Three other clones were isolated from pMMkatF3 or pM13-F2 that localized the *katF* gene to a 1.5 kb DNA segment (Figure 14). The first clone, labelled pM13-F4, was a 1.8 kb *Kpn*I-*Dra*I fragment from pMMkatF3 cloned into the *Kpn*I-*Eco*RV site of pM13KS(-). This recombinant did not complement the *katF*::Tn*10* mutation in UM258. A second clone isolated was composed of a 2.0 kb *Nru*I fragment from pM13-F2 cloned into the *Eco*RV site of pM13KS(-). This clone complemented the *katF* mutation and was named pM13-F5. These two clones, pM13-F4 and pM13-F5, localized one end of the *katF* gene to a 350 bp region between the *Nru*I and *Dra*I sites. A third clone named pM13-F6 was isolated from an *Apa*I-*Hin*cII (partial digest) of pM13-F2. This clone complemented the *katF* mutation indicating that the other end of the gene must lie between the *Hin*cII and *Acc*I sites.

Figure 12. Diagram showing the location of the Tn*10* in the 4.1 Kb *Cla*I fragment. The cross hatched region represents the Tn*10*.

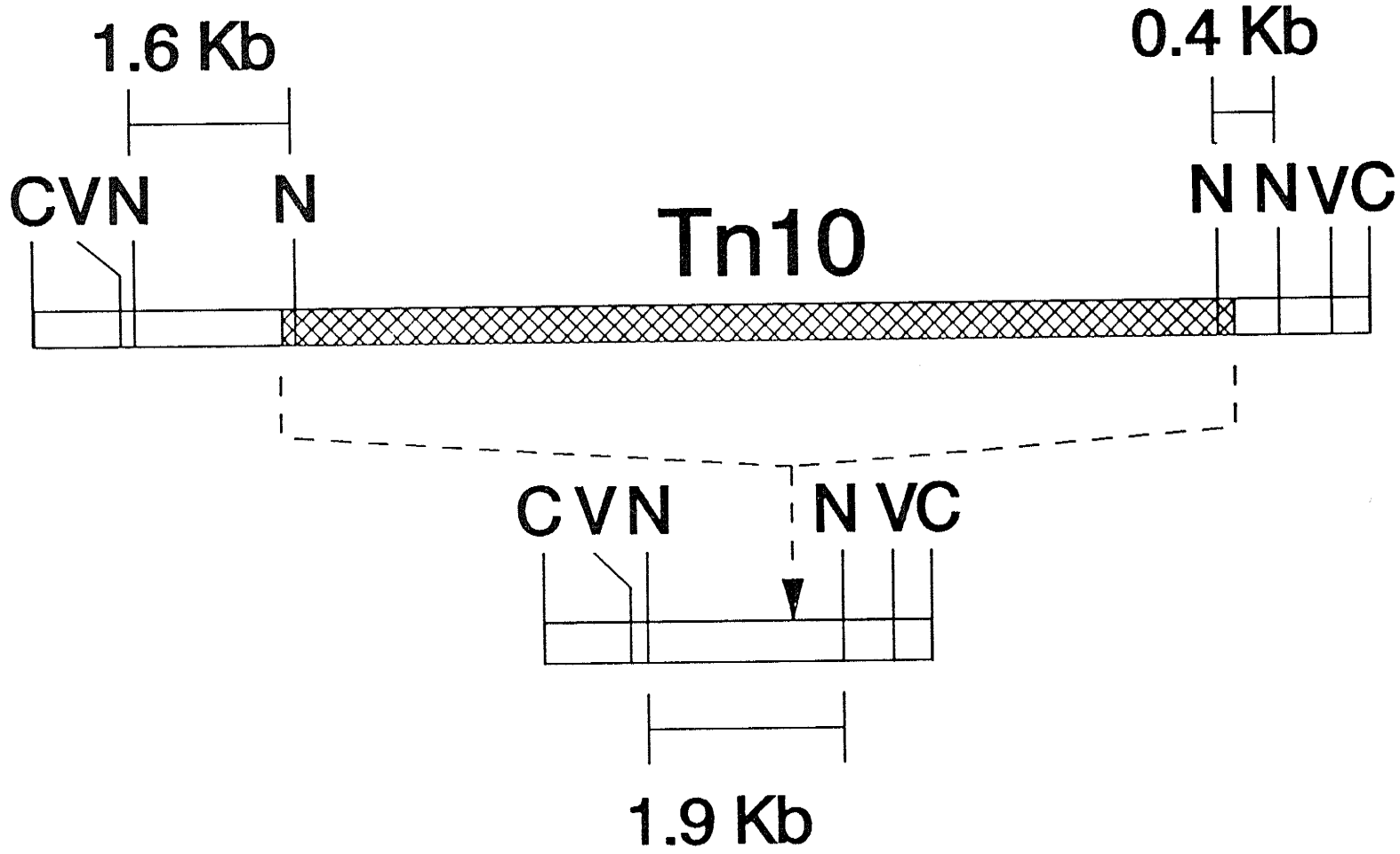
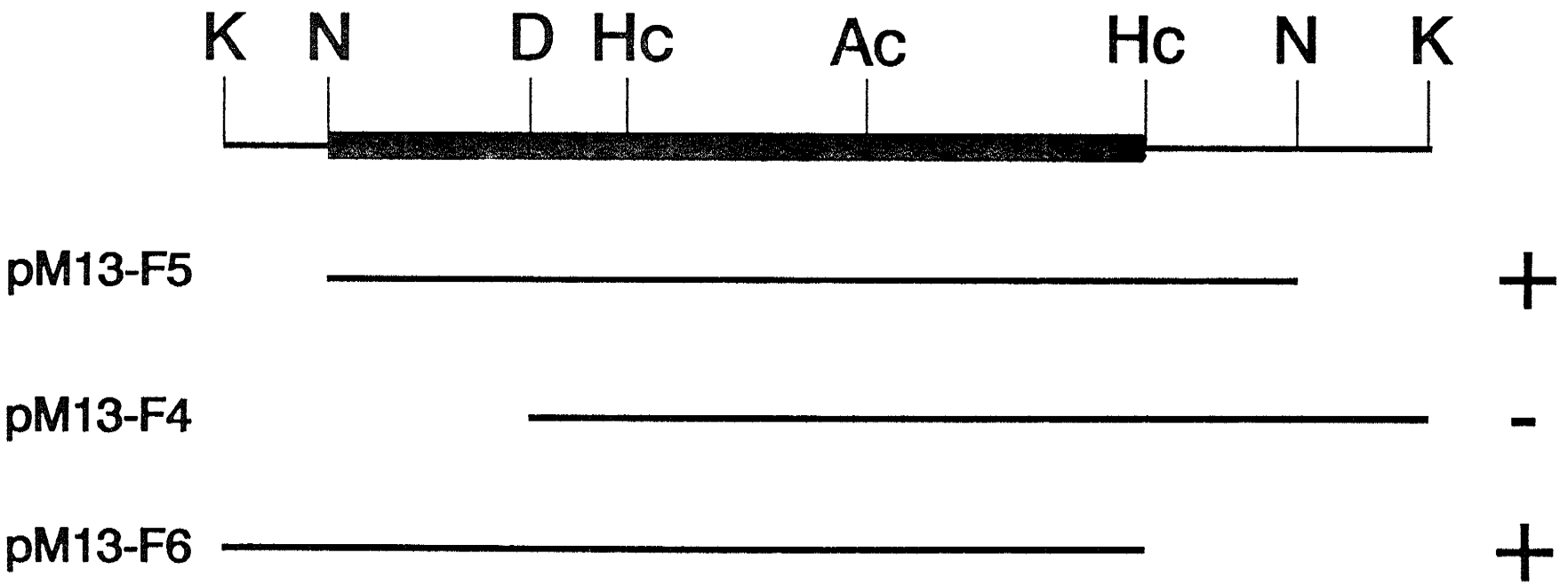

1.6 Kb
0.4 Kb
C V N
N
Tn10
N N V C
C V N
N V C
1.9 Kb

Figure 13. Restriction map of pM13-F2. The thick line represents the vector pM13. The multiple cloning region (MCR) is shown below the map. A new restriction enzyme abbreviation Ac for *Acc*I is used.

MCR
SacI
BstEII
SacII
NotI
EagI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
AccI
XhoI
ApaI
KpnI
MCR
D
D
P
K
V
N
D
H
Ac
H
P
N
K
P
0
kb
1
2
3
4
5
pM13F2

Figure 14. Defining the location of the *katF* gene. Thick line represents the location of the *katF* gene in the 2.3 kb *Kpn*I fragment. Positive or negative signs beside the various fragments denotes the presence (+) or absence (-) of catalase activity.


K
N
D
Hc
Ac
Hc
N
K
pM13-F5
+
pM13-F4
-
pM13-F6
+

4.11.2 Isolation of *Sau*3A Deletion Mutants for Sequencing

Partial *Sau*3A deletion mutants were constructed from the pM13-F2 plasmid. The plasmid was linearized by digestion with *Bam*HI and then partially digested with *Sau*3A. The deletion mutants were recircularized with DNA ligase and then digested with *Xho*I. This enzyme is located between the *Bam*HI site and the insert in the multiple cloning region (Figure 13). Any plasmids which do not contain a deletion would be linearized by this enzyme and could not be transformed (linear DNA does not enter the cell in a transformation). DNA was isolated from the transformants and digested with *Nru*I-*Xba*I to excise the insert. Four deletion mutants were isolated and named pΔ2, pΔ4, pΔ5, and pΔ6. The sizes of the plasmids and extent of the deletions are shown in Table 16.

To obtain *Sau*3A deletions in the reverse direction, a clone with an insert reverse to that of pM13-F2 was required. The clone pM13-F3 was selected. This clone is a *Kpn*I-*Pvu*II fragment from pMMkatF2 in the *Kpn*I-*Eco*RV site in pM13KS(-). The plasmid was linearized with *Bam*HI and then partially digested with *Sau*3A. The plasmids were resealed with ligase and then digested with *Eco*RI instead of *Xho*I. The *Xho*I site was lost in the construction of this plasmid and *Eco*RI was selected to linearize any plasmids which were not deleted. The DNA was isolated from the transformants and digested with *Xba*I to linearize the plasmids and *Xba*I-*Kpn*I to excise the insert. Three mutants of varying size were isolated and named pΔ15R, pΔ25R, and pΔ34R. The plasmid sizes are shown in Table 16.

Both of the initial plasmids used to construct the deletion mutants were in M13KS(-). Sequence analysis of the single stranded DNA produced from these plasmids would be from the *Kpn*I end that was not

Table 16

Plasmid Sizes of the *Sau*3A Deletion Mutants

Plasmid Name	Size of Plasmid (kb)	Size of Deletion (kb)
pM13-F2	5.3	-
pΔ2	5.1	0.2
pΔ4	4.76	0.54
pΔ5	4.42	0.88
pΔ6	4.24	1.06
pM13-F3	4.85	-
pΔ25R	3.81	1.04
pΔ15R	3.52	1.33
pΔ34R	3.3	1.55

deleted by the *Sau*3A. Therefore, all of the mutants were cloned into the M13mp19 vector. Inserts from pΔ2, pΔ4, pΔ5, pΔ6 were excised using *Nru*I-*Xba*I, separated on a 0.7% agarose gel and Genecleaned. The fragments were cloned into M13mp19 *Xba*I-*Hin*cII site and single stranded DNA was isolated for sequencing. The inserts from the reverse deletion mutants were obtained by digesting the plasmids with *Xba*I-*Kpn*I, separating the inserts on a 0.7% agarose gel, and then Genecleaning them. The fragments were then cloned into M13mp18 at the *Xba*I-*Kpn*I sites and sequenced.

Each of the deletion mutants was transformed into UM258 and assayed for catalase. All of the mutants were catalase negative.

4.11.3 Sequencing Strategy

As described above, a number of clones for sequencing were isolated using the *Sau*3A deletion technique. The remainder of the clones required for sequencing both strands of the gene were subcloned from pM13-F2 into the vectors M13mp18, M13mp19, M13KS(+), or M13KS(-). The multiple cloning regions (MCR) for these four vectors are shown in Figure 15. M13mp18 and 19 differ by the orientation of the MCR. The M13KS(+) and (-) vectors contain a different MCR than the M13mp vectors. The orientation of the MCR is the same in both of the (+) and (-) vectors. The two vectors differ by the strand of single stranded DNA that is produced. When single stranded DNA produced from M13KS(+) is sequenced, the M13 universal or SK primers are used. The reverse primer or KS primer is used when sequencing single stranded DNA produced from the M13KS(-) plasmid.

The complete sequencing strategy for the *katF* gene is shown in

Figure 15. The multiple cloning regions of the four vectors used in the sequence analysis of *katF*.

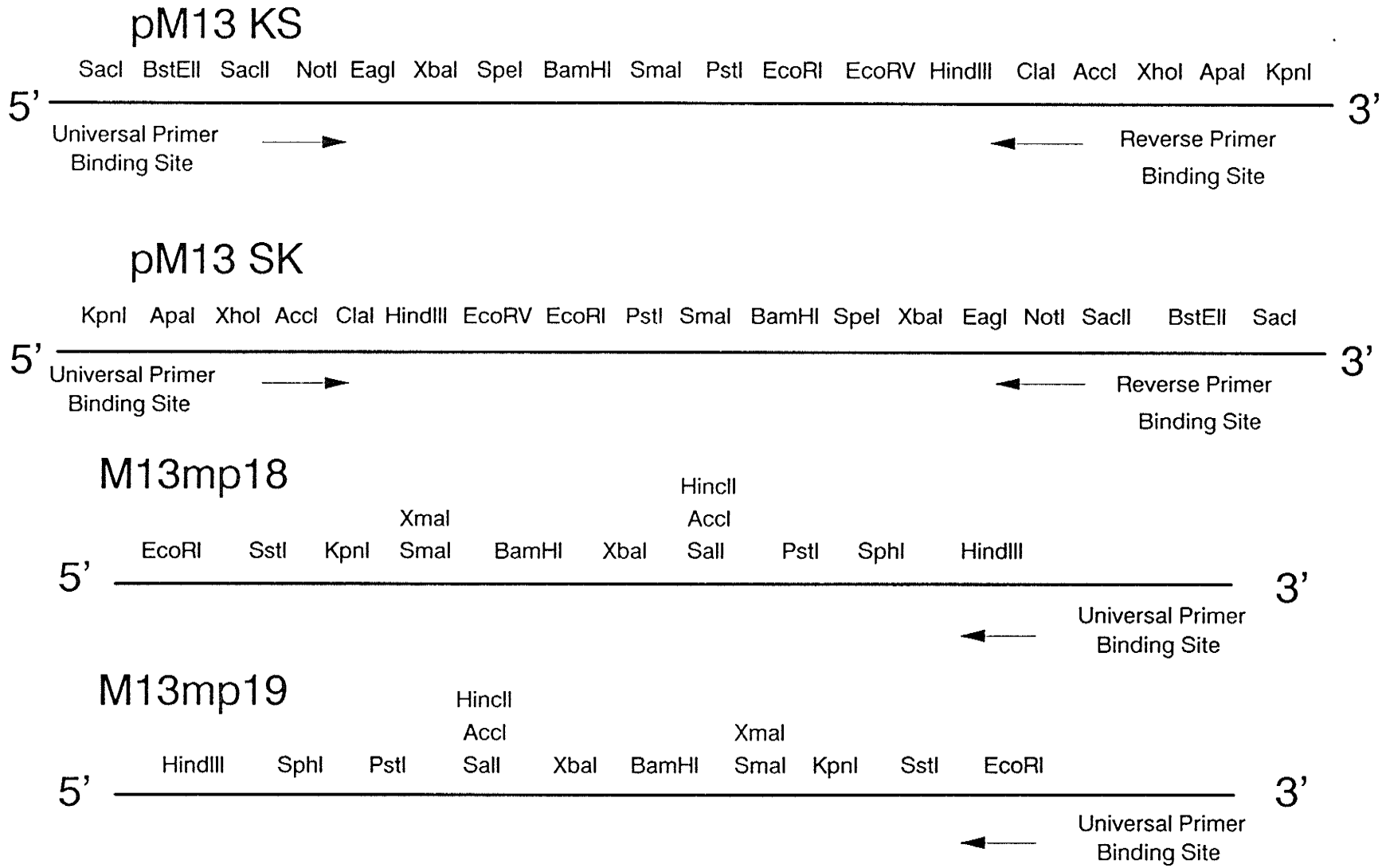

pM13 KS
SacI BstEII SacII NotI EagI XbaI SpeI BamHI SmaI PstI EcoRI EcoRV HindIII ClaI AccI XhoI ApaI KpnI
5'
3'
Universal Primer Binding Site
Reverse Primer Binding Site
pM13 SK
KpnI ApaI XhoI AccI ClaI HindIII EcoRV EcoRI PstI SmaI BamHI SpeI XbaI EagI NotI SacII BstEII SacI
5'
3'
Universal Primer Binding Site
Reverse Primer Binding Site
M13mp18
HincII AccI
XmaI SmaI
EcoRI SstI KpnI BamHI XbaI SalI PstI SphI HindIII
5'
3'
Universal Primer Binding Site
M13mp19
HincII AccI
XmaI SmaI
HindIII SphI PstI SalI XbaI BamHI KpnI SstI EcoRI
5'
3'
Universal Primer Binding Site

Figure 16. Sequencing strategy of *katF*. The origins of the arrows indicate the points at which sequencing began. The directions of the arrows indicate the direction of sequencing. Numbers above the arrows can be cross referenced to Table 17 to see the clone construction. New restriction enzyme abbreviations are as follows: *Hpa*II, Ha; *Rsa*I, R; *Sau*3A, S; *Hinc*II, H; and *Taq*I, T.

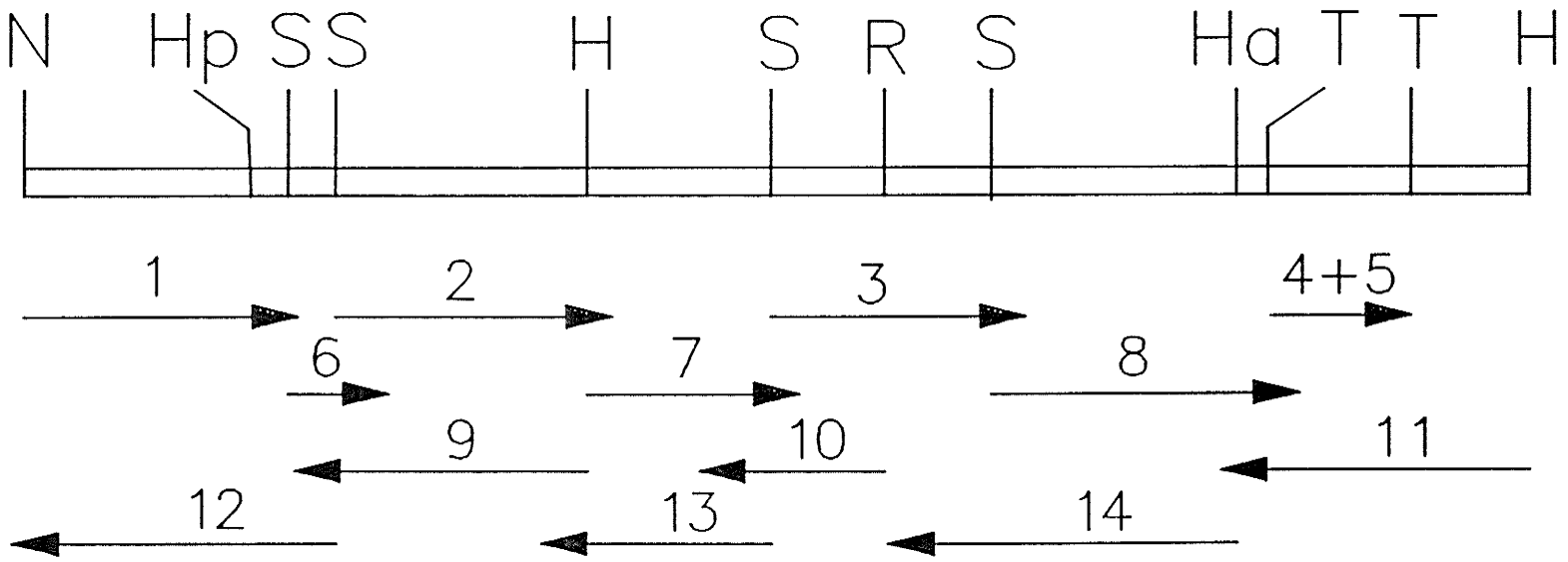

N
Hp
S
S
H
S
R
S
Ha
T
T
H
1
2
3
4+5
6
7
8
9
10
11
12
13
14

Table 17

Construction of Clones for Sequencing *katF*

Fragment Number	Name	Vector	Enzyme(s)	Origin	Enzyme(s)
1	*Nru* #1	M13KS(-)	*Eco*RV	pM13F2	*Nru*I
2	pΔ4*				
3	pΔ5*				
4	*Taq* #1	M13mp18	*Acc*I	pM13F2	*Taq*I
5	*Taq* #3	M13mp18	*Acc*I	pM13F2	*Taq*I
6	*Sau* #24	M13mp18	*Bam*HI	pMMkatF2	*Sau*3A
7	*Hin*c #4	M13mp19	*Hin*cII	pMMkatF2	*Hin*cII
8	pΔ6*				
9	*Hin*c #3	M13mp19	*Hin*cII	pMMkatF2	*Hin*cII
10	*Rsa* #10	M13mp18	*Hin*cII	pMMkatF2	*Rsa*I
11	*Hin*c #1	M13mp19	*Hin*cII	pMMkatF2	*Hin*cII
12	pΔ15R*				
13	pΔ25R*				
14	*Hae* #12	M13mp18	*Sma*I	pMMkatF2	*Hae*III

* construction previously described in Table 16

Figure 16. The sequenced fragments, indicated by arrows, are numbered to allow for cross referencing to Table 17 which contains the descriptions of their construction.

When subcloning into the M13mp vectors, white plaques were picked and single stranded DNA was isolated. The DNA was then T-tracked to determine if it actually contained an insert or if the white plaque was a result of a deletion. DNA found to contain inserts was then sequenced and recorded.

Subcloning into the M13KS(+) or (-) vectors required a different protocol to screen for inserts. Because this vector is a plasmid, white colonies were picked for further study instead of plaques. The double stranded plasmid DNA was isolated from these colonies and digested with restriction enzymes which would excise the insert. The digests were electrophoresed on agarose gels and the inserts were sized. Single stranded DNA was prepared from clones containing the appropriate insert. This DNA was then sequenced and recorded.

The entire 1.5 kb *Nru*I-*Hin*cII region was sequenced in both directions. All restriction sites were overlapped to ensure that no small fragments were missed.

4.11.4 The *katF* DNA Sequence

The complete nucleotide sequence of the 1483 bp *Nru*I-*Hin*cII fragment is shown in Figure 17. All of the predicted restriction sites corresponded quite well to the restriction map generated for pM13F2, although some of the fragment sizes differed by as much as 200 bp. The 1483 bp sequence contained only one open reading frame large enough to encode a protein with a molecular weight of 44 000 daltons. The 1086 bp

Figure 17. The *katF* DNA sequence and the predicted KatF protein sequence. Numbers on the right hand side of the sequence specify nucleotide number and those on the left indicate amino acid number. The potential promoter (-35 and -10), Shine-Dalgarno sequences (SD1 and SD2) and potential terminator sequences are underlined.

***Nru*I** 60
TCGCGACCTGAGATGGCCGGTTGTTTAGTGCTGGTAATCGTTGTGGGGTGACTGGTAAGT

120
CTGATTATCATGAAATCATAATGATGATTCAGCCTGAGTAGCCTTACGCCCATAACCGAC

180
ACAAGTGCTGGTCCGGGAACAACAAGAAGTTAAGGCGGGGAAAAAAATAGCGACCATGGG

240
-35
TAGCACCGGAACCATTCAACACGCTGCATTTTGAAATTCGTTACAAGGGGAAATCCGTAA

-10 SD1 300
ACCCGCTGCGTTATTTGCCGCAGCGATAAATCGGCGGAACCAGGCTTTGCTTGAATGTTC
M F

SD2 360
CGTCAAGGGATCACGGGTAGGAGCCACCTTATGAGTCAGAATACGCTGAAAGTTCATGAT
R Q G I T R G S H L M S Q N T L K V H D
3

420
TTAAATGAAGATGCGGAATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAGCCGTTA
L N E D A E F D E N G V E V F D E K P L
23

480
GTAGAACAGGAACCCAGTGATAACGATTTGGCCGAAGAGGAACTGTTATCGCAGGGAGCC
V E Q E P S D N D L A E E E L L S Q G A
43

540
ACACAGCGTGTGTTGGACGCGACTCAGCTTTACCTTGGTGAGATTGGTTATTCACCACTG
T Q R V L D A T Q L Y L G E I G Y S P L
63

600
TTAACGGCCGAAGAAGAAGTTTATTTTGCGCGTCGCGCACTGCGTGGAGATGTCGCCTCT
L T A E E E V Y F A R R A L R G D V A S
83

660
CGCCGCCGGATGATCGAGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGTTATGGC
R R R M I E S N L R L V V K I A R R Y G
103

720
AATCGTGGTCTGGCGTTGCTGGACCTTATCGAAGAGGGCAACCTGGGGCTGATCCGCGCG
N R G L A L L D L I E E G N L G L I R A
123

780
GTAGAGAAGTTTGACCCGGAACGTGGTTTCCGCTTCTCAACATACGCAACCTGGTGGATT
V E K F D P E R G F R F S T Y A T W W I
143

840
CGCCAGACGATTGAACGGGCGATTATGAACCAAACCCGTACTATTCGTTTGCCGATTCAC
R Q T I E R A I M N N Q T R T I R L P I
163

900
ATCGTAAAGGAGCTGAACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAGCTGGAC
I V K E L N V Y L R T A R E L S H K L D
183

960
CATGAACCAAGTGCGGTAGAGATCGCAGAGCAACTGGATAAGCCAGTTGATGACGTCAGC
H E P S A V E I A E Q L D K P V D D V S
203

1020
CGTATGCTTCGTCTTAACGAGCGCATTACCTCGGTAGACACCCCGCTGGGTGGTGATTCC
R M L R L N E R I T S V D T P L G G D S
223

1080
GAAAAAGCGTTGCTGGACATCCTGGCCGATGAAAAAGAGAACGGTCCGGAAGATACCACG
E K A L L D I L A D E K E N G P E D T T
243

1140
CAAGATGACGATATGAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCCAAACAG
Q D D D M K Q S I V K W L F E L N A K Q
263

1200
CGTGAAGTGCTGGCACGTCGATTCGGTTTGCTGGGGTACGAAGCGGCAACACTGGAAGAT
R E V L A R R F G L L G Y E A A T L E D
283

1260
GTAGGTCGTGAAATTGGCCTCACCCGTGAACGTGTTCGCCAGATTCAGGTTGAAGGCCTG
V G R E I G L T R E R V R Q I Q V E G L
303

1320
CGCCGTTTGCGCGAAATCCTGCAAACGCAGGGGCTGAATATCGAAGCGCTGTTACCGCGA
R R L R E I L Q T Q G L N I E A L L P R
323

1380
GTAAGTAAGCATCTGTCAGAAAGGCCAGTCTCAAGCGAGGCTGGTTTTTTCTGTGCACAA
V S K H L S E R P V S S E A G F F C A Q
343 (362)

1440
TAAAAGGTCCGAATGCCCATCGGACCTTTTTATTAAGGTCAAATTACCGCCCATACGCAC
TERMINATION

*Hinc*II (1483)
ACAGGTAATTAAGAATCCGGTAAAACCGAGAATGGTCGTTAAC

of the KatF protein was determined by maxicell analysis.

The amino acid composition predicted by the DNA sequence is summarized in Table 18. There is high number of charged amino acids with an excess of acidic (17.7%) over basic residues (13.8%). The N terminal region between residue numbers 22 and 56 is extremely rich in acidic amino acids (45.7%) and contains only a single basic residue. The codon usage analysis suggests the KatF protein is in the category of a moderately to highly expressed protein (Table 19).

The *katF* sequence was compared to the GenBank (1986) of DNA sequences using the computer program Microgenie. No homologous DNA sequences were found.

4.11.5 Control Sequences of the *katF* Gene

4.11.5.1 Mapping of the katF Promoter

The RNA initiation site of *katF* was mapped using reverse transcriptase mapping. The method involved the isolation of *katF* RNA from cells with the *katF* gene on a high copy number plasmid. An end labelled primer (complementary to the amino acid initiation point) was then annealed to the RNA and reverse transcriptase was used to synthesize a DNA strand. The length of the newly synthesized DNA corresponded to the length of RNA from the primer to the beginning of the RNA. The fragment was run along side DNA sequence originating from the same primer and the location of the RNA start point was determined.

Two strong bands corresponding to transcriptional start sites were apparent. The two were separated by a single base and were located 22 and 24 bp away from the first AUG codon. A third weaker band was

Table 18. Amino Acid Composition of the KatF Protein

Amino Acid	Number of Residues[a]	% of Total[b]
Alanine	25	6.9 (7.1)
Arginine	37 (35)	10.2 (10.0)
Asparagine	13	3.6 (3.7)
Aspartate	23	6.4 (6.6)
Cysteine	1	0.3 (0.3)
Glutamine	17 (16)	4.7 (4.6)
Glutamate	41	11.3 (11.7)
Glycine	22 (20)	6.1 (5.7)
Histidine	6 (5)	1.7 (1.4)
Isoleucine	20 (19)	5.5 (5.4)
Leucine	47 (46)	12.9 (13.1)
Lysine	13	3.6 (3.7)
Methionine	6 (5)	1.7 (1.4)
Phenylalanine	11 (10)	3.0 (2.8)
Proline	11	3.0 (3.1)
Serine	18 (17)	5.0 (4.8)
Threonine	18 (17)	5.0 (4.8)
Tryptophan	3	0.8 (0.9)
Tyrosine	7	1.9 (2.0)
Valine	23	6.3 (6.6)

[a] The numbers in parentheses are the number of residues in the shorter 350 amino acid KatF.
[b] The numbers in parentheses represent the % composition in the shorter 350 amino acid KatF.

Table 19. Codon Usage in *katF*

TTT Phe 5 (1.4)	**TCT Ser 1 (0.3)**	**TAT Tyr 3 (0.8)**	**TGT Cys 1 (0.3)**
TTC Phe 6 (1.7)	**TCC Ser 2 (0.6)**	**TAC Tyr 4 (1.1)**	**TGC Cys 0 (0.0)**
TTA Leu 5 (1.4)	TCA Ser 4 (1.1)	TAA End 1 (0.3)	TGA End 0 (0.0)
TTG Leu 9 (2.5)	TCG Ser 2 (0.6)	TAG End 0 (0.0)	TGG Trp 3 (0.8)
CTT Leu 6 (1.7)	**CCT Pro 0 (0.0)**	**CAT His 4 (1.1)**	**CGT Arg 19 (5.2)**
CTC Leu 1 (0.3)	**CCC Pro 1 (0.3)**	**CAC His 2 (0.6)**	**CGC Arg 11 (3.0)**
CTA Leu 0 (0.0)	CCA Pro 4 (1.1)	CAA Gln 6 (1.7)	CGA Arg 3 (0.8)
CTG Leu 26 (7.1)	CCG Pro 6 (1.7)	CAG Gln 11 (3.0)	CGG Arg 2 (0.6)
ATT Ile 10 (2.8)	**ACT Thr 2 (0.6)**	**AAT Asn 4 (1.1)**	**AGT Ser 5 (1.4)**
ATC Ile 10 (2.8)	**ACC Thr 7 (1.9)**	**AAC Asn 9 (2.5)**	**AGC Ser 4 (1.1)**
ATA Ile 0 (0.0)	ACA Thr 3 (0.8)	AAA Lys 6 (1.7)	AGA Arg 0 (0.0)
ATG Met 6 (1.7)	ACG Thr 6 (1.7)	AAG Lys 7 (1.9)	AGG Arg 2 (0.6)
GTT Val 8 (2.2)	**GCT Ala 1 (0.3)**	**GAT Asp 14 (3.9)**	**GGT Gly 11 (3.0)**
GTC Val 4 (1.1)	**GCC Ala 7 (1.9)**	**GAC Asp 9 (2.5)**	**GGC Gly 4 (1.1)**
GTA Val 8 (2.2)	GCA Ala 7 (1.9)	GAA Glu 26 (7.1)	GGA Gly 3 (0.8)
GTG Val 3 (0.8)	GCG Ala 10 (2.8)	GAG Glu 15 (4.1)	GGG Gly 4 (1.1)

The codons which are boldfaced are those which show bias in usage in highly expressed genes. Underlined codons are rarely used in in highly expressed genes Grosjean and Fiers (1982).

located 15 bp from the first AUG codon.

4.11.5.2 Control Sequences of the *katF* Gene

E. coli RNA polymerase (holoenzyme) binds at a specific site called the promoter sequence. This sequence is composed of a Pribnow box or -10 region and a -35 region. These sequences vary according to which σ factor is associated with the core polymerase. The -10 and -35 consensus sequences for the major sigma factor (σ^{70}) in *E. coli* are TATAAT and TTGACA respectively. These two sequences are spaced between 16 and 18 bp apart. Initiation of transcription often occurs at an A that may be the central base in the triplet CAT which is usually found 5-9 bp downstream from the -10 sequence (Harley and Reynolds, 1987). Examination of the DNA sequence 5-9 bp upstream from the RNA start site did not reveal any homology to the -10 consensus for σ^{70}. The sequence 7 bp upstream from the start site was homologous to another promoter consensus sequence in *E. coli*. The flagellar regulon has a -35 and -10 consensus sequence TAAA N_{15} GCCGATAA (Helmann and Chamberlin, 1987) and the *katF* sequence 7 bp from the RNA initiation was TAAA N_{15} GCCGCAGC (Figure 19). This strongly suggests the *katF* gene may be controlled by σ^{F}, the sigma factor that controls some 39 flagellar, motility, and chemotactic genes in *E. coli*.

The Shine-Dalgarno consensus sequence, AGGAGG, is usually found 3-12 bp upstream of the first amino acid codon and is required for the initiation of translation (Shine and Dalgarno, 1974). Two potential translational start sites exist in the *katF* gene (Figures 17 and 19).

The first coding sequence extends from base 295 to base 1380 and encodes a 362 amino acid protein. There is a short Shine-Dalgarno sequence, AGG, thirteen base pairs upstream of the potential initiation

Figure 18. The control sequences of the *katF* gene.
1). The promoter sequence. The *E. coli* promoter consensus sequence for σF is shown in (a). Capital letters represent strongly conserved bases. The *katF* promoter sequence is shown in (b). Lines in between (a) and (b) indicate identical bases. The two asterisks represent the two potential RNA start sites.
2). Shine-Dalgarno Sequences (SD). The two potential SD sequences of *katF* are shown below the *E. coli* consensus sequence. Conserved bases are joined by dashed lines.
3). The Termination Sequence. A potential rho dependent termination sequence for *katF* is shown with the predicted secondary structure of the RNA below.

(1) Promoter Sequence

```
                        -35      15 bp       -10    5-9 bp
a)   Flagellar Regulon   TAAA..............GCCGATAA
                         ||||              ||||        * *
b)   katF promoter       TAAAcccgctgcgttatttGCCGCAGCgataaatcg
```

(2) Shine-Dalgarno Sequences

```
        AGGAGG
           |||
   282  ACCAGGctttgcttgaATG
          SD1           MET

        AGGAGG
        |||||
   319  AGGAGCcaccttATG
         SD2        MET
```

(3) Termination Sequence

```
   1375  GCACAATAAAAGGTCCGAATGCCCATCGGACCTTTTTATTAA
         ALAEND       PALINDROMIC SEQUENCE

                          C
                       G   C
                       U   C
                       A   A
                        A:U
                        G|C
                        C|G
                        C|G
                        U:A
                        G|C
                        G|C
                        A:U
                        A:U
                        A:U
                        A:U
                    GCACAAU   UAUUAA
```

codon. A second initiation site may exist because the methionine at residue 13 of the protein is preceded by a stronger ribosome binding sequence of AGGAG. This potential shorter protein would be 350 amino acids in length and have a predicted size of 40.1 kDa.

All *E. coli* termination sequences contain a palindromic sequence that results in base pairing of the mRNA. This sequence is usually G-C rich and is followed by a poly T tail in rho independent terminators (Rosenberg and Court, 1979). The *katF* sequence contains an element of two fold symmetry beginning at the end of the termination codon TAA (Figure 19). The 7 bp hyphenated dyad could form a stem-loop structure with a predicted stability of -21.0 kcal/mol which is followed by an eight basepair stretch containing 7 T's. Three of these T's could be included in the stem-loop structure increasing its predicted stability to -24.6 kcal/mol.

4.11.6 Determination of the Direction of Transcription

A T7 polymerase vector system (Tabor and Richardson, 1985) was employed to confirm the direction of transcription of *katF*. This system requires two plasmids. The first plasmid, pT7-5, contains an Amp^R gene and a small multiple cloning site downstream from a T7 promoter (Figure 20). The second plasmid, pGP1-2, contains a Kan^R gene, a different origin of replication from that of pT7-5 and the T7 RNA polymerase gene downstream from the λ promoter P_L controlled by the heat sensitive λ cI857 repressor (Figure 20).

The two plasmids, pMMkatF2 and pMMkatF3, contain the *katF* gene on a 4.1 kb *Cla*I fragment in opposite orientations. The 4.1 kb insert was excised from both of these plasmids by digestion with *Hin*dIII and *Eco*RI. The fragments varied only by the orientation of the restriction sites.

Figure 19. Diagram of the two overexpression vectors pGP1-2 and pT7-5.

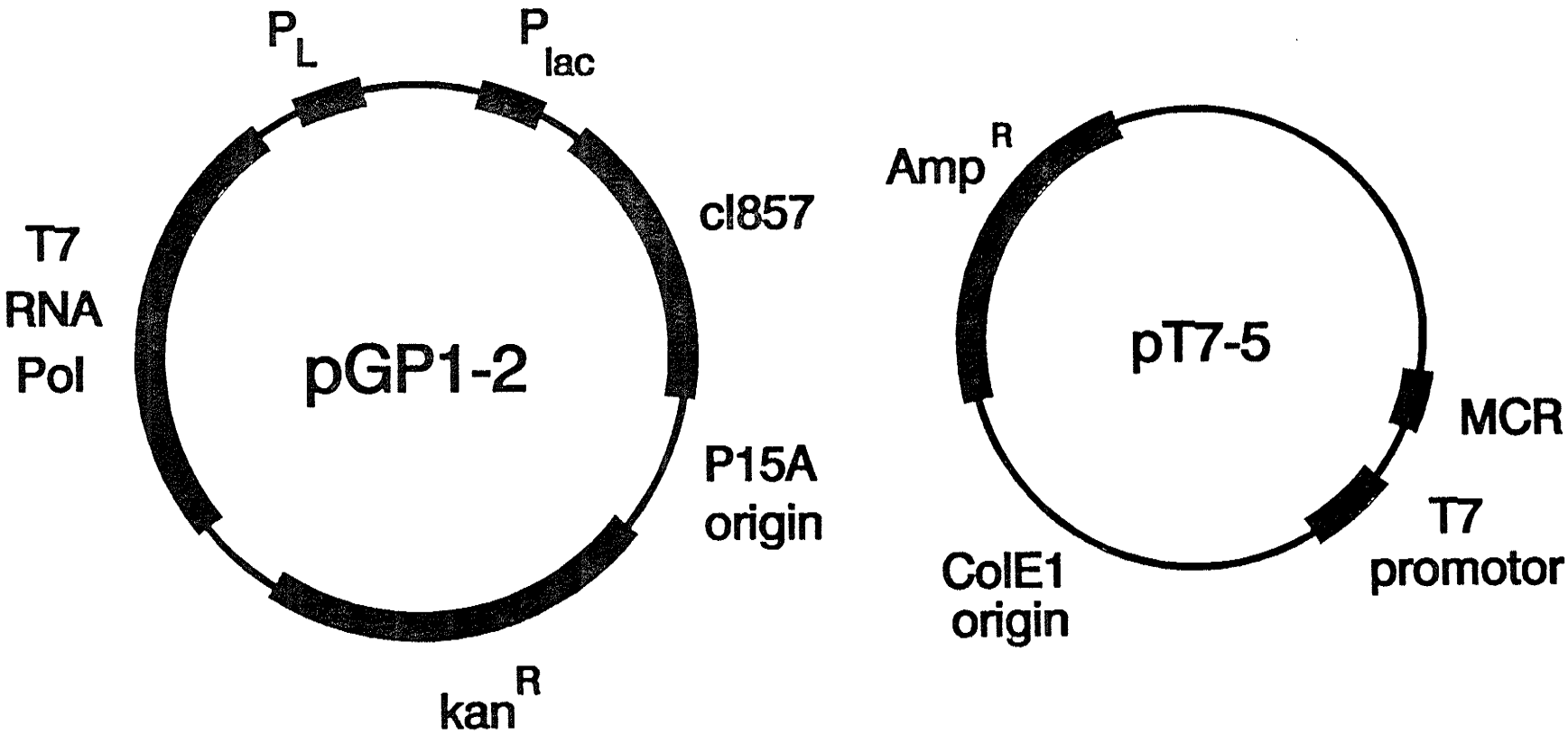
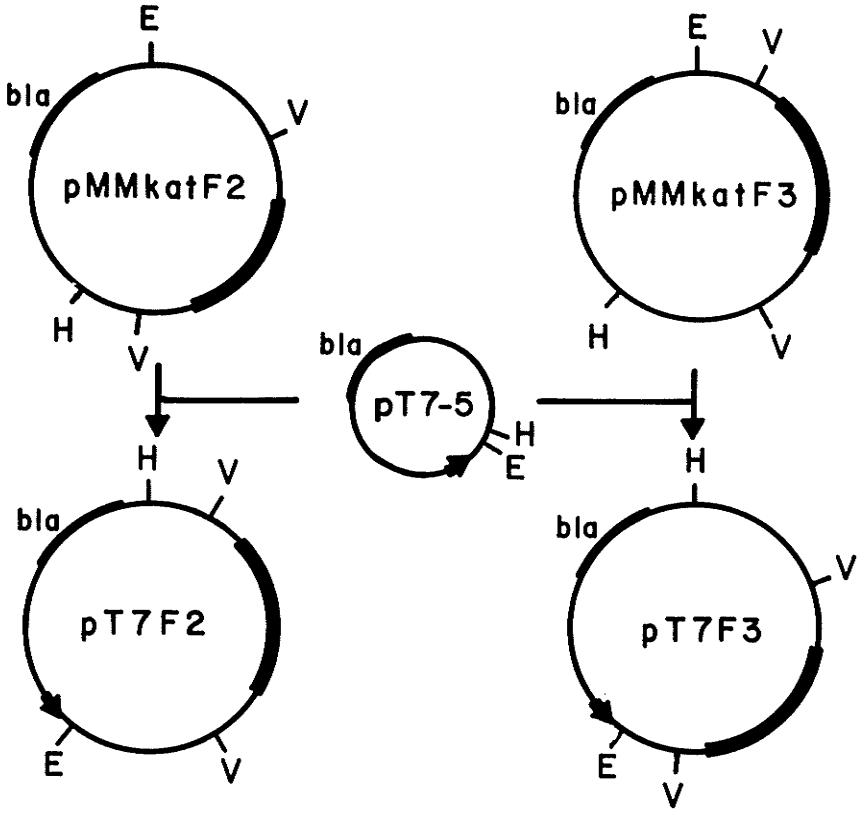
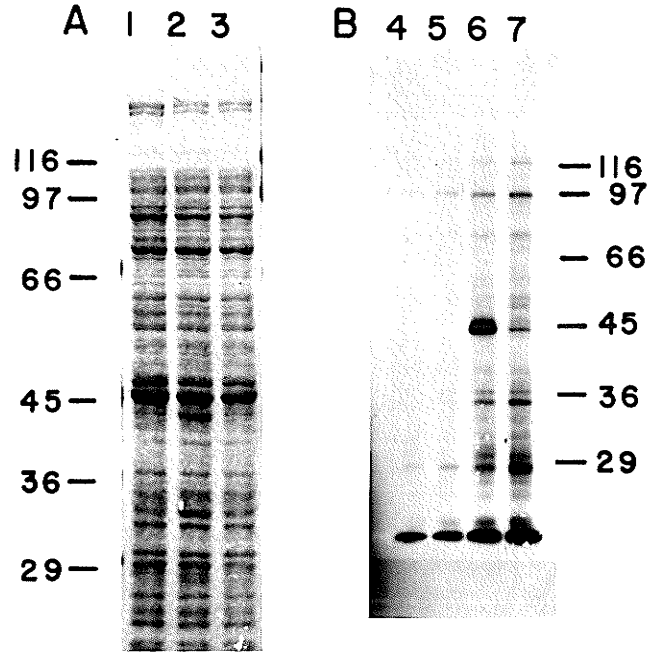

P L
P lac
cI857
T7
RNA
Pol
pGP1-2
P15A
origin
kan R
Amp R
pT7-5
MCR
T7
promotor
ColE1
origin

Figure 20. Construction of clones with *katF* adjacent to a T7 promoter and analysis of the expressed proteins. A 4.1 kb fragment generated by *Hin*dIII and *Eco*RI from either pMMkatF2 or pMMkatF3 was transferred into pT7-5 generating the plasmids pT7F2 and pT7F3. Extracts from induced cells were separated on SDS polyacrylamide gels and either stained with Coomasie brilliant blue (A) or subjected to autoradiography (B). Extracts from cells containing pMMkatF2, pT7F2 (pGP1-2), and pT7F3 (pGP1-2) were run in lanes 1, 2, 3 respectively. Extracts without the plasmid, with pT7-5, pT7F2 (pGP1-2), and pT7F3 (pGP1-2) were run in lanes 4, 5, 6, and 7 respectively. Location and sizes (x 10^{-3}) of various molecular weight markers are indicated along side the gels.


E
bla
V
pMMkatF2
H
V
E
V
bla
pMMkatF3
H
V
bla
pT7-5
H
E
H
V
bla
pT7F2
E
V
H
bla
V
pT7F3
E
V


A 1 2 3
B 4 5 6 7
116
97
66
45
36
29
116
97
66
45
36
29

Both of the inserts were cloned into the *Hin*dIII-*Eco*RI sites of pT7-5 producing the plasmids pT7F2 and pT7F3 (Figure 20). These two plasmids differed only by the orientation of the inserts.

Heat shock treatment of the cells containing pGP1-2 and either pT7F2 or pT7F3 resulted in production of T7 RNA polymerase from pGP1-2 which in turn transcribed DNA downstream from its promoter on the pT7 plasmids. Rifampicin was added to inhibit *E. coli* RNA polymerase and [^{14}C]amino acids or [^{35}S]methionine were added to label any protein produced in the cells. Of the two plasmids containing *katF*, only pT7F2 yielded a significant band with an apparent size of 44 kDa (Figure 20). The expression of KatF from pT7F2 is consistent with the direction of expression implied by the open reading frame in the nucleotide sequence.

Because the T7 RNA polymerase is extremely efficient, this vector system can also be used to overexpress genes. Crude extracts from induced cells containing pGP1-2 and pT7F2 were separated by SDS-PAGE and then stained with Coomassie blue. The over production of the KatF protein was so great that a visible band of protein was apparent at 44 kDa (Figure 20A).

4.11.7 Comparison of the KatF Protein to Other Regulatory Proteins

As stated above, a comparison of the nucleotide sequence with GenBank (1986) did not reveal any significant homology to any sequenced gene. However, when the predicted amino acid sequence was compared to a group of *E. coli* regulatory proteins termed sigma factors, substantial homology existed (Figure 21). An overall comparison between KatF and the *E. coli* major sigma factor (σ^{70}) revealed 30% identical amino acids and 45% conservative replacements. Comparison of KatF to the heat-shock

Figure 21. Comparison of the predicted amino acid sequence of KatF with portions of the sequences of σ^{70} (*rpoD*) and σ^{32} (*htpR*). Identical amino acids are indicated by = and conservative matches are indicated by *. Region 2, 3, and 4 are regions that have been conserved in a large number of sigma factors (Helmann and Chamberlin, 1988). The conservative amino acid changes are defined as any within the following groups: (I,L,V,M), (H,K,R), (D,E,N,Q), (A,G), (F,Y,W), (S,T), (P), and (C) (Helmann and Chamberlin, 1988).

```
katF     1  MFRQGITGRSHLMSQNTLKVHDLNEDAEFDENGVEVFDEKPLVEQEPSDNDLAE
                     =  * *    * =*  *   *    *          *=   *  =*
rpoD    29  DHLPEDIVDSDQIEDIIQMINDMGIQVMEEAPDADDLMLAENTADEDAAEAAAQ

htpR     1  MADKMQSLALAPVGGLDSYIRAANAWPMLSADEERALAEKLHYHGDLEAAKTLI
                =      * * * =*      =*=*=*==   = *    ==*   * *=
katF    55  EELLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVYFARRDLR-GDVASRRRMI
                *    =  =   *=* =*=   === = =*  =*=    =    ** =*
rpoD    83  VLSSVESEIGRTTDPVRMYMREMGTVELLTREGEIDIAKRIED-GINQAKKEMV

               [-----------------------------REGION 2------------
htpR    55  LSHLRFVVHIARNYAGYGLPQADLIQEGNIGLMKAVRRFNPEVGVRLVSFAVHW
             = == ==*=== =*  ==   ===*===*==**== *=*== = =  **=  =
katF   108  ESNLRLVVKIARRYGNRGLALLDLIEEGNLGLIRAVEKFDPERGFRFSTYATWW
            = =====* ==**= ====  ====*===*==**==*==*  ==**========
rpoD   381  EANLRLVISIAKKYTNRGLQFLDLIQEGNIGLMKAVDKFEYRRGYKFSTYATWW

            ------------------------------]           [------------
htpR    109  IKAEIHEYVLRNWRIVKVATTKAQRKLFFNLRKTKQRLGWFNQDEV-EMVAREL
             =*  =   ** * = ***          *  =  **     * *     *= *=
katF    162  IRQTIERAIMNQTRTIRLPIHIVKELNVYL-RTARELSHKLDHEPSAVEIAEQL
             === = = = *= ====*=*=**  *=    =  =**   * *==*  =*== *
rpoD    435  IRQAITRSIADQARTIRIPVHMIETINKLN-RISRQMLQEMGREPTPEELAERM

            ------REGION 3-------------------------]
htpR    162  GVTSKDVREMESRMAAQDMTFDLSSDDDSDSQPMAPVLYLQDKSSNFADGIEDD
                 ==  =  =*     * =     ==*          =*    *  *= ==
katF    215  DKPVDDVSRM-LRLNERITSVDTPLGGDSEK------ALLDILADEKENGPEDT
               = = * ** =**     =**==*= = *         == * =   * = =*
rpoD    488  LMPEDKIRKV-LKIAKEPISMETPIGDDEDS------HLGDFIEDTTLELPLDS

                         [------------------REGION 4----------
htpR    216  NWEEQAANRLTDAMQGLDERSQDIIRARW-LDEDNKSTLQELADRYGVSAERVR
               ***  * *   *  =* * ***  =* =   *  == ***   =** ====
katF    262  TQDDDMKQSIVKWLFELNAKQREVLARRFGLLGYEAATLEDVGREIGLTRERVR
                * **      =  = =*   ==  ===*      ===*==**  *====*=
rpoD    535  ATTESLRAATHDVLAGLTAREAKVLRMRFGIDMNTDYTLEEVGKQFDVTRERIR

            ------------------------]
htpR    269  QLEKNAMKKLRAAIEA (284)
             =** *****==  **
katF    316  QIQVEGLRRLREILQTQGLNIEALLPRVSKHLSERPVSSEAGFFCAQ (362)
             ==*  *==*==    * *=
rpoD    589  QIEAKALRKLRHPSRSEVLRSFLDD (613)
```

sigma factor σ^{32} revealed less homology.

The amino acid sequences of twelve sigma factors has been compared (Helmann and Chamberlin, 1988). The sigma factors appear to share four major regions of homology, some of which have been subdivided into smaller regions. If one compares the KatF protein to these sigma factors (Figure 22 and Table 20) it is quite evident that KatF falls between the groupings of major and minor sigma factors. The major sigma factors σ^{70} (*E. coli*), σ^{43} (*B. subtilis*), and σ^{80} (*M. xanthus*) are very homologous to each other. The homology does not drop below 80% in regions 2, 3, and 4 when compared to σ^{70} (Table 20). If one compares the other sigma factors to σ^{70} the homology is usually below 60% in regions 2, 3, and 4 (with a few exceptions in the subregions for some sigma factors). The KatF protein has consistently higher homology to σ^{70} than do the other minor sigma factors. However, the KatF protein lacks any strong homology to region 1 which is found in all the major sigma factors. The KatF protein has the longest carboxyl terminus of all the sigma factors and a longer N-terminal portion than the minor sigma factors. However because the protein lacks region 1, it is smaller than the major sigma factors.

4.12 Isolation of the KatF Protein

The similarity of KatF to known sigma factors suggested that it was also a sigma factor. In order to confirm this hypothesis it became necessary to purify the protein but sigma factors are usually found in very low levels in the cell. In order to circumvent this problem, I proposed to employ the T7 system originally used to confirm the direction of transcription. A large portion of the RNA

Figure 22. Alignment of bacterial sigma factors in region 2, 3, and 4. The number next to the factor name indicates the starting amino acid number. Bold face letters identify those amino acids which

REGION 2

		[----2.1----] [-----2.2-----]
KatF	111	LRLVVKIARRYGN.....RGLAL....LDLIEEGNLGLIRAV
σ^{70}	384	LRLVISIAKKYTN.....RGLQF....LDLIQEGNIGLMKAV
σ^{43}	143	LRLVVSIAKRYVG.....RGMLF....LDLIHEGNMGLMKAV
σ^{80}	421	LRLVVSIAKKYTN.....RGLQF....LDLIQEGNIGLMKAV
σ^{32}	58	LRFVVHIARNYAG.....YGLPQ....ADLIQEGNIGLMKAV
σ^{29}	67	LRLVVYIARKFEN.....TGINI....EDLISIGTIGLIKAV
IIAC	42	MRLVWSVVQRFLN.....RGYEP....DDLFQIGCIGLLKSV
σ^{30}	42	RNFVRAKARSYFL.....IGADR....EDIVQEGMIGLYKSI
σ^{37}	39	TNLVDMLAKKYS.....KGKSF...HEDLRQVGMIGLLGAI
σ^{28}	21	PKAGDDLMRRYMPLVTYHYGRISVGLPKSVHKDDLMSLGMLGLYMMP

REGION 2

		[-------2.3------][--------2.4------]
KatF	144	EKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKELNVYL
σ^{70}	417	DKFEYRRGYKFSTYATWWIRQAITRSIADQARTIRIPVHMIETINKLN
σ^{43}	176	EKFDYRKGYKFSTYATWWIRQAITRAIADQARTIRIPVHMVETINKLI
σ^{80}	512	DKFEYKRGYKFSTYATWWIRQAITRAIADQARTIRIPVHMIETINKLI
σ^{32}	91	RRFNPEVGVRLVSFAVHWIKAEIHEYVLRNWRIVKVATTKAQRKLFFN
σ^{29}	100	NTFNPEKKIKLATYASRCIENEILMYLRRNNK.IRSEVSFDEPLNIDW
IIAC	75	DKFDLTYDVRFSTYAVPMIIGEIQRFIRDDG.TVKVSRSLKELGNKIR
σ^{30}	75	RDFKEDKLTSFKAFAELCITRDIITAIKTATRQKHIPLNSYASLDKPI
σ^{37}	72	KRYDPVVGKSFEAFAIPTIIGEIKRFLRDKTWSVHVPRRIKELGPRIK
σ^{28}	68	LKNLTQPDLKFDTYASFRIRGAIIDGLRKEDWLPRTSREKTKKVEAAI

REGION 3

KatF		DHEPSAVEIAEQLDKPVDDVSR..MLRLNERITSVDTPLGGDSEKALL
σ^{70}	475	GREPTPEELAERMLMPEDKIRK..VLKIAKEPISMETPIGDDEDSHLG
σ^{43}	234	GREPTPEEIAEDMDLTPEKVRE..LLKIAQEPVSLETPIGEEDDSHLG
σ^{80}	570	GREPTPEEIAEKMELPLDKVRK..VLKIAKEPISLETPIGEEEDSHLG
σ^{32}	149	FNQDEVEMVARELGVTSKDVRE.MESRMAAQDMTFDLSSDDDSDSQPM
IIAC	132	GRVPTVQEIADHLEIEAEDVV..LAQEAVRAPSSIHETVYENDGDPIT
σ^{37}	130	QRSPKVEEIAEFLDVSEEEVLETMEMGKSYQALSVDHSIEADSDGSTV
σ^{28}	123	LRNVSPAEIAEELGMTVQDVVSTMNEGFFANLLSIDEKLHDQDDGENI

REGION 4

KatF	LNAKQREVLARRFGLLGYEAATLEDVGREIGLTRERVRQIQVEGLRRLREILQTQGLNIEALLPRVS
σ^{70}	LTAREAKVLRMRFGIDMNTDYTLEEVGKQFDVTRERIRQIEAKALRKLRHPSRSEVLRSFLDD*
σ^{43}	LTDREENVLRLRFGLDDGRTRTLEEVGKVFDGTRERIRQIEAKALRKLRHPSRSKRLKDFLE*
σ^{80}	LTPREEKVLRMRFGIGEKSDHTLEEVGQDFEVTRERIRQIEAKALRKLRHPSRSKRLRSFVES*
σ^{32}	LDERSQDIIRARWLDEDNKS.TLQELADRYGVSAERVRQLEKNAMKKLRAAIEA*
IIAC	LEEREKLIVYLRYYKDQ....TQSEVAERLGISQVQVSRLEKKILKQIKVQMDHTDG*
σ^{37}	LSDREKQIIDLTYIQNK....SQKETGDILGISQMHVSRLQRKAVKKLREALIEDPSMELM*
σ^{28}	LSEKEQLVVSLFYKEEL....TLTEIGQVLNLSTSRISQIHSKALFKLKNLLEKVIQ*

Table 20. Comparison of various sigma factors to σ^{70} [a]

	Region 2								
	2.1		2.2		2.3		2.4		Overall (65 aa[b])
	13aa	%	15aa	%	18aa	%	19aa	%	%
σ^{80}	13	100	15	100	18	100	19	100	100
σ^{43}	11	85	14	93	18	100	18	95	94
KatF	11	85	15	100	16	89	14	74	86
σ^{32}	8	62	14	93	9	50	9	47	62
σ^{29}	11	85	11	73	9	50	8	42	60
σ^{SPIIAC}	8	62	10	67	10	56	11	58	60
σ^{37}	8	62	10	67	7	39	10	53	54
σ^{28}	4	31	7	47	8	44	7	37	40

	Region 3		Region 4				
			4.1		4.2		Overall (49 aa)
	46aa	%	17aa	%	28aa	%	%
σ^{80}	44	96	13	77	27	96	86
σ^{43}	40	87	13	77	26	93	80
KatF	27	59	10	59	23	82	67
σ^{32}	19	41	8	47	21	75	61
σ^{SPIIAC}	23	50	9	53	14	50	47
σ^{37}	21	46	9	53	16	57	51
σ^{28}	22	48	10	59	18	64	57

[a] percentages were calculated including conservative amino acid replacements as in Figure 22.

[b] amino acids

polymerase from cells which overexpress the KatF protein via the pT7 vector system should be charged with the KatF sigma factor and it should be possible to purify KatF from the polymerase. This procedure has been used successfully in the isolation of many sigma factors including σ^{70} (Burgess and Jendrisak,1975), σ^{32} (Grossman *et al.*, 1984), σ^{F} (Arnosti and Chamberlin, 1989), and σ^{E} (Erickson and Gross, 1989).

The RNA polymerase was isolated from approximately 30 g of induced cells. After precipitation with PEG and ammonium sulfate, the extracts were fractionated on a BioGel 1.5 M column (2 cm x 100 cm). Fractions of the column eluate were assayed for the ability to incorporate [^{3}H]ATP into acid precipitable product using the plasmid pAMKatE72 as a template (Figure 23). At least two peaks of polymerase activity were consistently observed between fractions 34 and 49. Initially, each peak was pooled separately for further purification, but no difference in protein composition was observed in either peak. Fractions which contained peak polymerase activity (fractions 34 to 49) were pooled and concentrated by ultrafiltration. The sample was diluted until the conductivity equalled that of 0.1 M KCl and was again concentrated to a volume of approximately 10 ml. The protein was loaded onto a heparin-Sepharose column and eluted with a 0.1-1.0 M KCl gradient. Fractions were assayed for polymerase activity (Figure 24) and every third fraction was separated on SDS-polyacrylamide gels to identify the proteins present. Examination of the gel revealed a peak of RNA polymerase between fractions 26-30, which corresponded with peak transcriptional levels. The polymerase concentration was estimated from the intensities of the characteristic ß and ß′ subunits of the RNA polymerase found at 165 and 155 kDa respectively. A number

Figure 23. Elution profile from the Biogel 1.5 M column. Squares represent protein from various fractions (absorbance at 280 nm) and the triangles represent RNA polymerase activity in counts per minute (cpm).

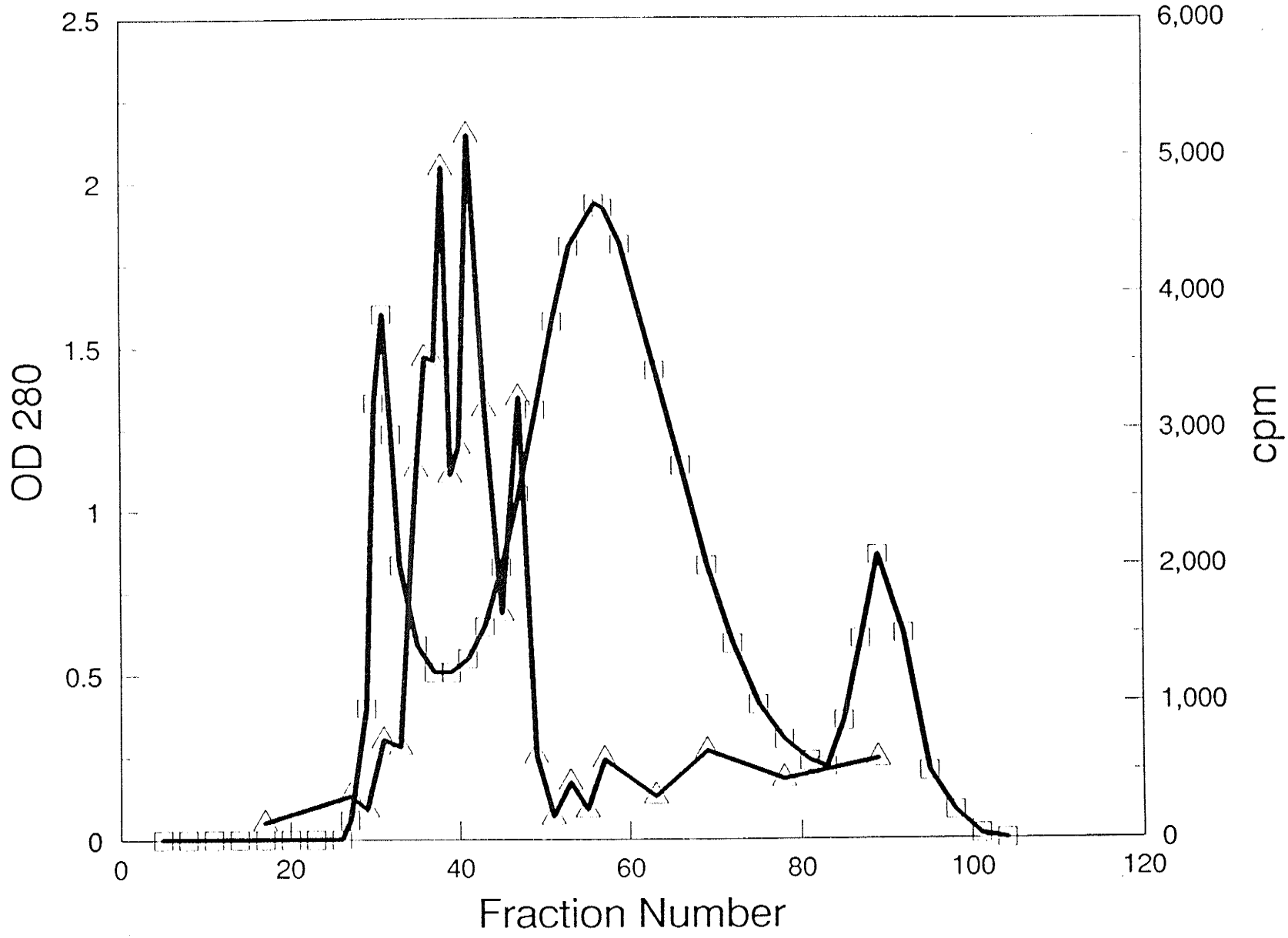
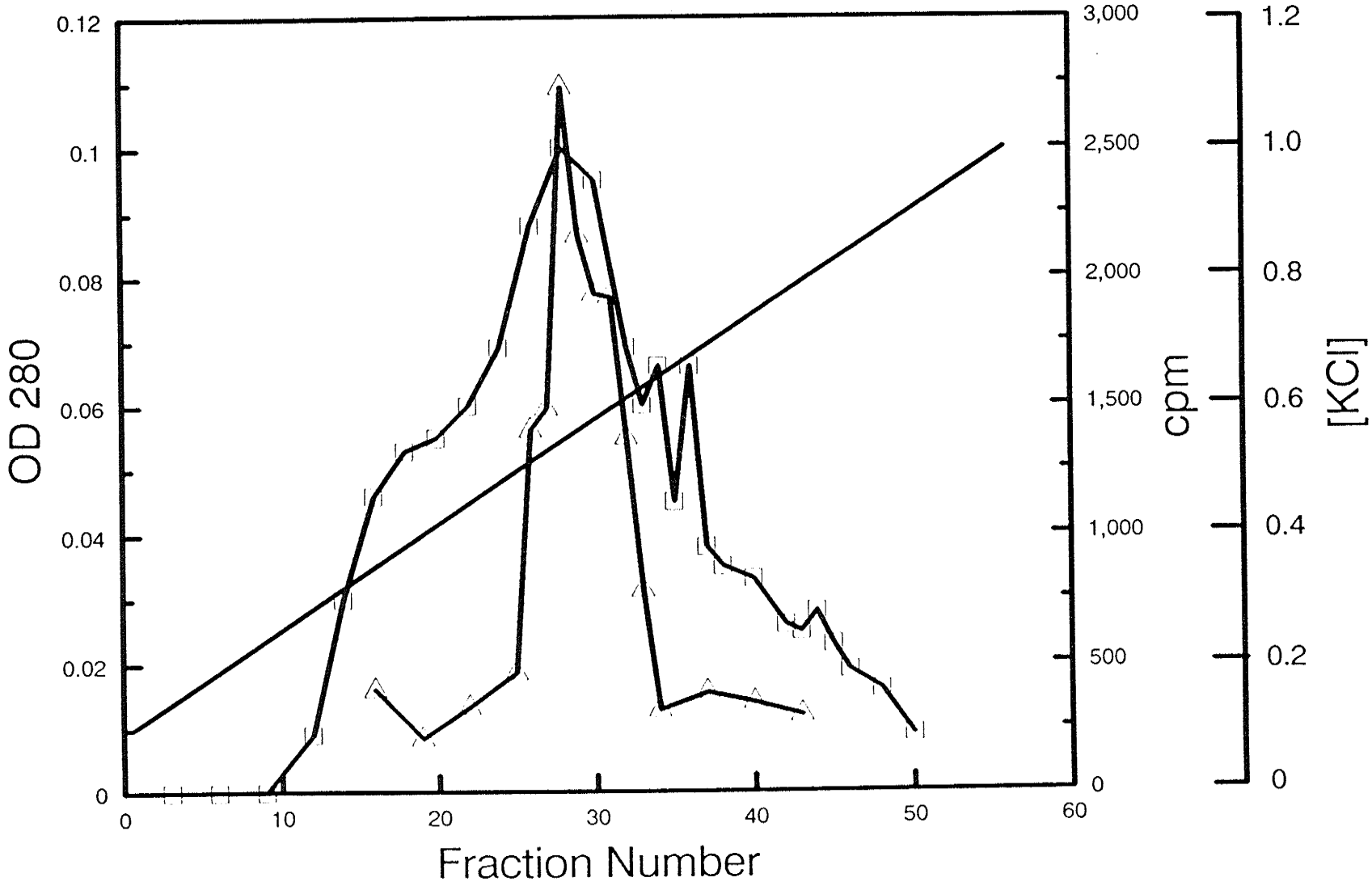

2.5
2
1.5
1
0.5
0
OD 280
6,000
5,000
4,000
3,000
2,000
1,000
0
cpm
0
20
40
60
80
100
120
Fraction Number

Figure 24. Elution profile from the heparin-Sepharose column. Squares represent protein content from various fractions ($O.D._{280}$) and triangles represent RNA polymerase activity (cpm). The diagonal line in the graph represents the KCl gradient run through the column.


0.12
0.1
0.08
0.06
0.04
0.02
0
OD 280
3,000
2,500
2,000
1,500
1,000
500
0
cpm
1.2
1.0
0.8
0.6
0.4
0.2
0
[KCl]
0
10
20
30
40
50
60
Fraction Number

of contaminating bands eluted with the RNA polymerase from the heparin-Sepharose column (Figure 25, lane 6). To remove these bands the salt concentration was adjusted to 0.1 M KCl using membrane filtration as above and the protein was loaded onto a DNA cellulose column from which the RNA polymerase was eluted with a linear KCl gradient from 0.1- 1.5 M. The RNA polymerase eluted as a broad peak between 0.8 and 1.2 M. Analysis of the eluted polymerase on SDS-PAGE revealed four protein bands corresponding to the ß, ß′, σ^{70}, and α subunits, as well as a fifth band below the α subunit at about 44,000 daltons. Commercial RNA polymerase (Pharmacia) was run along side the preparation as a control (Figure 25 lane 8). The α band from these gels ran at approximately 45,000 daltons which was larger than the expected size of 39,000.

4.13 Regulation of the *katE* and *katF* Genes

4.13.1 Construction of the *katE* and *katF* Fusion Plasmids

The *lac* fusion vector pRS415 was employed to study the regulation of both the *katE* and *katF* genes (Simons *et al.*, 1987). The vector contains a multiple cloning site (*Eco*RI-*Sma*I-*Bam*HI) upstream of a promoterless *lac* operon (Figure 26). Also contained on the plasmid is a gene coding for ampicillin resistance to allow for selection of the plasmid. When a DNA fragment containing a promoter sequence is inserted in the correct orientation in the multiple cloning site, the *lac* genes are expressed allowing for selection of desired recombinants and assay of ß-galactosidase levels (Miller, 1972) in the *lac*$^-$ strain NM522.

The fusion plasmid containing the *katE* promoter, pRSkatE16, was constructed by inserting the 1.4 kb *Sma*I-*Eco*RI fragment from pAMkatE72

Figure 25. SDS-polyacrylamide gel showing the various purification steps in the isolation of RNA polymerase. Lane 1) crude extract; 2) Biogel 1.5 M load; 3) heparin-Sepharose load; 4) heparin-Sepharose flow-through; 5) DNA-cellulose load; 6) peak RNA polymerase activity eluted from the DNA-cellulose column; 7) molecular weight markers; 8) Commercial RNA polymerase (Pharmacia). Sizes of the molecular weight markers are as follows; Myosin 205,000 Da; ß-galactosidase 116,000 Da; phosphorylase b 97,400; bovine albumin, 66,000 Da; egg albumin, 45,000 Da, and carbonic anhydrase, 29,000 Da.

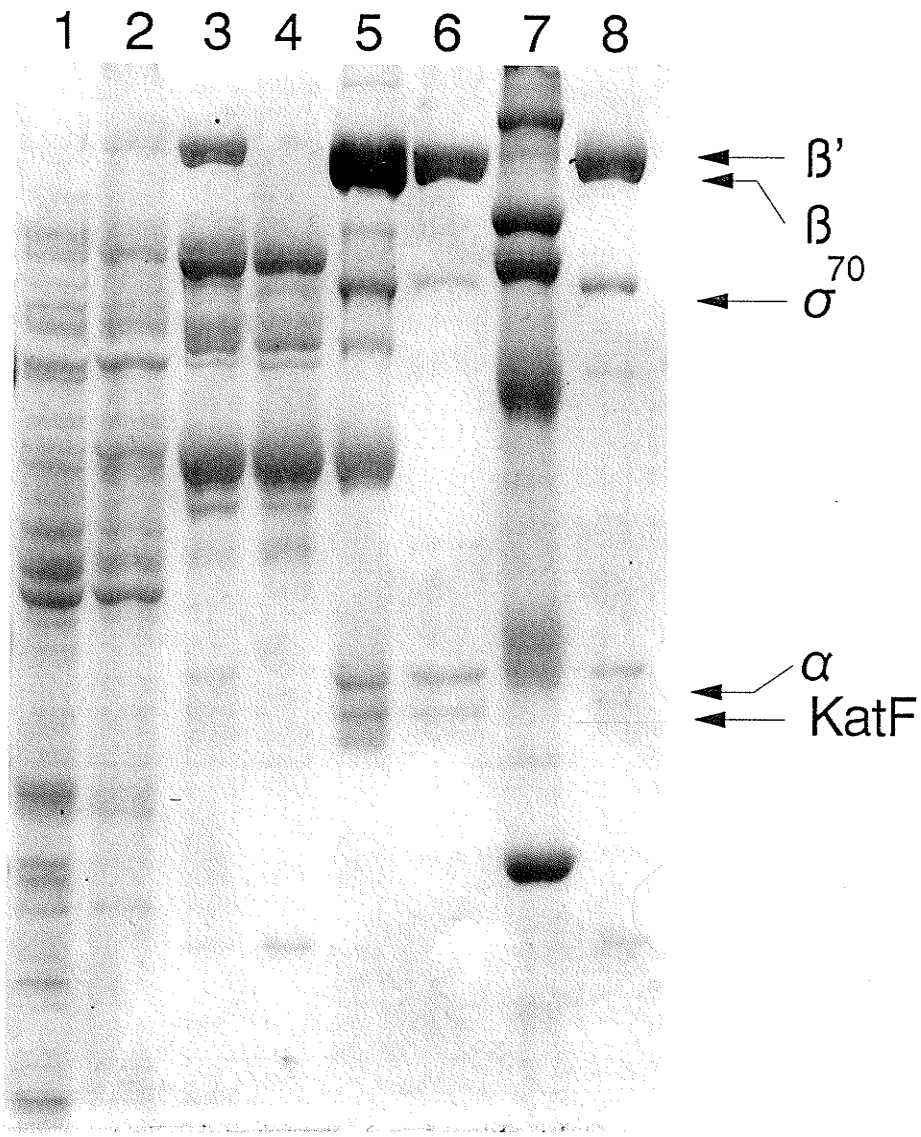
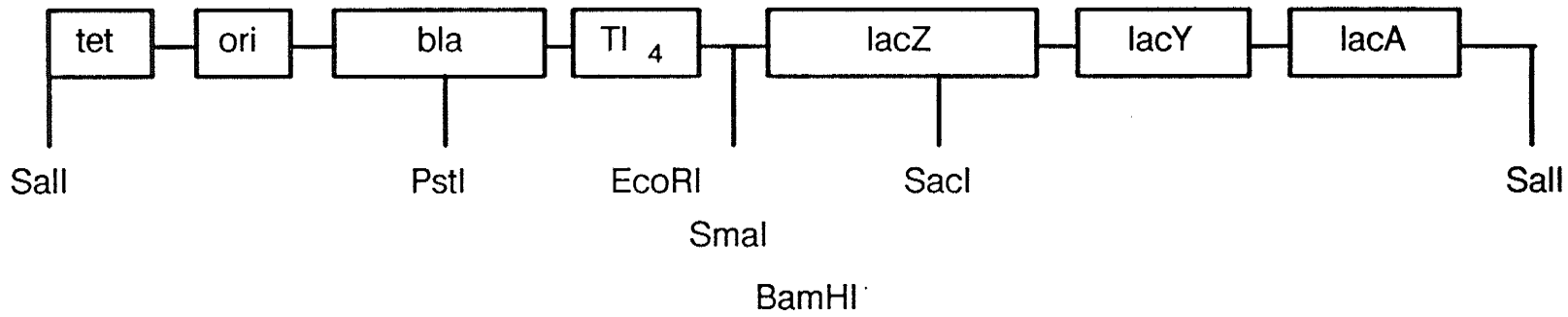

1 2 3 4 5 6 7 8
β'
β
σ70
α
KatF

Figure 26. The *lac*-fusion vector pRS415.


tet
ori
bla
Tl 4
lacZ
lacY
lacA
SalI
PstI
EcoRI
SmaI
BamHI
SacI
SalI

into the *Sma*I site of pRS415 (Figure 27a). The fusion plasmid containing the *katF* promoter, pRSkatF5, was constructed by inserting the 600 bp *Eco*RI-*Dra*I fragment from pM13F2 into pRS415 cut with *Eco*RI and *Sma*I (Figure 27b). The nucleotide sequences of both genes confirmed that a portion of the coding regions as well as the upstream sequence sufficient for gene expression were contained on the cloned fragments (I. von Ossowski, personal communication for *katE*; Mulvey and Loewen, 1989 and Figure 17 for *katF*).

The fusion plasmids were transformed into NM522 which is a *lac*$^-$ mutant producing undetectable amounts of ß-galactosidase activity (Table 21). When the *katE* and *katF* fusion vectors were transformed into NM522 the levels of ß-galactosidase increased to 14,568 and 3,993 units for pRSkatE16 and pRSkatF5 respectively.

Because the cloned fragment in pRSkatE16 was quite large, 1.4 kb, it was possible that there were other promoters present on the fragment that might influence expression of the *lacZ* gene. Therefore, strain UM315 was constructed, which was isogenic to NM522 except for a transposon *Tn*10 insertion in *katF*. The fusion plasmids were transformed into this strain and the ß-galactosidase levels were measured as the cells entered stationary phase. Expression of the *katE* fusion vector was quite low (194 units) as compared to the expression in the KatF$^+$ NM522 (14568 units)(Table 21), confirming that expression from the cloned *katE* promoter remained under positive control of *katF*. Expression from the *katF* promoter in UM315 was similar to levels found in NM522.

Figure 27. Construction of the fusion plasmids containing *lacZ* under the control of the *katE* (a) and *katF* (b) promoters. In (a), a 1.4 kb fragment (solid broad line) was cut from pAMkatE72 with *Sma*I (S) and *Eco*RV (V) and ligated into the *Sma*I site of pRS415 to generate pRSkatE16. The direction of transcription from the *katE* promoter is indicated by the arrow. The cross hatched region indicates the coding region of *katE*. In (b), a 600 bp fragment (solid broad line) was cut from pMF2 with *Eco*RI (R) and *Dra*I (D) and ligated into pRS415 cut with *Eco*RI and *Sma*I to generate pRSkatF5. The direction of transcription from the *katF* promoter is indicated by the arrow. The cross hatched region indicates the coding region of *katF*.

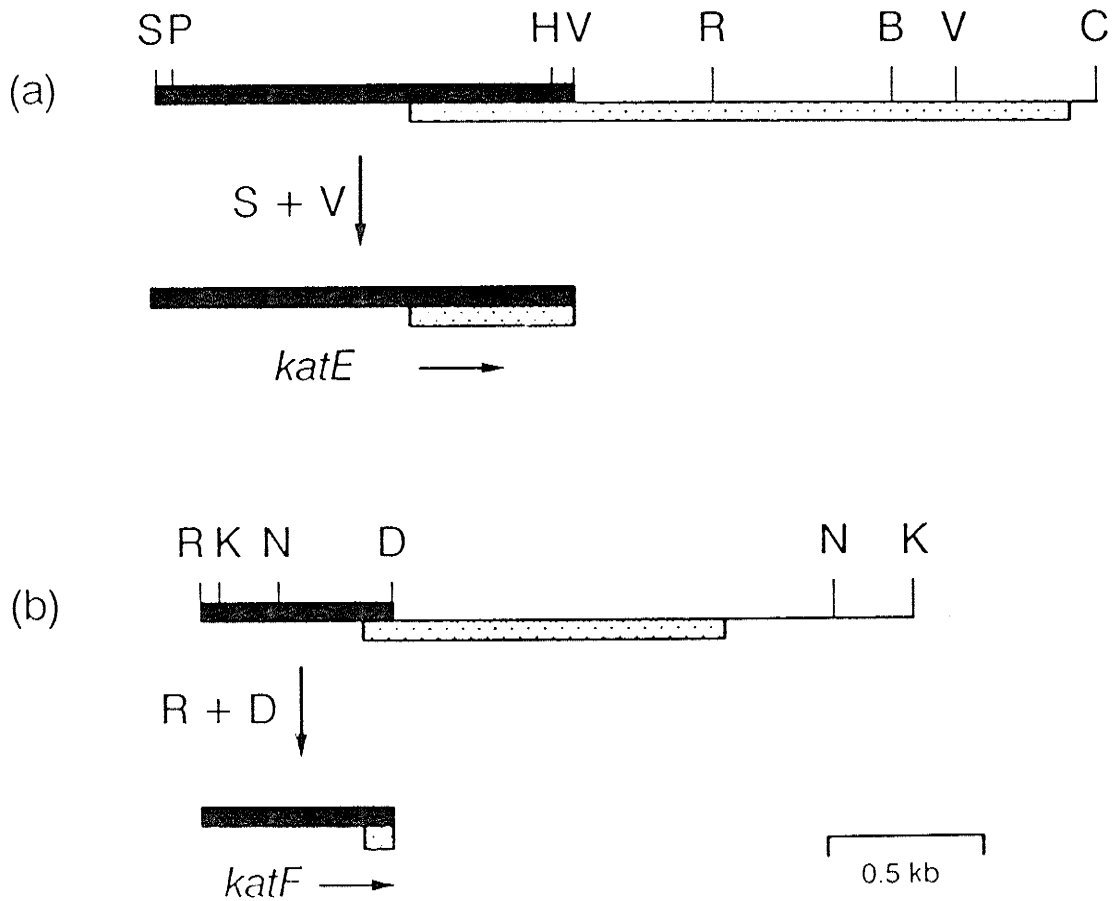

SP
HV
R
B
V
C
(a)
S + V
katE
R K N
D
N
K
(b)
R + D
katF
0.5 kb

Table 21 Expression of fusion plasmids

Strain and plasmid	β-galactosidase (Miller units)
NM522	N.D.[a]
NM522 [pRSkatE16]	14568
NM522 [pRSkatF5]	3993
UM315	N.D.
UM315 [pRSkatE16]	194
UM315 [pRSkatF5]	4196

[a] N.D. Not detectable

4.13.2 Effect of Carbon Source on *katE* and *katF* Expression

The carbon source has been shown to affect the level of HPII expression in *E. coli* (Loewen *et al.*, 1985; Meir and Yagil, 1990). Therefore, the effect of growth medium on the expression of the *katE* and *katF* genes were examined using the fusion vectors.

4.13.2.1 Growth in LB Medium

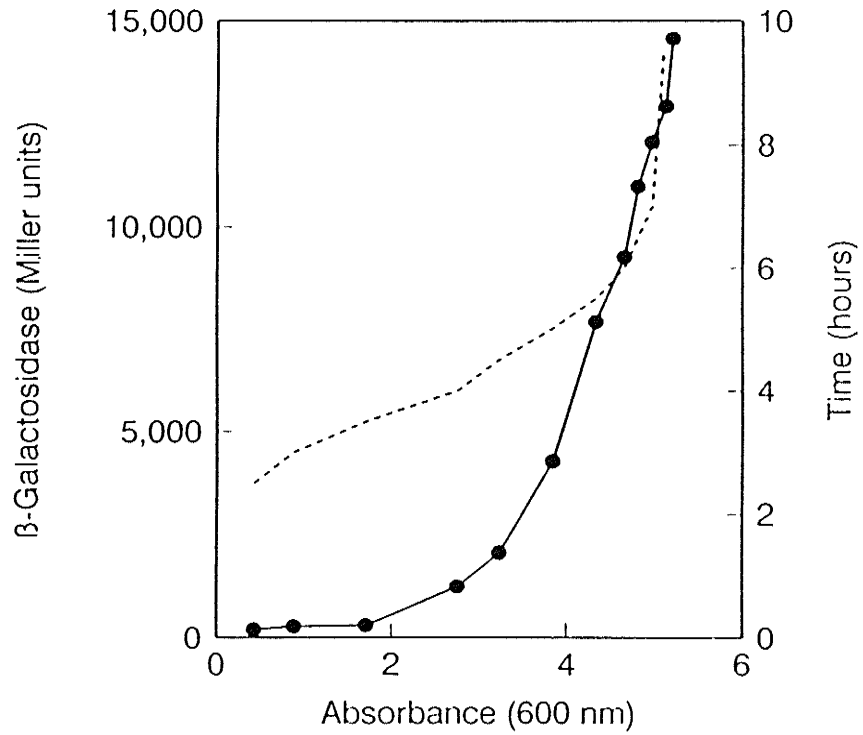
The synthesis of ß-galactosidase controlled by *katE* and *katF* in cells growing in LB medium buffered at pH 7.0 are shown in Figure 28. Both cultures grew rapidly to an optical density of 4.5 to 5 at 600 nm in approximately seven hours. Expression from the *katE* promoter remained low (200-400 Miller units) during exponential growth but as the cells approached stationary phase (OD_{600} of 3) the ß-galactosidase levels increased significantly to approximately 15,000 units. The changes in ß-galactosidase activity controlled by the *katE* promoter essentially paralleled the changes in HPII levels observed during similar growth experiments (Loewen *et al.* 1985, Sak *et al.* 1989). Expression from the *katF* promoter differed in that it commenced in early exponential phase and increased across the growth curve into stationary phase to a maximum level of 2,900 units.

4.13.2.2 Growth in Minimal Medium

Growth in glucose minimal broth resulted in different growth characteristics as well as changes in the expression of both the *katE* and *katF* promoter clones. The growth rates and ß-galactosidase levels for the *katE* and *katF* promoter clones are shown in Figure 29 and 30 respectively. The cultures in M9-glucose medium entered stationary

Figure 28. ß-Galactosidase synthesis from *lacZ* under the control of the *katE* promoter (pRSkatE16) (a) and the *katF* promoter (pRSkatF5) (b) expressed as a function of cell density (A_{600}). Growth for both cultures is indicated by the dashed line which represent cell density (A_{600}) as a function of time.

A


15,000
10,000
5,000
0
0
2
4
6
10
8
6
4
2
0
ß-Galactosidase (Miller units)
Time (hours)
Absorbance (600 nm)

B

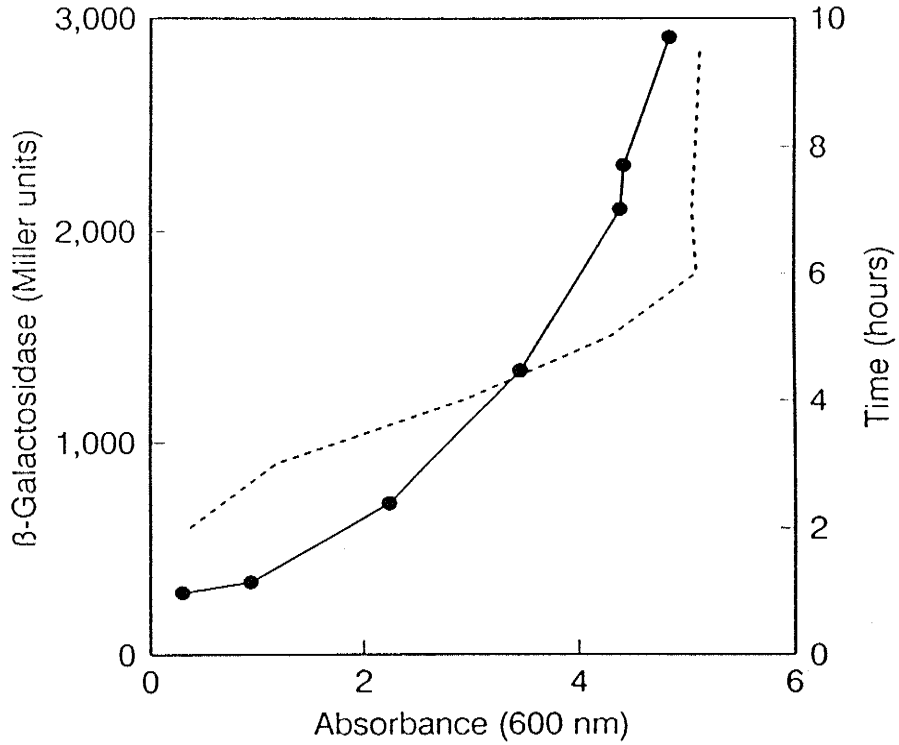

3,000
2,000
1,000
0
0
2
4
6
10
8
6
4
2
0
ß-Galactosidase (Miller units)
Time (hours)
Absorbance (600 nm)

Figure 29. ß-Galactosidase synthesis from *lacZ* under the control of the *katE* promoter. ß-Galactosidase was assayed in cultures growing in M9 minimal medium supplemented with 20 mM glucose (•), 20 mM succinate (■) or 20 mM glucose and 0.2% (w/v) of a mixture of 20 amino acids (▲). Growth for the glucose and succinate cultures is indicated by the dashed and dotted lines respectively which represent cell density (A_{600}) as a function of time.

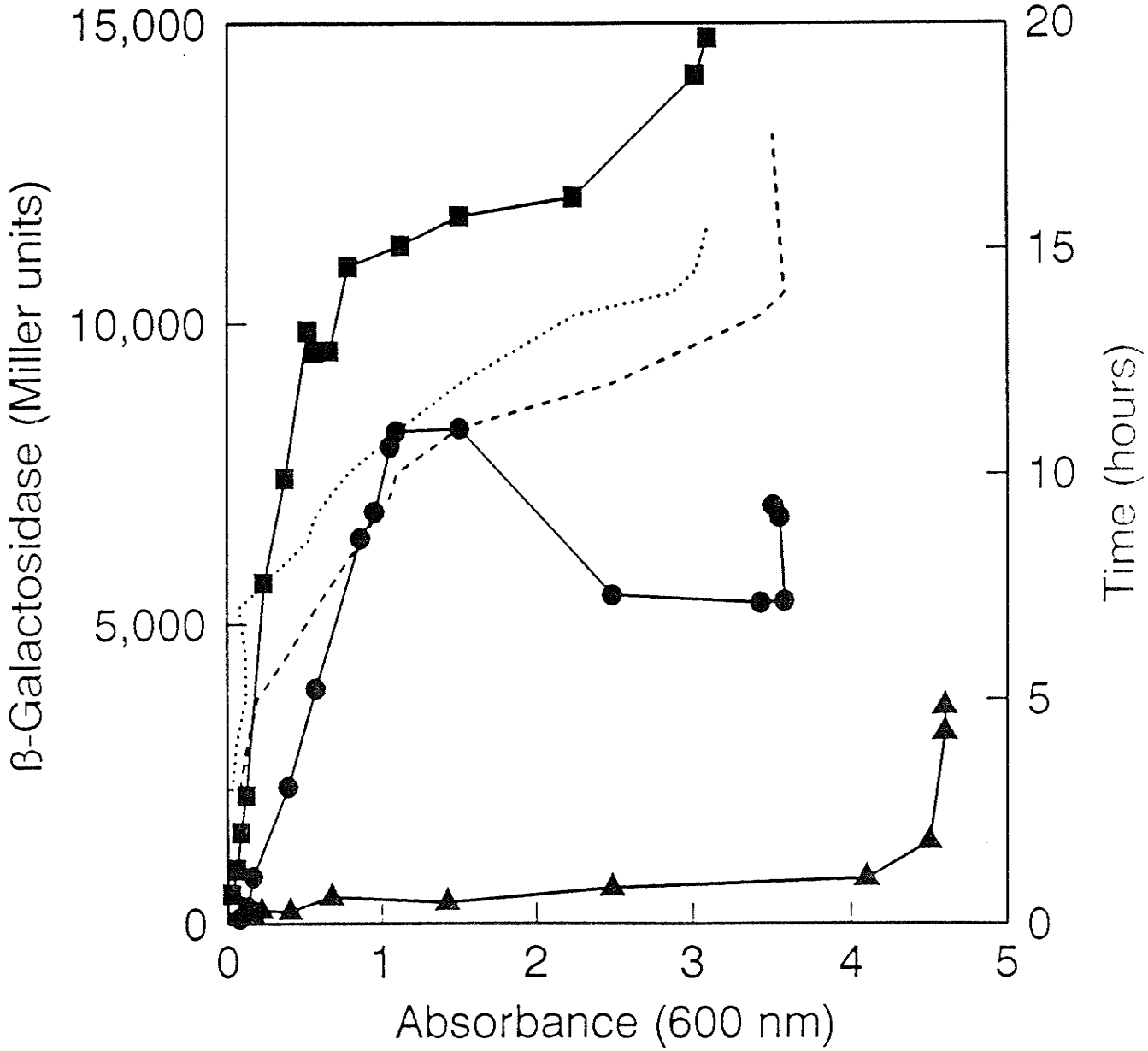
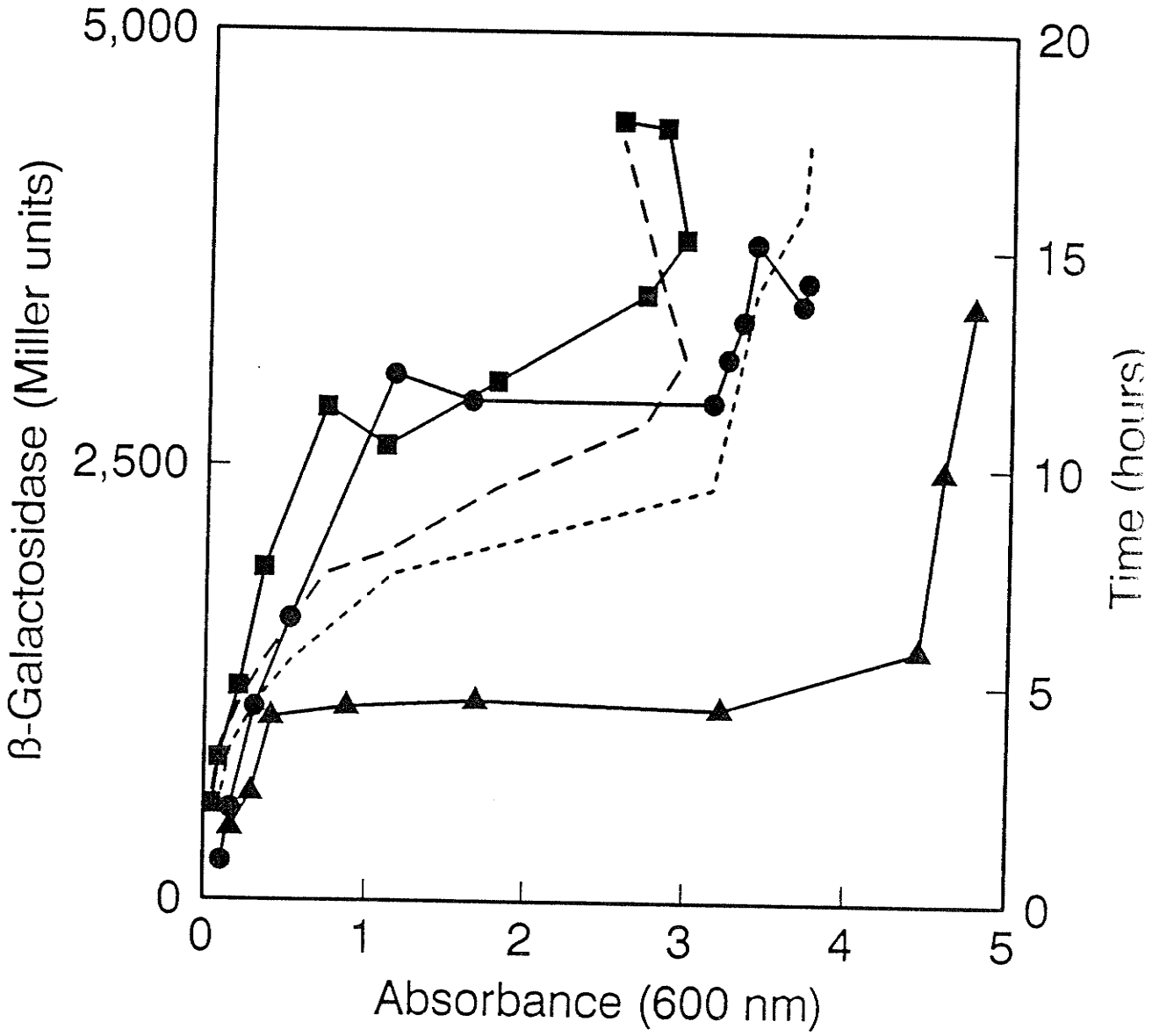

15,000
10,000
5,000
0
ß-Galactosidase (Miller units)
20
15
10
5
0
Time (hours)
0
1
2
3
4
5
Absorbance (600 nm)

Figure 30. ß-Galactosidase synthesis from *lacZ* under the control of the *katF* promoter. ß-Galactosidase was assayed in cultures growing in M9 minimal medium supplemented with 20 mM glucose (•), 20 mM succinate (■) or 20 mM glucose and 0.2% (w/v) of a mixture of 20 amino acids (▲). Growth for the glucose and succinate cultures is indicated by the dashed and dotted lines respectively which represent cell density (A_{600}) as a function of time.


5,000
2,500
0
β-Galactosidase (Miller units)
0
1
2
3
4
5
Absorbance (600 nm)
20
15
10
5
0
Time (hours)

phase at an OD_{600} of 3 as compared to OD_{600} of 5 in LB and they grew more slowly, taking 14 hours to reach stationary phase. In the *katE* fusion plasmid, pRSkatE16, ß-galactosidase levels increased to 8,000 units by the time the culture had reached an OD_{600} of 1 and then declined to about 6,000 units between 2 to 3.5 OD_{600} units. As the cells entered stationary phase (OD_{600} of 3.5) the ß-galactosidase levels remained at approximately 7,000 units. ß-Galactosidase synthesis from the *katF* promoter fusion also increased during the lag phase before growth reaching a level of 2,600 units at OD_{600} of 1 and remained at this level during log phase growth and into stationary phase. The low initial levels of ß-galactosidase in these cultures were the result of using an inoculum from a mid-log phase culture grown in LB.

Growth rates and expression for *katE* and *katF* promoter clones in succinate minimal medium are also shown in Figure 29 and 30 respectively. The growth rates on succinate were somewhat slower than in M9-glucose but the synthesis of ß-galactosidase directed by the *katE* promoter increased more rapidly and reached levels that were two-fold higher than those observed in the glucose grown culture almost equivalent to levels reached in LB medium (15,000 Miller units). Expression from the *katF* promoter was similar to ß-galactosidase levels found in cultures grown in glucose minimal.

The low initial levels of ß-galactosidase in these cultures were the result of using an inoculum from a mid-log phase culture grown in LB medium. If an inoculum with high ß-galactosidase levels from either stationary phase cells in LB or glucose minimal medium was used, the enzyme levels in both glucose- and succinate-minimal medium remained at a high level throughout growth.

Amino acids are a major component of LB medium and addition of a mixture of all 20 amino acids to M9-glucose medium repressed expression from both promoters until growth approached stationary phase (Figure 29 for *katE* and Figure 30 for *katF*). If amino acids were used as the sole carbon source, expression was slightly elevated throughout growth (1469 units at OD_{600} of 0.58 for *katE* and 1116 units at OD_{600} of 0.49 for *katF*). As the cells entered stationary phase (OD_{600} of 1.0) *katE* and *katF* levels increased to fully induced levels (15,997 units for *katE* and 4,021 units for *katF*). This indicated that a combination of both glucose and amino acids were required to maintain low levels of expression (Table 22). The addition of a single amino acid, either aspartate (acidic), alanine (neutral), or arginine (basic), at the concentration of 0.2% w/v to glucose minimal medium did not serve to lower expression suggesting that it was not a simple buffering effect that was repressing expression when the amino acids were present (Table 22).

4.13.2.3 The Effect of Glucose on *katE* and *katF* Expression

Meir and Yagil (1990) have reported that LB supplemented with 1% glucose lowers the levels of HPII found in *E. coli* by as much as 88% of wild-type levels. The data presented above have also shown a two-fold difference in *katE* expression in cultures grown on glucose and succinate in M9 medium, although no change was observed in *katF* levels. If a lower concentration of glucose, 0.8 mM, was used such that the cells entered stationary phase at a lower cell density (A_{600} of 0.35), the expression from both *katE* and *katF* promoters increased to the same extent as in cultures with 16 mM glucose (Table 23). Increasing the

Table 22. Effect of amino acids in minimal medium on *katE* and *katF* expression.

Medium	ß-Galactosidase (Miller units)	
	pRSkatE16	pRSkatF5
glucose[a]	4005 (0.87)[b]	3040 (1.03)
amino acid mixture[a]	1469 (0.58)	1116 (0.49)
amino acid mixture	15997 (0.97)	4021 (1.05)
glucose + amino acid mixture	848 (0.61)	839 (0.39)
glucose + aspartate[a]	5278 (0.88)	2944 (0.82)
glucose + alanine[a]	5406 (0.69)	2722 (0.66)
glucose + arginine[a]	6245 (1.20)	2760 (0.72)

[a] The following concentrations of components were used: glucose, 16 mM; amino acid mixture, 0.2% (w/v) of mixture containing equal amounts of 20 common amino acids; aspartate, alanine and arginine, 0.2% (w/v).

[b] The values in parentheses are the A_{600} (absorbance at 600 nm) readings of cell density. Cells growing in M9+glucose medium grew to an A_{600} of 3.4 in stationary phase. All of the cultures in this table were in mid-log phase.

glucose concentration in the M9 medium to 100 mM had little effect on the expression of *katE*, with ß-galactosidase remaining similar to those in M9 medium with 16 mM glucose (Table 23).

High concentrations of glucose (100 mM) in unbuffered LB medium prevented any increase in HPII activity in stationary phase cells consistent with the findings of Meir and Yagil (1990). This finding led the two researchers to suggest that catabolite repression was involved in the regulation of HPII. The observation that high glucose reduced *katE* expression was confirmed using pRSkatE16 but 50 mM lactose and 200 mM glycerol also reduced the expression of *katE* suggesting a more general phenomenon related to growth on high concentrations of any carbon source was involved (Table 23). Cultures growing on the high carbon source concentration in unbuffered medium grew more slowly and did not grow beyond a cell density equivalent to that achieved in unsupplemented media, possibly because of a drop in pH of the medium. The low expression from the *katE* promoter in unbuffered media may be a result of pH inhibition of normal growth patterns including gene expression associated with cells entering stationary phase. If the LB medium was buffered at pH 7.0, 16 mM or 100 mM glucose reduced the final level of ß-galactosidase expression from the *katE* promoter by 50%. Furthermore, the turn-on of ß-galactosidase expression and the entry into stationary phase were delayed to a higher cell density indicating that pH was a significant factor in cell growth and gene expression (Figure 31 for *katE* expression and Figure 32 for *katF* expression and Table 23). The enhanced growth in the presence of glucose suggested that the carbon source was limiting in normal LB medium and supplementing the medium with glucose allowed the cells to grow to a

Table 23. Effect of glucose, glycerol and lactose on *katE* and *katF* expression in various media.

Medium	ß-Galactosidase (Miller units)			
	pRSkatE16		pRSkatF5	
M9 + glucose (0.8 mM)	9783	(0.36)[a]	2864	(0.35)
M9 + glucose (16 mM)	8283	(1.05)	3049	(0.99)
M9 + glucose (16 mM)	6068	(3.41)	3302	(3.43)
M9 + glucose (100 mM)	5631	(0.57)	-[c]	
LB	7323	(3.86)	-	
LB + glucose (100 mM)	985	(4.90)	-	
LB + glycerol (200 mM)	2618	(5.57)	-	
LB + lactose (50 mM)	973	(6.46)	-	
LB (pH 7)[b]	8339	(4.74)	2695	(4.74)
LB (pH 7) + glucose (100 mM)	7010	(9.39)	3079	(7.14)
LB (pH 7) + glycerol (200 mM)	6423	(8.77)	3734	(9.28)
LB (pH 7) + lactose (50 mM)	5729	(6.98)	3597	(8.28)

[a] The values in parentheses are the A_{600} readings of cell density. Cells growing on 16 mM glucose grew to an A_{600} of 3.4 in stationary phase whereas cells growing on 0.8 mM glucose reached stationary phase at an A_{600} of 0.36.

[b] The medium was adjusted to pH 7 with in the presence of 50 mM PIPES.

[c] The dash indicates that the assay was not carried out.

Figure 31. Effect of high glucose on ß-galactosidase synthesis from *lacZ* under the control of the *katE* promoter expressed as a function of cell density (A_{600}). ß-Galactosidase was assayed in cultures growing in LB medium (•) and LB medium supplemented with 16 mM glucose (■) or 100 mM glucose (▲). All media were adjusted to pH 7.0 with 50 mM PIPES buffer prior to inoculation. Growth was followed in LB medium and LB supplemented with 100 mM glucose is indicated by the small dashed and large dashed lines respectively which represent cell density (A_{600}) as a function of time. The growth curve in medium supplemented with 16 mM glucose was the same as that shown for 100 mM glucose.

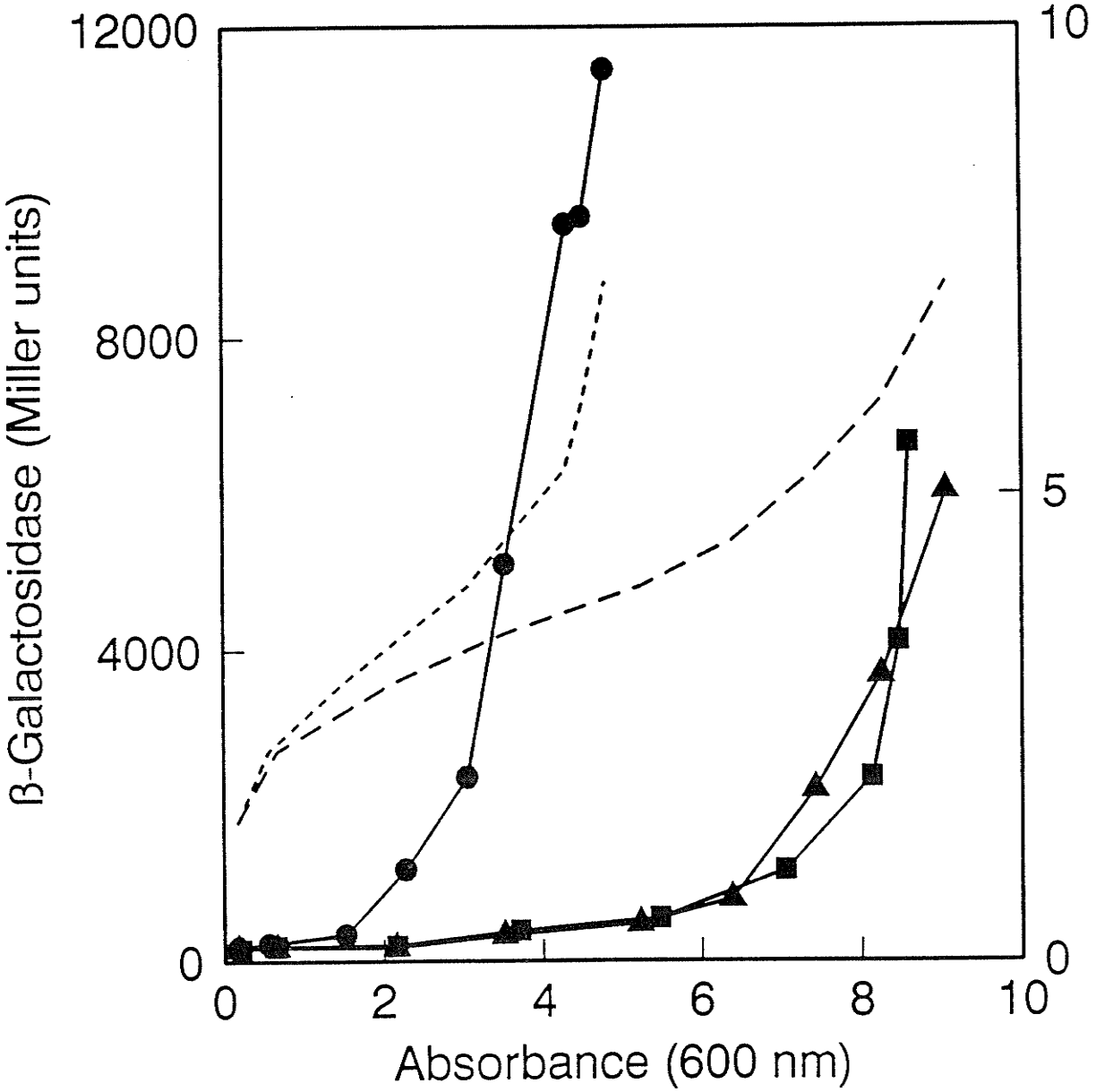
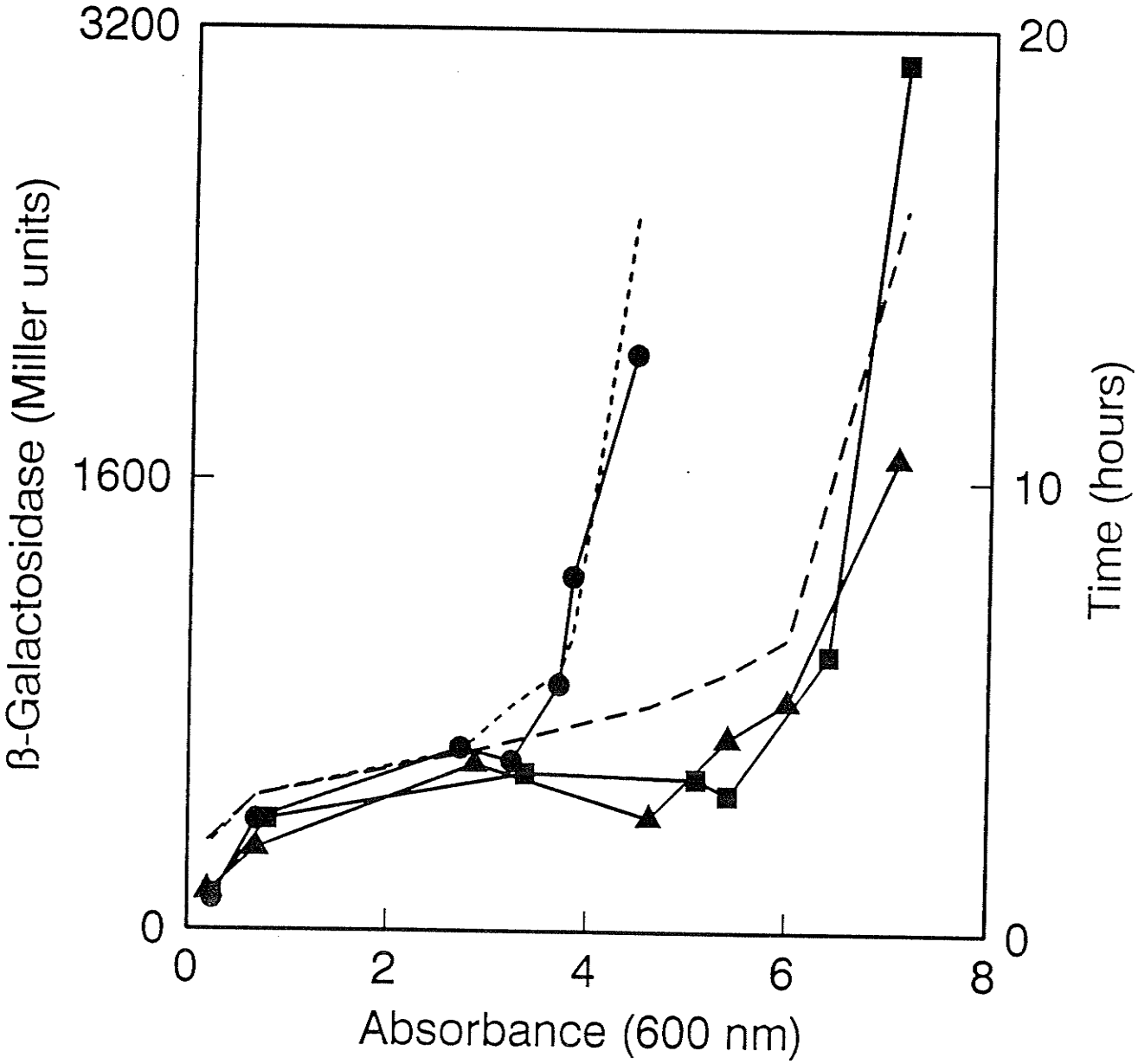

12000
8000
4000
0
β-Galactosidase (Miller units)
10
5
0
Time (hours)
0
2
4
6
8
10
Absorbance (600 nm)

Figure 32. Effect of high glucose on ß-galactosidase synthesis from *lacZ* under the control of the *katF* promoter (A_{600}). ß-Galactosidase was assayed in cultures growing in LB medium (•) and LB medium supplemented with 16 mM glucose (■) or 100 mM glucose (▲). All media were adjusted to pH 7.0 with 50 mM PIPES buffer prior to inoculation. Growth was followed in LB medium and LB supplemented with 100 mM glucose is indicated by the small dashed and large dashed lines respectively which represent cell density (A_{600}) as a function of time. The growth curve in medium supplemented with 16 mM glucose was the same as that shown for 100 mM glucose.


3200
1600
0
20
10
0
0
2
4
6
8
β-Galactosidase (Miller units)
Time (hours)
Absorbance (600 nm)

higher density. With either 16 mM and 100 mM glucose supplements, the cultures entered stationary phase at the same cell density suggesting that another component besides the carbon source was limiting growth, or that a byproduct of metabolism was accumulating in the medium causing growth inhibition and that this was coupled with the induction of *katE* and *katF*.

4.13.3 Effect of starvation on *katE* and *katF* expression.

One explanation for the increase in *katE* expression just prior to stationary phase is that nutrient depletion acts as a signal for gene induction. That is, when glucose or other medium components become limiting (starvation for a particular growth requirement) the *katE* gene is induced. A number of proteins have already been shown to be induced under nutrient limited conditions (Schultz *et al.*, 1988). The fact that the addition of glucose to LB (buffered at pH 7.0) allows the cells to grow to a higher density and delays the induction of the two genes supports the idea that glucose limitation induces the gene. Furthermore, the fact that the cells stop growing at the same density whether the LB was supplemented with either 16 mM or 100 mM glucose supports the explanation that limitation of any growth requirement (carbon, nitrogen, or phosphate) may induce the genes.

To test this idea, starvation was induced by transferring cells from a mid-log culture grown on minimal medium supplemented with both glucose and amino acids to a new medium lacking either carbon (ammonium ion as nitrogen source) or nitrogen (glucose as carbon source). Cell growth was prevented in both cases but only starvation for nitrogen allowed the induction of *katE*, while carbon starvation prevented *katE* expression and lowered *katF* expression (Table 24). This experiment

TABLE 24 Effect of removing all carbon or nitrogen from M9 medium on *katE* and *katF* expression.

Medium[a]	ß-Galactosidase (Miller units)	
	pRSkatE16	pRSkatF5
M9 + glucose + NH_4Cl	6951 (3.8)[b]	2853 (3.7)
M9 + NH_4Cl	311 (0.08)	1264 (0.13)
M9 + glucose	5613 (0.10)	2781 (0.15)

[a] The following concentrations of supplements were used: glucose, 16 mM; NH_4Cl, 19 mM.

[b] The numbers in parentheses are the A_{600} values of cell density following incubation for 20 hours.

seems to contradict the earlier observation that glucose starvation induces the *katE* gene but the differences between the two experiments are significant. In the latter all sources of carbon were removed from the medium, whereas in the former starvation occurred without drastic changes in the medium. Because carbon removal prevents *katE* expression, it is proposed that some form of carbon metabolism, even in the absence of cell growth, is required for induction, possibly through the production of an inducing component.

4.13.4 The Effect of pH on the Expression of the *katE* and *katF* Genes

The effects of external pH on the levels of *katE* and *katF* were examined at pH 6, 7, and 8. The *katF* fusion plasmid produced similar induction patterns at pH 6.0, 7.0, and 8.0 (Figure 35) with the levels gradually increasing to approximately 2800 units. Levels of ß-galactosidase from the *katE* promoter were similar in LB at pH 7.0 and 8.0 increasing dramatically as the cells approached stationary phase to levels of approximately 15000 units (Figure 34), but at pH 6.0 the induction was delayed and reached levels that were about one-third the levels achieved at either pH 7.0 or 8.0 (Figure 34).

Schellhorn and Hassan (1988) have proposed that there may be a link between the induction of an acid-sensitive regulon by non-metabolizable weak acids, such as benzoate, and the induction of HPII. The two promoter clones pRSkatE16 and pRSkatF5 were used to test this hypothesis. Cells containing either the *katE* or *katF* promoter fusion were grown in LB buffered at pH 7.0 containing 20 mM benzoate and assayed at various times for ß-galactosidase activity. The results of the experiment are shown in Figure 36 for expression from the *katE* promoter and Figure 37 for *katF* expression. ß-Galactosidase levels from

Figure 33. ß-Galactosidase synthesis from *lacZ* under the control of the *katE* promoter expressed as a function of cell density. ß-Galactosidase was assayed in cultures growing in LB medium buffered at pH 6.0 (■), pH 7.0 (•) and pH 8.0 (▲). Growth at pH 7 is indicated by the dashed line which represents cell density (A_{600}) as a function of time. Growth curves at the other pHs were very similar and are not shown.

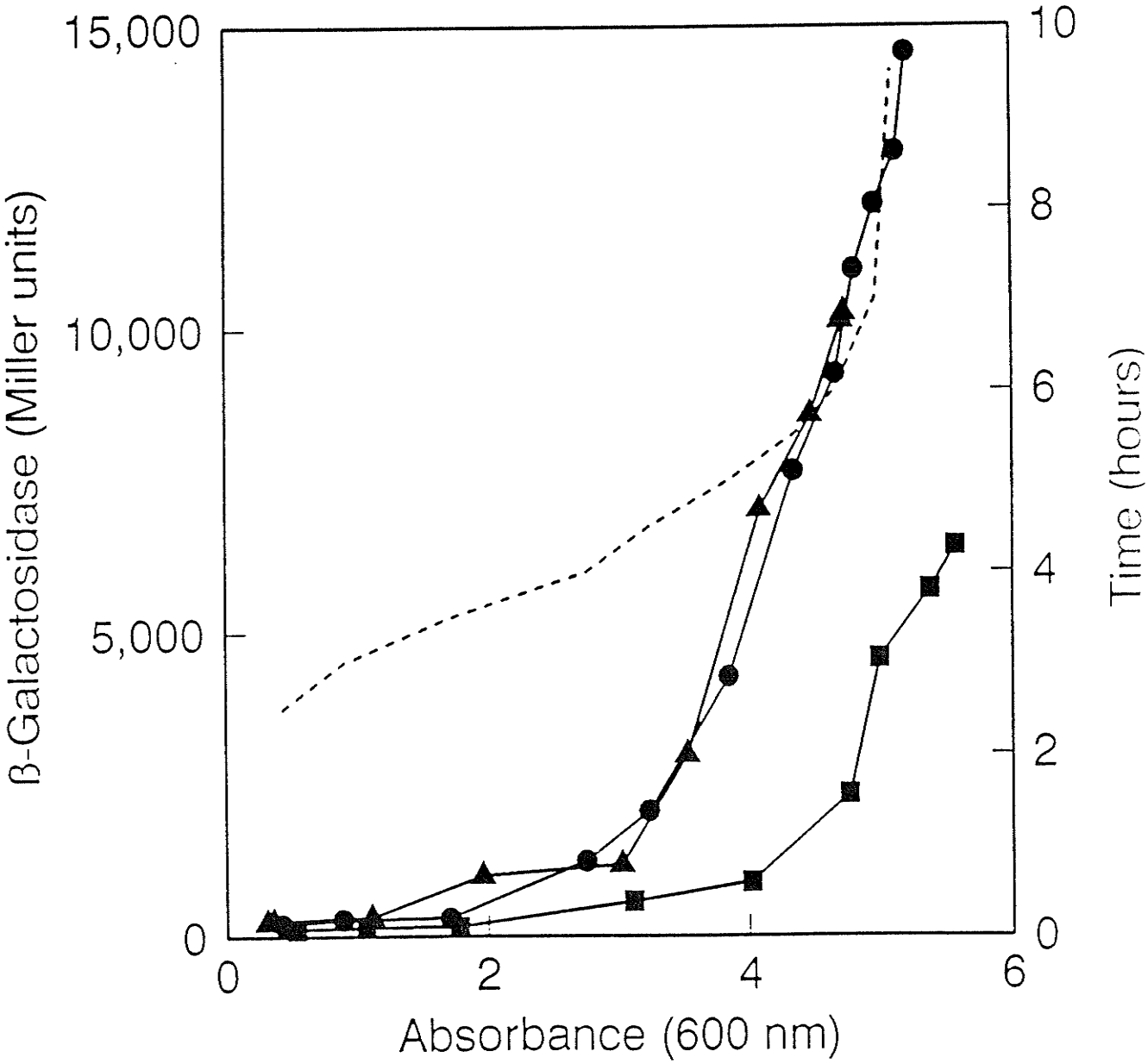

15,000
10,000
5,000
0
β-Galactosidase (Miller units)
10
8
6
4
2
0
Time (hours)
0
2
4
6
Absorbance (600 nm)

Figure 34. ß-Galactosidase synthesis from *lacZ* under the control of the *katF* promoter expressed as a function of cell density. ß-Galactosidase was assayed in cultures growing in LB medium buffered at pH 6.0 (■), pH 7.0 (•) and pH 8.0 (▲). Growth at pH 7 is indicated by the dashed line which represents cell density (A_{600}) as a function of time. Growth curves at the other pHs were very similar and are not shown.

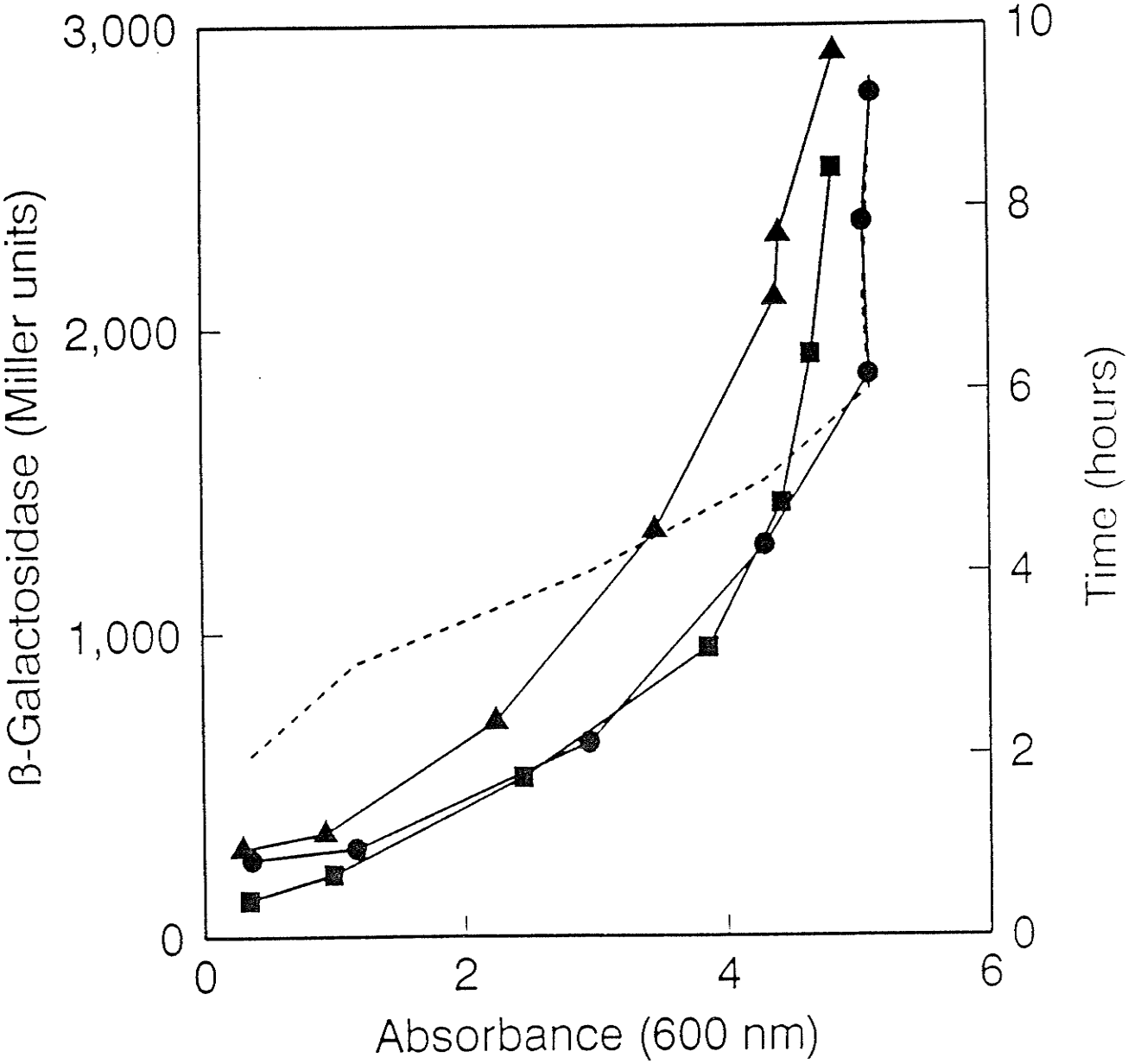

3,000
2,000
1,000
0
ß-Galactosidase (Miller units)
10
8
6
4
2
0
Time (hours)
0
2
4
6
Absorbance (600 nm)

Figure 35. Effect of benzoate on ß-galactosidase synthesis from *lacZ* under the control of the *katE* promoter (pRSkatE16) expressed as a function of cell density (A_{600}). ß-Galactosidase was assayed in cultures grown in LB medium (•) and LB medium with sodium benzoate added to final concentration of 20 mM prior to inoculation (■). Growth without and with benzoate is indicated by the short dashed and long dashed lines respectively which represent cell density (A_{600}) as a function of time.

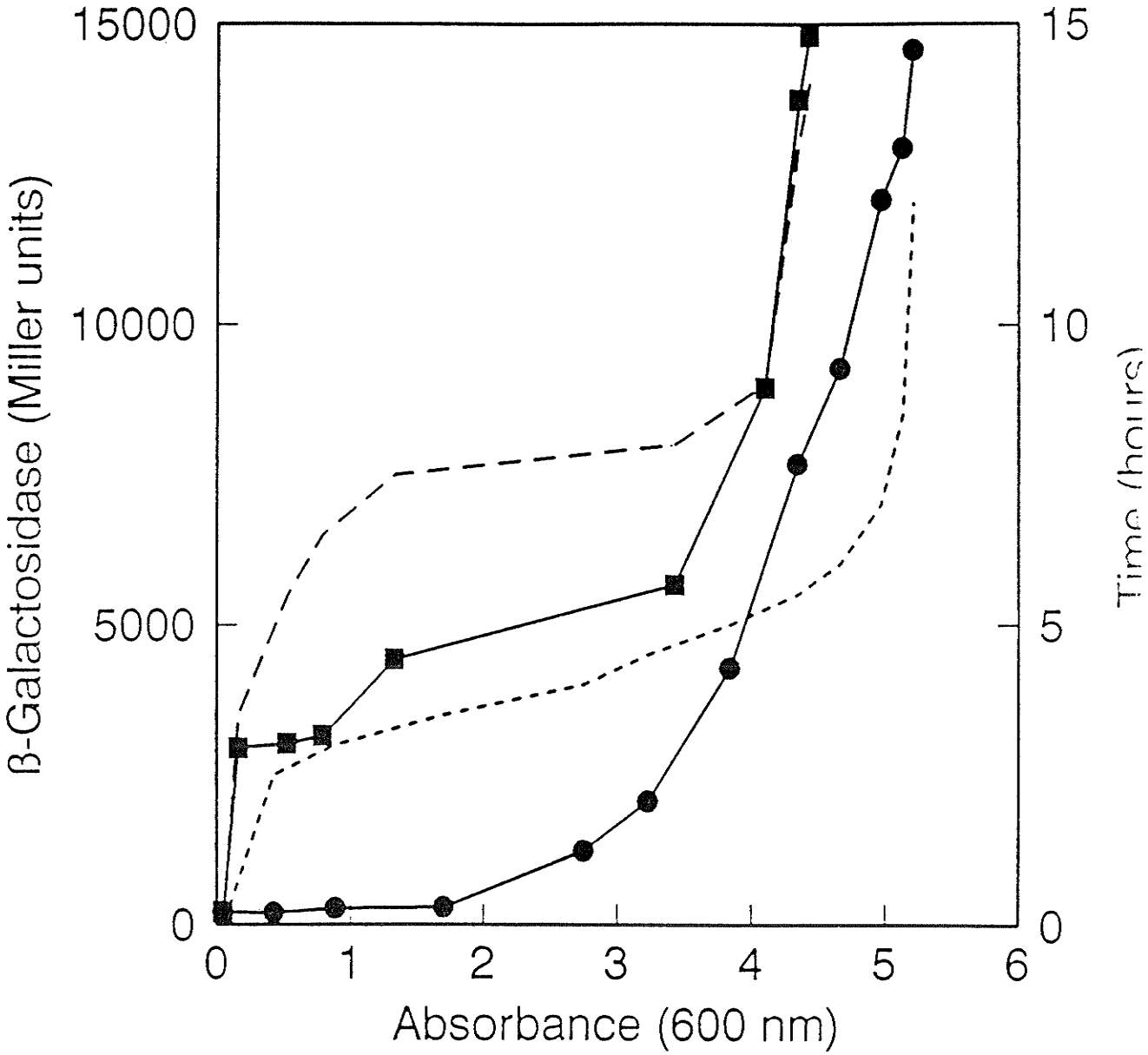
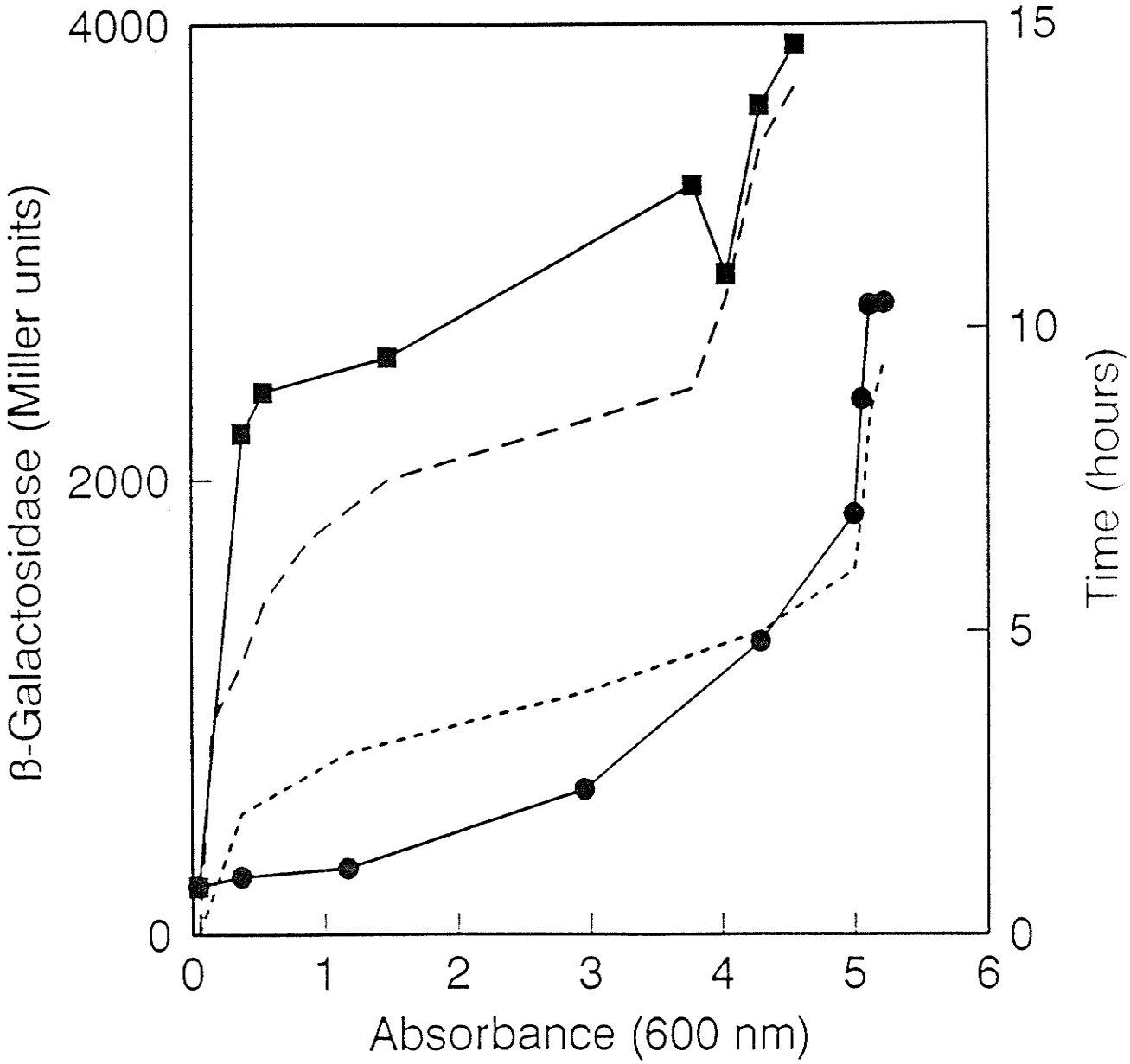

15000
10000
5000
0
β-Galactosidase (Miller units)
15
10
5
0
Time (hours)
0
1
2
3
4
5
6
Absorbance (600 nm)

Figure 36. Effect of benzoate on ß-galactosidase synthesis from *lacZ* under the control of the *katF* promoter (pRSkatF5) expressed as a function of cell density (A_{600}). ß-Galactosidase was assayed in cultures grown in LB medium (•) and LB medium with sodium benzoate added to final concentration of 20 mM prior to inoculation (■). Growth without and with benzoate is indicated by the short dashed and long dashed lines respectively which represent cell density (A_{600}) as a function of time.


4000
2000
0
15
10
5
0
0
1
2
3
4
5
6
β-Galactosidase (Miller units)
Time (hours)
Absorbance (600 nm)

the *katE* fusion increased to approximately 4,000 units at an OD_{600} of 1.0, almost 10 fold higher than in LB grown cultures at the same cell density lacking benzoate. The levels remained constant as cells grew logarithmically, but at the onset of stationary phase *katE* expression increased another 3 to 4 fold. The pattern of ß-galactosidase expression from the *katF* fusion plasmid differed by increasing dramatically to almost fully induced levels (approximately 2500 units) early in the growth phase and remaining relatively unchanged during entry to stationary phase.

A similar effect on the expression of both fusion plasmids was observed with *o*-hydroxybenzoate (another nonmetabolizable weak acid) as well as the related metabolizable weak acid *p*-aminobenzoate, but not with *o*-aminobenzoate (Table 25). Potassium fluoride, which also lowers internal pH, did not affect the expression from the *katE* promoter. The fact that *o*-aminobenzoate and potassium fluoride have no effect on *katE* induction argues against the theory that lowering internal pH causes the induction of *katE* and *katF*. Another possibility is that an inducing component that resembles benzoate is produced as the cells approach stationary phase or when all growth is limited.

This conclusion is consistent with the observation of Schellhorn and Hassan (1988) that a component of spent medium was responsible for the induction of *katE* expression. This experiment was repeated using the two fusion plasmids confirming that *katF* as well as *katE* is induced by resuspending mid-log cells in medium from a stationary phase culture (Table 26). If the spent medium from a stationary phase culture was dialysed against SM buffer before the addition of mid-log cells, the

TABLE 25. Effect of various supplements on *katE* expression.

Medium + supplement[a]	ß-Galalactosidase (Miller units) pRSkatE16
LB	244 (0.76)[b]
LB + acetate	179 (0.54)
LB + malate	275 (0.57)
LB + succinate	252 (0.57)
LB + fumarate	251 (0.74)
LB + glutamate	240 (0.76)
LB + phenylalanine	469 (0.84)
LB + benzoate	3155 (0.80)
LB + *o*-hydroxybenzoate	6684 (0.10)
LB + *p*-aminobenzoate	6851 (0.66)
LB + *o*-aminobenzoate	544 (0.66)

[a] All supplements were added to 20 mM except *o*-hydroxybenzoate which was at 10 mM. The medium was buffered to pH 7 in the presence of 50 mM PIPES.

[b] The numbers in parentheses are the cell densities in A_{600} units at the time of assaying for ß-galactosidase. Stationary phase in LB medium was reached at an A_{600} of approximately 5.0.

TABLE 26. Effect of spent LB medium on *katE* and *katF* expression.

Medium + supplement	ß-Galactosidase (Miller units)	
	pRSkatE16	pRSkatF5
LB	462 (1.93)[a]	628 (2.18)
LB (spent)	6867 (0.55)	2414 (0.65)
LB (spent dialyzed 16 h)	2499 (0.49)	1864 (0.54)

[a] The numbers in parentheses are the cell densities in A_{600} units at which the assay was carried out. Cells growing in LB medium reached stationary phase at an A_{600} of approximately 5.0.

extent of induction was significantly reduced indicating that the active component had been inactivated by dialysis or was relatively small and had passed out of the dialysis bag. Storage of the spent medium for 48 hours or boiling for 3 minutes did not reduce its ability to induce *katE* (Table 26). Attempts to identify the medium component by supplementing LB medium with various metabolic components which had previously been associated with elevated levels of HPII (Loewen *et al.*, 1985), including acetate, pyruvate, malate, succinate, and fumarate, had no effect on levels of expression (Table 25).

4.13.5 Effect of anaerobiosis on *katE* and *katF* expression

The literature contains conflicting reports of the effect of anaerobiosis on the expression of HPII. Loewen *et al* (1985) have shown there to be little HPII synthesis during anaerobic growth in minimal medium with nitrate or fumarate as the terminal electron source. Schellhorn and Hassan (1988) showed that the synthesis of HPII during anaerobic growth in LB medium increased as the cells entered stationary phase in a pattern similar to that observed for aerobic cultures but to a level that was only one third of the induced aerobic level. Meir and Yagil (1989) reported an induction of HPII synthesis within an hour of shifting a culture to anaerobic conditions in LB medium and concluded that the increase in HPII levels in aerobic stationary phase cells was the result of anaerobiosis during the onset of stationary phase.

Because an anaerobic effect was not consistent with the model involving pH or metabolite signals suggested above, the effect of anaerobiosis on *katE* and *katF* expression was investigated. Shifting cultures to anaerobic growth did not cause an increase in expression from either promoter even after 3 hours (Figure 37 for *katE* expression

Figure 38. Effect of anaerobic growth on ß-galactosidase synthesis from *lacZ* under the control of *katE* (pRSkatE16) expressed as a function of cell density (A_{600}). ß-Galactosidase was assayed in cultures growing in LB medium flushed with nitrogen and sealed (•) and in cultures that was grown aerobically for two hours to an A_{600} of approximately 0.2 (at the point indicated by the arrow) when it was flushed thoroughly with nitrogen and sealed (■). Growth in the anaerobic cultures and in the cultures shifted to anaerobic growth are indicated by the long dashed and short dashed lines respectively which represent cell density (A_{600}) as a function of time.

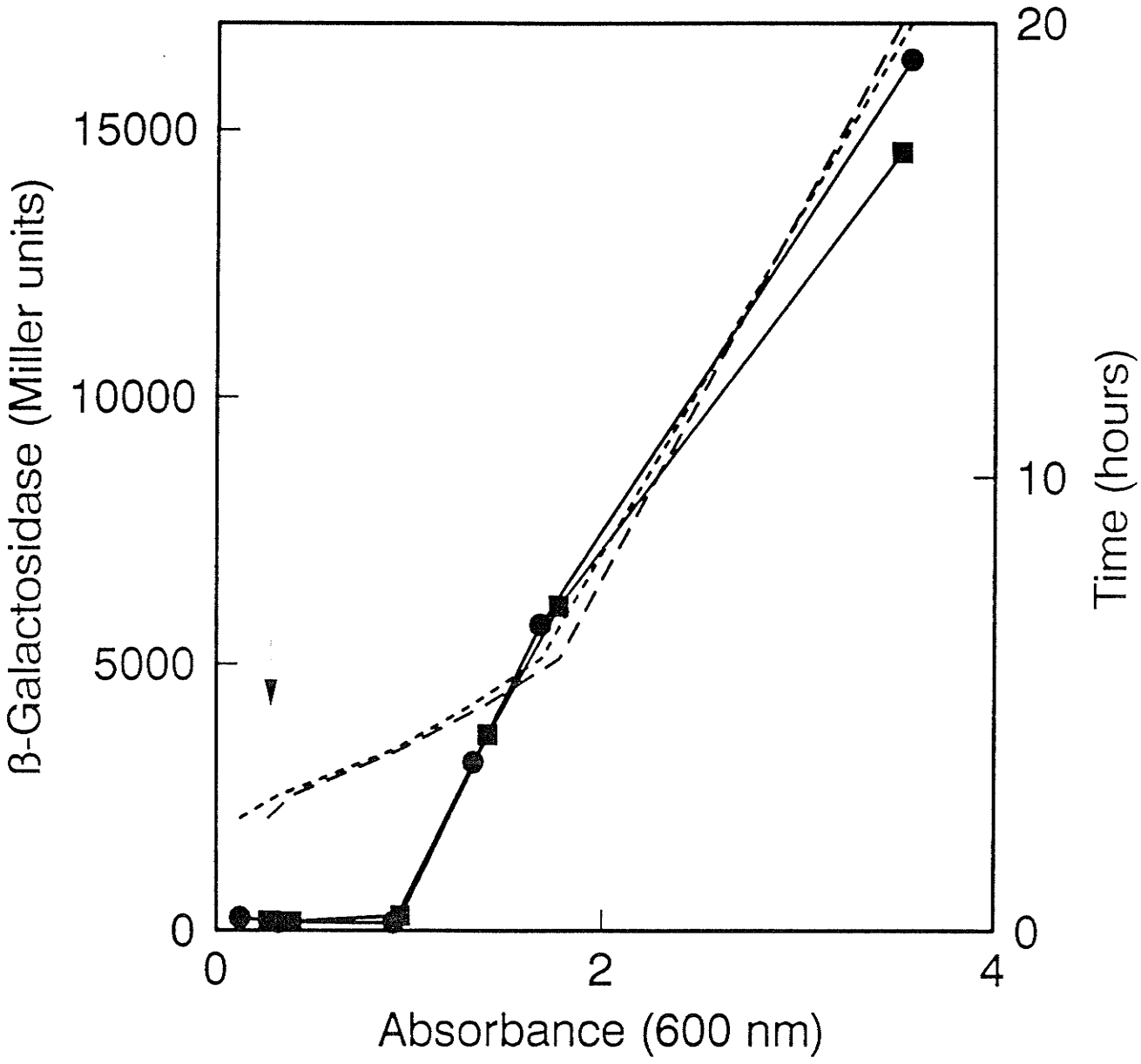
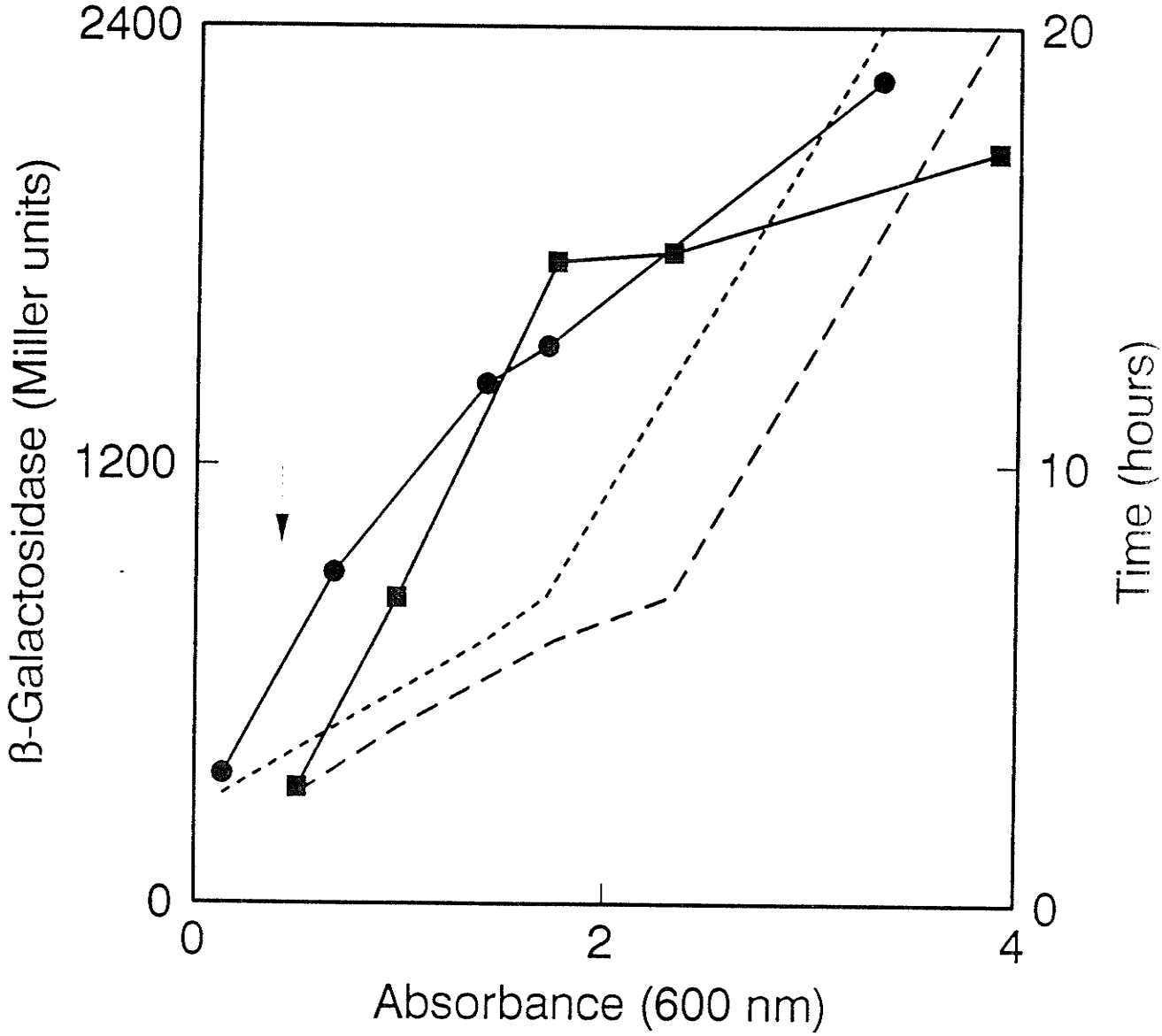

15000
10000
5000
0
0
2
4
20
10
0
β-Galactosidase (Miller units)
Absorbance (600 nm)
Time (hours)

Figure 39. Effect of anaerobic growth on ß-galactosidase synthesis from *lacZ* under the control of *katF* (pRSkatF5) expressed as a function of cell density (A_{600}). ß-Galactosidase was assayed in cultures growing in LB medium flushed with nitrogen and sealed (•) and in cultures that was grown aerobically for two hours to an A_{600} of approximately 0.2 (at the point indicated by the arrow) when it was flushed thoroughly with nitrogen and sealed (■). Growth in the anaerobic cultures and in the cultures shifted to anaerobic growth are indicated by the long dashed and short dashed lines respectively which represent cell density (A_{600}) as a function of time.


2400
1200
0
0
2
4
20
10
0
β-Galactosidase (Miller units)
Time (hours)
Absorbance (600 nm)

and Figure 39 for *katF* expression) but there was a turn-on of expression as the cells approached stationary phase.

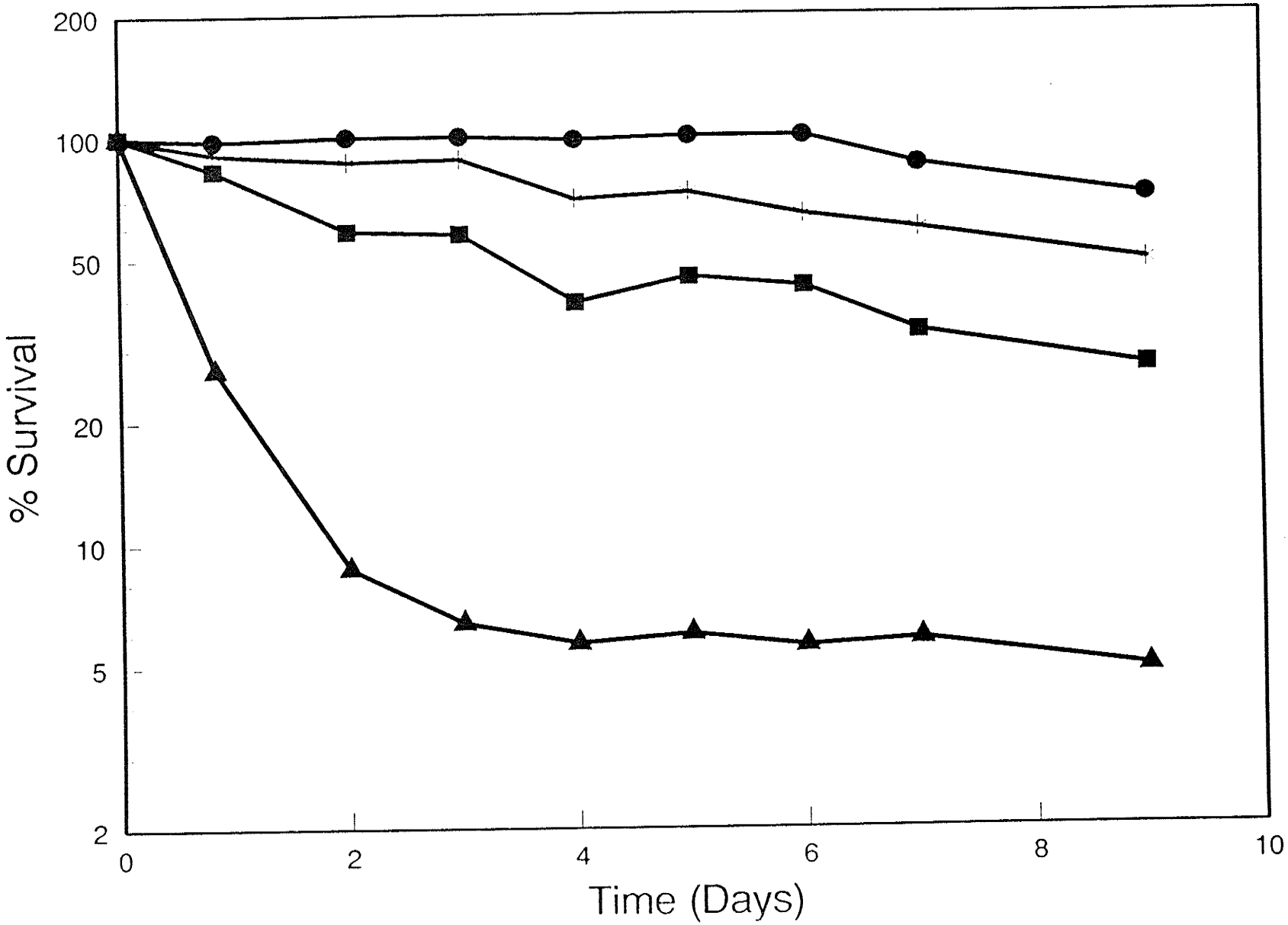
4.14 Effect of *katE*, *katF*, and *katG* on Cell Survival

Jenkins *et al.* (1988) has shown that starvation for a carbon source induces cross protection to heat and oxidative stress. The work described above has shown that *katE* and *katF* are induced under carbon and nitrogen limitation. Therefore, an experiment was devised to test the effects of deficiencies in the various catalase genes (wild type, *katE*::Tn*10*, *katF*::Tn*10*, and *katG*::Tn*10*) on survival during starvation.

Each strain was inoculated into 15 ml of minimal medium supplemented with 0.8 mM glucose to ensure the carbon source would be limiting. Each 125 ml fluted flask was then shaken at 37°C for 9 days. At 24 hr intervals, samples were removed from each culture, diluted in SM buffer, and plated in duplicate on LB plates containing the appropriate antibiotics. The plates were incubated inverted overnight and then counted. The average of the two plates were calculated and the percent survival was determined.

The results of the survival curves are shown in Figure 40. After a period of nine days, 71% of the MP180 cells were still viable. The decrease in viability only began to occur at day 7. The strain lacking *katG*, the structural gene for the HPI catalase (UM202) gradually decreased in viability to 49% after nine days. The viability of strain UM120, which lacks *katE*, the structural gene for HPII, decreased at a faster rate than UM202 with only 27% of the cells remaining viable after 9 days. The viability of strain UM122, which lacks the *katF* gene, decreased dramatically to only 6% after 4 days and then remained constant at about 5% for the remaining period of the experiment.

Figure 40. Effect of various catalase mutants on the survival patterns during long incubation at 37°C under aerobic conditions. Strains MP180 (wild-type), circles; UM202 (*katG*), asterisks; UM120 (*katE*), squares; and UM122 (*katF*), triangles; were grown for nine days.


200
100
50
20
10
5
2
% Survival
0
2
4
6
8
10
Time (Days)

DISCUSSION

5.0 DISCUSSION

Two genes, *katE* and *katF*, are required for the synthesis of HPII (Loewen, 1984; Loewen and Triggs, 1984). This catalase is not inducible by ascorbate nor is it under the control of the *oxyR* regulon. To further understand the regulation and the role of the catalase HPII in *E. coli*, the cloning of the two genes, *katE* and *katF*, was undertaken.

Initial attempts to shotgun clone the genes into the vector pAT153 using the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I and *Sal*I were unsuccessful. Therefore, the *katE* and *katF* genes were cloned using a multistep procedure utilizing the Tn*10* insertions in the strains UM120 (*katE*::Tn*10*) and UM122 (*katF*::Tn*10*). The two libraries, from UM120 and UM122, were probed with the plasmid pBT107 (Moyed *et al.*, 1983) to isolate *katE*::Tn*10* and *katF*::Tn*10* DNA sequences. DNA adjacent to the Tn*10* was then cloned and used as probes to isolate wild-type *katE* and *katF* sequences from an MP180 library. The restriction map of the *katE* clones revealed sites recognized by the restriction enzymes listed above in close proximity to the transposon insertion site, which is inserted in a location that prevented the production of a final product from *katE*. This explains the lack of success in shotgun cloning *katE*. The lack of success in shotgun cloning *katF* can be attributed to the fact that the enzymes used in the cloning attempt produce only large fragments (approximately 9 Kb) which would reduce the frequency of insertion in pAT153 relative to smaller fragments.

The size of DNA containing the *katF* gene was further reduced to a 4.1 kb *ClaI* fragment. Insertion of the fragment in either orientation complemented the *katF*::Tn*10* mutation implying that the entire gene was located on this fragment. Southern blots verified that the *katF* gene had been cloned from the same chromosomal location as the Tn*10*. Furthermore, the restriction map deduced by Kohara *et al.* (1987) was in

close agreement with the map of this *Cla*I fragment in the 59 min region of the *E. coli* chromosome.

It was still unclear what role the *katF* gene was playing in the synthesis of HPII. Because of its small size (44 KDa), it was possible the protein was regulatory in nature. This hypothesis was further supported by the findings of Sammartano *et al.* (1986) that showed *katF* mutants were more sensitive than *katE* mutants to NUV. This finding also suggested that *katF* controlled other genes involved in NUV resistance. In an attempt to answer the question of the function of *katF*, the gene was sequenced. An open reading frame of 1086 bp coding for a 362 amino acid protein was located on the 1483 bp *Nru*I-*Hin*cII fragment. Thirteen bp upstream from the initial codon there exists a potential ribosome binding site of AGG. The predicted molecular weight of the protein is 41 500 and is similar to the 44 000 dalton size of the KatF protein determined by maxicell analysis. A second initiation site may exist because the methionine at residue 13 of the protein is preceded by a stronger ribosome binding sequence of AGGAG. This potential shorter protein would be 350 amino acids in length and have a predicted size of 40.1 kDa. One possibility for the discrepancy in protein size could be the fact that the KatF protein is highly charged. There is one particular stretch of amino acids near the N-terminus (residues 25 to 56) that contains an acidic amino acid content of 47%. The direction of transcription of *katF* was confirmed by the T7 polymerase vector system (Tabor and Richardson, 1985).

A potential termination sequence exists just following the final termination codon. The 7 bp hyphenated dyad could form a stem-loop structure with a predicted stability of -21.0 kcal/mol which is followed by an eight base pair stretch containing 7 T's. Three of these T's could be included in the stem-loop structure increasing its predicted

stability to -24.6 kcal/mol.

Comparison of the nucleotide sequence of *katF* with Genbank (1986) did not reveal any significant homology to any sequenced gene. However, when the predicted amino acid sequence was compared to the group of proteins termed sigma factors, substantial homology existed. An overall comparison between KatF and the *E. coli* major sigma factor (σ^{70}) (Burton *et al.*, 1981) revealed 30% identical amino acids and 45% conservative replacements. Comparison of thirteen amino acid sequences from other bacterial and phage sigma factors has revealed four regions of homology (Helmann and Chamberlin, 1988). Very strong homology exists between KatF and the other sequences in region 2. In subregion 2.2, the KatF protein is identical (using conservative amino acid replacements) to σ^{70}. This subregion may be involved in core binding suggesting that KatF and σ^{70} may have similar affinities for core polymerase. Only one other sigma factor shares 100% homology in this region with σ^{70}, the major σ factor from *M. xanthus*, σ^{80}. Region 3 is weakly conserved among sigma factors and is thought to be involved in structural functions. KatF shares only 59% homology with σ^{70} in this region. Region 4 is made up of a weakly conserved subregion, 4.1, and a strongly conserved subregion 4.2, which is predicted to form a DNA binding helix-turn-helix motif. The KatF predicted amino acid sequence is also predicted to form a helix-turn-helix DNA binding structure in this region.

A single base addition or deletion during the sequencing process can shift the inferred protein sequence out of frame, making confirmation by direct protein sequencing of a segment of the protein advisable. In the case of KatF the protein was not available to be sequenced but the strong homology throughout the KatF sequence with the

other sigma factors supports the argument that the correct reading frame has been established.

Because the predicted protein sequence of the *katF* gene is homologous to the sigma factors, RNA polymerase isolated from cells that overproduce the protein should be charged with the KatF protein. This methodology has been used successfully in the isolation of many sigma factors including σ^{70} (Burgess and Jendrisak, 1975), σ^{32} (Grossman *et al.*, 1984), σ^{F} (Arnosti and Chamberlin, 1989), and σ^{E} (Erickson and Gross, 1989). The T7 polymerase system (Tabor and Richardson, 1985) which was used to determine the direction of transcription was also used to overexpress the KatF protein. It is interesting to note that several peaks of RNA polymerase were observed from the Biogel 1.5 M column, each peak being separated by only one or two fractions. These peaks were pooled separately and the RNA polymerase was isolated from each. No difference between each peak was observed on SDS polyacrylamide gels. The low RNA polymerase activity in fractions between the peaks may be due to the copurification of a ribonuclease at these points. The ribonuclease would interfere with the transcriptional assays resulting in lower RNA levels from the transcriptional assay.

RNA polymerase isolated by this procedure was composed of five bands when separated on a SDS-polyacrylamide gel. The bands corresponded to the ß (165 KDa), ß'(155 KDa) , σ^{70} (90 KDa), and α (39 KDa) subunits of RNA polymerase and a protein with a molecular weight of approximately 43,000. Labelled KatF protein comigrated with the 43,000 molecular weight protein strongly suggesting that the 43,000 dalton protein is the KatF sigma factor. The α band was sized at an apparent molecular weight of 45,000 which is larger than the expected size of

39,000 daltons. It is unknown why the α protein migrated in this manner. Commercial RNA polymerase (Pharmacia) also contains a 43,000 dalton band but at a much lower concentration and it is possible this protein is the KatF protein because stationary phase cells, when *katF* expression is greatest, are most likely used for the isolation of the RNA polymerase at Pharmacia.

From the data presented above, the KatF protein would appear to be a sigma factor. These proteins usually control multiple unlinked genes and/or operons which form regulons. If this is the case, what other genes does KatF control? Sak *et al.* (1989) has shown that both *katE* and *xthA* are regulated by the *katF* gene product. Both *katE* and *xthA* map at around 38 minutes on the *E. coli* chromosome. Sak *et al.* (1989) has also found that an *xthA* point mutant lacks exoIII activity as well as having a slight decrease in HPII activity. However, the *katE*::Tn*10* mutation lacks HPII activity but retains ExoIII activity. These findings have led the researchers to propose that the *xthA* and *katE* genes form an operon transcribed from *xthA* to *katE*. However, if one lines up the two genes on the physical map of *E. coli* produced by Kohara *et al.* (1987) one finds them separated by approximately 25 kb of DNA and the direction of the two genes is from *katE* to *xthA* not *xthA* to *katE* as proposed by Sak *et al.* (1989). Furthermore, DNA sequence analysis of the *xthA* gene (Saporito *et al.*, 1988) and *katE* (I. von Ossowski, unpublished data) suggests that both genes are transcribed monocistronically, each containing promoter and terminator sequences. This strongly suggests that KatF protein controls the two genes producing two separate transcripts forming a regulon and not an operon.

Saporito *et al.* (1988) reported that the transcriptional start

point for *xthA* had a -10 but no -35 consensus sequence for the major σ^{70} factor of *E. coli*. Since the *katF* gene product resembles a sigma factor (Mulvey and Loewen, 1989), the promoter regions of *xthA* and *katE* may contain a consensus promoter sequence specifically for KatF sigma factor. Alignment of the two sequences upstream of the transcriptional start sites of both genes is shown below.

```
          -35                          -10
xthA  GTAAGC-------15 bp-------CCATCC  5 bp to start
       * ***                    * ***
katE  TTTAGC-------17 bp-------ACGTCC  7 bp to start
```

Both sequences share similar bases in the putative -10 and -35 regions, but it will be necessary to examine other genes that are regulated by the KatF protein before a consensus promoter sequence emerges.

To further study the regulation of the *katE* and *katF* genes, the promoters from the two genes were fused to the promoterless *lac* operon vector pRS415 producing the two plasmids pRSkatE16 and pRSkatF5. The two promoter clones were used to study the effect of various growth conditions including high glucose concentrations, anaerobiosis, medium supplements, and pH alterations on expression from the two promoters. The changes in ß-galactosidase activity controlled by the *katE* promoter essentially paralleled the changes in HPII levels observed during similar growth experiments in LB (Loewen *et al.* 1985, Sak *et al.* 1989). Expression from the *katF* promoter differed in that it commenced in early exponential phase and increased across the growth curve into stationary phase.

Schellhorn and Hassan (1988) have found that spent LB medium induces the *katE* gene. This has led to the proposal that there may be a link between the induction of an acid-sensitive regulon by non-

metabolizable weak acids such as benzoate and the induction of HPII. These weak acids are thought to cross the membrane in the protonated form releasing the proton in the interior thus lowering the internal pH (Slonczewski *et al.*, 1987). The finding that spent medium induces the *katE* gene has been confirmed in this work and it has been shown the *katF* gene is also induced by spent medium. The *katF* levels were almost fully induced using the non-metabolizable weak acids benzoate and *o*-hydroxybenzoate. Expression from *katE* increased in concert with the increase in expression from *katF* resulting in ß-galactosidase levels that were 10 fold higher (approximately 5000 units) than in un-induced cells. As the cells recovered from the benzoate and began to grow logarithmically, *katE* expression maintained ß-galactosidase levels at 5000 units until the cell reached stationary phase when further *katE* expression raised the ß-galactosidase levels another three- to four-fold to levels normally attained in LB.

From these results it seems clear that *katF* can be fully induced with only partial *katE* induction, such as in the presence of benzoate in LB medium or glucose in minimal medium suggesting that another factor or factors, besides *katF*, are involved in the regulation of *katE*. These factors could be additional regulatory proteins or inducer molecules which are responsible for the final three- to four-fold induction of *katE*. Alternatively, the KatF protein could be produced in a low activity form, which causes an initial ten-fold increase in *katE* expression and as the cells approach stationary phase, the low activity form of KatF is altered to a high activity form to cause the further three- to four-fold increase. Starving the cells for nitrogen or allowing the cells to exhaust the glucose in the medium seems to induce the *katE* and *katF* genes. However, if the glucose is rapidly removed

from the medium no induction takes place. This suggests that some form of carbon metabolism may be required for the production of an inducing component. Whether or not the putative component accumulates or not may depend on the growth rate of the cells. For example, growth on M9-glucose medium, requiring the cells to make amino acids, was sufficiently slow to allow accumulation of the putative inducing component. The same argument can be made for succinate which is an even poorer carbon source than glucose. In LB medium or M9-glucose-amino acid medium, the growth rate is rapid enough to prevent the accumulation of the inducing component. An alternate explanation, that it is the lack of a specific component causing the turn-on, is not consistent with the lack of response in the absence of all carbon.

If there is an inducing factor accumulating in the cell under suboptimal growth conditions, what might the signal be? The fact that weak acids benzoate, *o*-hydroxybenzoate, and *p*-aminobenzoate induce the *katE* gene suggests that lowering the internal pH may cause turn-on of the genes. This explanation has not been entirely ruled out but the fact that acetate and fluoride, which also lower internal pH, had little or no inducing activity supports the idea that internal pH changes do not affect *katE* and *katF* expression. Therefore, if lowering internal pH does not induce the *katE* and *katF* genes it would seem likely that the compound which putatively accumulates in the cell during suboptimal growth conditions is similar to benzoate or has the same effect as benzoate on the cell.

Recent studies have produced somewhat conflicting data with regard to what affects the synthesis of catalase HPII in *E. coli*. For example, anaerobiosis has been reported either to reduce (Loewen *et al.*,

1985 and Schellhorn and Hassan, 1988) or enhance (Meir and Yagil, 1990) HPII synthesis and glucose has been reported either to reduce (Meir and Yagil, 1990) or to have no effect on HPII synthesis (Loewen *et al.*, 1985). Anaerobiosis, either as a result of a shift to anaerobic growth or as a result of continuous anaerobic growth, did not cause an induction of either promoter in early log phase growth and did not change the normal pattern of increasing expression during late log growth. This is consistent with the findings of Schellhorn and Hassan (1988) but contradicts the findings of Meir and Yagil (1990). Possible explanations for the discrepancies may lie in the bacterial strains used or the methodology of the experiment. Meir and Yagil used a strain which contained both HPI and HPII and to discriminate between the two, they measured HPII activity at pH 10.5. It is possible that another enzyme induced by anaerobiosis has a catalase activity at pH 10.5. The finding that anaerobiosis induces HPII led the two researchers to search for a regulatory protein that controls its induction. A mutation in the *fnr* locus was found to affect the synthesis of HPII when measured at pH 10.5 suggesting that HPII is controlled by *fnr*. However, when I examined the *katE* and *katF* sequences for the *fnr* binding consensus sequence, no homology was found. This strongly suggests the *fnr* gene is not involved in the regulation of the *katE* or *katF* genes.

As mentioned above, Meir and Yagil (1990) have also reported that high glucose concentrations lowered the synthesis of catalase such that on solid media the synthesis of both HPI and HPII is completely repressed. I have confirmed this finding using the strain NM522 but when the wild-type strain MP180 was used some catalase was produced, albeit at lower than normal levels. Expression from the *katE* and *katF*

promoters was also lower in liquid LB medium containing high glucose. This phenomenon is not just related to glucose but high concentrations of other sugars (glycerol and lactose) also prevent the expression of *katE*. If the high glucose medium was buffered, *katE* and *katF* were induced normally in late log phase. The pH in unbuffered media drops during growth on high glucose and the inhibition of *katE* expression by low pH may be the result of a general inhibition of cellular metabolism preventing synthesis of the inducing component. However, if the high glucose medium was buffered to prevent the drop in pH, normal growth to a higher cell density was possible and the *katE* and *katF* genes were induced normally in late log phase. Consequently, the glucose (or high concentration of carbon source) effect is relatively small when pH effects are eliminated.

Recently, a number of genes have been identified in *E. coli* (Groat and Matin, 1986) and *S. typhimurium* (Spector *et al.*, 1988) that are induced by starvation for carbon, nitrogen, or phosphate. As discussed above, it seems likely that the *katE* and *katF* genes are also induced by starvation and their gene products may play a significant role in starvation survival. In particular, inactivation of the KatF protein significantly reduced the ability of cells to survive starvation, remarkably similar to the effect of mutations inactivating several peptidases that are also required for survival (Reeve *et al.*, 1984). It seems likely from these data that the *katF* gene product is a sigma factor that is involved in the regulation of at least two genes, *katE* and *xthA*. The KatF protein may be playing a role in the overall response to starvation, inducing HPII and exonuclease III as a protective response. The HPII protein degrades toxic hydrogen peroxide

which may damage DNA and cellular components while exonuclease III repairs the DNA damaged by oxidation. The mechanism of induction seems complex and may involve other regulatory proteins and possibly small aromatic compounds that resemble benzoate. These compounds may accumulate as the cell experiences suboptimal growth conditions to ensure the presence of the protective enzymes as medium depletion increases to full starvation. Alternatively, the onset of starvation may produce internal pH fluctuations causing changes in the proton motive force. These changes may be the signal for *katF* induction.

Research involving the *katF* gene can now proceed in a number of directions. Firstly, work can begin on *katF in vitro* mutagenesis to further understand the interaction of the KatF protein with the core polymerase as well as with the *katE* promoter. It appears as if another factor is involved in the regulation of *katE*. It will be interesting to locate a fragment of *katE* DNA which still retains the -10 and -35 promoter region but has lost any upstream sequences. This fragment could then be inserted into the pRS415 vector to determine if any potential regulatory protein binding regions have been lost. The *katE* -10 and -35 promoter regions can also be mutagenized to further understand which bases are important in polymerase recognition. Secondly, if the inducing component of *katF* is accumulating in the medium, further experiments can been developed to characterize this factor. Thirdly, to determine if the *katF* gene is part of the flagellar regulon, a *lacZ flaI* double mutant could be constructed. If the ß-galactosidase levels are lower in this strain harbouring the plasmid pRSkatF5 than in its isogenic *lacZ* parent, the results would further support the RNA mapping studies presented in this thesis.

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6.0 References

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