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Physical characterization of *katG*, encoding catalase HPI of *Escherichia coli*

(Recombinant DNA; transposon Tn*5*; promoter cloning; BAL 31 exonuclease; maxicell analysis; peroxidase)

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SUMMARY

The gene encoding the bifunctional catalase-peroxidase HPI from *Escherichia coli* was located on a 3.8-kb *Hin*dIII fragment of the Clarke and Carbon plasmid pLC36-19 using transposon Tn*5* insertions. This fragment was subcloned into the *Hin*dIII site of pAT153 to create pBT22. The size of the insert was reduced by BAL 31 digestion of one end to an apparent minimum size for catalase expression of approx. 2.5 kb as determined by complementation and expression in maxicell strains. Further reduction in size or digestion from the opposite end inactivated the gene. The location and orientation of the promoter at the 0 kb end of the insert in pBT22 was confirmed by cloning a 320-bp *Bgl*II fragment into the promoter-cloning vector pKK232-8. Differences in the Southern blots of genomic DNA from a wild-type strain and a *katG*17::Tn*10* mutant digested with *Hin*cII and probed with pBT22 confirmed that the transposon previously mapped in *katG* was located in the 2.5-kb coding region for HPI.

INTRODUCTION

Two different catalases or hydroperoxidases, HPI and HPII, are produced by *E. coli*. HPI is a bifunctional enzyme containing both catalase and hydroperoxidase activities and it has been purified and partially characterized as a tetramer of identical 84-kDa subunits (Claiborne and Fridovich, 1979). HPI could be separated electrophoretically into two isoenzyme variants, HPI-A and HPI-B, and the synthesis of both isoenzymes was affected by the locus *katG* located at 89.2 min on the *E. coli* chromosome (Loewen et al., 1985b). The synthesis of HPI was induced by hydrogen peroxide (Finn and Condon, 1975; Hassan and Fridovich, 1978; Richter and Loewen 1981; Loewen et al., 1985a) and *oxyR* was implicated as a possible positive regulatory element in its induction (Christman et al., 1985). A plasmid from the Clarke and Carbon (1976) library, pLC36-19, was found to encode HPI but the 19.2-kb chro-

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Abbreviations: Ap, ampicillin; bp, base pair(s); Cm, chloramphenicol; HP, catalase-hydroperoxidase; *katG*, gene encoding catalase-hydroperoxidase HPI; kb, kilobase pairs or 1000 bp; Km, kanamycin; ^{R}, resistant; ^{S}, sensitive; SDS, sodium dodecyl sulphate; Sm, streptomycin; Tc, tetracycline; Tn, transposon; ::, novel joint; [ ], designates plasmid-carrier state.

mosomal DNA insert in the plasmid was much larger than the 2.4 kb required to encode the 84-kDa subunit of HPI (Loewen et al., 1983). This paper describes the subcloning and subsequent characterization of the catalase-encoding DNA on a 2.5-kb insert. The direction of transcription has been determined and the identity of the DNA fragment as *katG* has been unequivocally confirmed.

MATERIALS AND METHODS

(a) Bacterial strains, plasmids and media

A list of the *E. coli* K-12 strains and plasmids used in this work is given in Table I.

LB medium (Miller, 1972) contained 10 g of tryptone (Difco), 5 g of yeast extract (Difco) and 10 g of NaCl per litre. Lambda broth contained 10 g of tryptone and 2.5 g of NaCl per liter. Lambda YM broth was prepared by supplementing λ broth with 0.01% yeast extract and 0.2% maltose. Solid media were prepared with 1.5% agar. Ten μg Ap/ml, 80 μg Sm/ml, 25 μg Km/ml, 15 μg Tc/ml and 10 μg Cm/ml were added as required.

(b) Catalase activity

Catalase activity on plates was tested by applying a drop of 30% H_2O_2 with a syringe on the edge of the colony. Catalase-positive colonies evolved oxygen bubbles. Catalase activity in liquid medium was determined by the method of Rørth and Jensen

TABLE I

Escherichia coli strains and plasmids

Strain or plasmid	Description or genotype	Reference or source[a]
JA200	F$^+$ *recA trp thr leu*	Clarke and Carbon (1976)
UM53	*leuB*6 *proC*83 *purE*42 *trpE*28 *his*-208 *argG*77 *ilvA*681 *met*-160 *thi*-1 *ara*-14 *lacY*1 *galK*2 *xyl*-5 *mtl*-1 *azi*-6 *rpsL*109 *tonA*23 *tsx*-67 *supE*44 *malA*38 *xthA katE*2 *katG*15 *recA*	Loewen (1984)
HB101	*pro leu rpsL hsdM hsdR endI recA lacY*	Boyer and Roulland-Dussoix (1969)
CSR603	*thr*-1 *leuB*6 *proA*2 *phr*-1 *recA*1 *argE*3 *thi*-2 *uvrA*6 *ara*14 *lacY*1 *galK*2 *xyl*5 *mtl*-1 *gyrA*98 *ppsL*31 *tsx*33 λ^- *supE*44 *thi*-1 *HfrH*	C.G.S.C.[b]
MP180	*thi*-1 *HfrH*	Pearson (1972)
UM123	as HB101 but *kat*-18	Nitrosoguanidine mutagenesis
UM202	as MP180 but *katG*17::Tn*10*	Loewen et al. (1985)
UM228	as HB101 but *kat*-19	Nitrosoguanidine mutagenesis
pAT153	Ap^{R}Tc^R	Twigg and Sherratt (1980)
pLC36-19	ColE1^R	Clarke and Carbon (1976)
pKK232-8	Ap^R Cm^R	Brosius (1984)
pA30	pLC36-19::Tn*5*; Km^R	λNK467 × JA200[pLC36-19]
pC1	pLC36-19::Tn*5*; Km^R	λNK467 × JA200[pLC36-19]
pE6	pLC36-19::Tn*5*; Km^R	λNK467 × JA200[pLC36-19]
pD31	pLC36-19::Tn*5*; Km^R	λNK467 × JA200[pLC36-19]
pBT1	Km^R *katG*20	*Hin*dIII deletion from pC1
pBT2	Km *katG*$^+$	*Hin*dIII deletion from pC1
pBT5	Ap^R Tc^S *katG*21	pLC36-19 · *Sph*I + pAT153 · *Sph*I
pBT22	Ap^R Tc^S *katG*$^+$	pLC36-19 · *Hin*dIII + pAT153 · *Hin*dIII
pBT24	Ap^R Tc^S *katG*22	*Eco*RI deletion from pBT22
pBT28	Ap^R Tc^S *katG*$^+$	BAL 31 deletion of pBT22
pBT29	Ap^R Tc^S *katG*23	BAL 31 deletion of pBT22
pBT30	Ap^R Tc^S *katG*24	BAL 31 deletion of pBT22
pBT54	Ap^R Tc^S *katG*$^+$	pLC36-19 · *Hin*dIII + pAT153 · *Hin*dIII
pGprml	Ap^R Cm^R	pKK232-8 · *Bam*HI + pBT22 · *Bgl*II

[a] Symbol × indicates genetic cross. Symbol · *Sph*I or · *Hin*dIII specifies digestion of given plasmid with the indicated enzyme; symbol + specifies ligation.

[b] Coli Genetic Stock Center, B. Bachmann, Curator.

(1967) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase was defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min at 37°C.

(c) Transposon Tn*5* insertions

The strain JA200[pLC36-19] grown to 5×10^8 cells/ml in YM broth was infected with phage λNK467 (*b*221, *rex*::Tn*5*, *c*I857, *O*am29 *P*am80) (DeBruijn and Lupski, 1984) and Km-resistant colonies were selected. Approx. 1000 Km-resistant colonies were harvested from the agar plates and grown overnight in LB broth. A 1-ml volume of a log-phase culture of the Tn*5* inserts was mixed with 1 ml of a log-phase culture of UM53 followed by shaking for 1 h at 37°C. Aliquots were plated on LB plates supplemented with Sm and Km. Individual colonies were picked and used for small-scale plasmid preparations to screen for possible transposon insertions. The location of the transposon was determined by restriction-enzyme mapping.

(d) DNA manipulations

Large-scale plasmid preparations were done by the method of Birnboim and Doly (1979). Small-scale plasmid isolations were done by the procedure of Birnboim (1983) and plasmid preparations for the purpose of screening only were done using the direct loading procedure of Maniatis et al. (1982). Enzymes for DNA analyses were purchased from Boehringer Mannheim and Bethesda Research Laboratories. Restriction-enzyme analysis, transformations, ligations, nick translations, Southern blotting and hybridizations were done as described by Maniatis et al. (1982).

(e) BAL 31 deletion analysis

Ten μg of pBT22 DNA was digested with *Sal*I. The DNA was precipitated, resuspended in BAL 31 incubation buffer and treated with BAL 31 exonuclease as described by Frey et al. (1984) except that the times of sample removal varied in different experiments. After reprecipitation, the DNA was resuspended, treated with ligase overnight and used to transform UM228. Ap-resistant colonies were picked and screened for catalase activity. Direct loading plasmid preparations were made to identify deletion plasmids which were then characterized by restriction nuclease analysis.

(f) Expression of proteins in maxicells

The proteins encoded by the various plasmids were identified in maxicell extracts as described by Sancar et al. (1979). Strain CSR603 was transformed with the various plasmids and the selected transformants were grown in liquid medium. The cells were irradiated with a UV lamp for 12 s at a distance of 36 cm. The culture was labelled with [^{14}C]amino acids and the labelled proteins were analyzed by polyacrylamide gel electrophoresis in the presence of SDS followed by fluorography of the dried gels. Preparation for fluorography was as described by Boulnois and Timmis (1984).

RESULTS AND DISCUSSION

(a) Location of the catalase gene in pLC36-19

Because pLC36-19 contained almost 17 kb in excess of what was required to code for HPI, it was necessary to identify the coding region. Transposon Tn*5* insertions in pLC36-19 were isolated in an attempt to correlate catalase activity and the physical location of the catalase gene. Four plasmids, pA30, pC1, pD31 and pE6, containing Tn*5* insertions were isolated, mapped (Fig. 1a) and used to transform the catalase deficient, *recA* strain, UM53. Transformants from all four plasmids complemented the catalase deficiency (Table II), although pD31 directed a lower level of catalase possibly as a result of the Tn*5* insertion position being close enough to the catalase gene to interfere with its transcription.

Deletion mutations of pC1 were made by *Hin*dIII digestion followed by ligase treatment and transformation of catalase deficient, UM123. Km-resistant transformants were screened for plasmids with sizes smaller than pC1 and two plasmids pBT1 and pBT2 were selected for further study. The larger plasmid, pBT2 (Fig. 1b), contained three *Hin*dIII fragments including the 3.8-kb fragment originally in pLC36-19 and it complemented the catalase deficiency in UM53. The smaller plasmid, pBT1, differed physi-

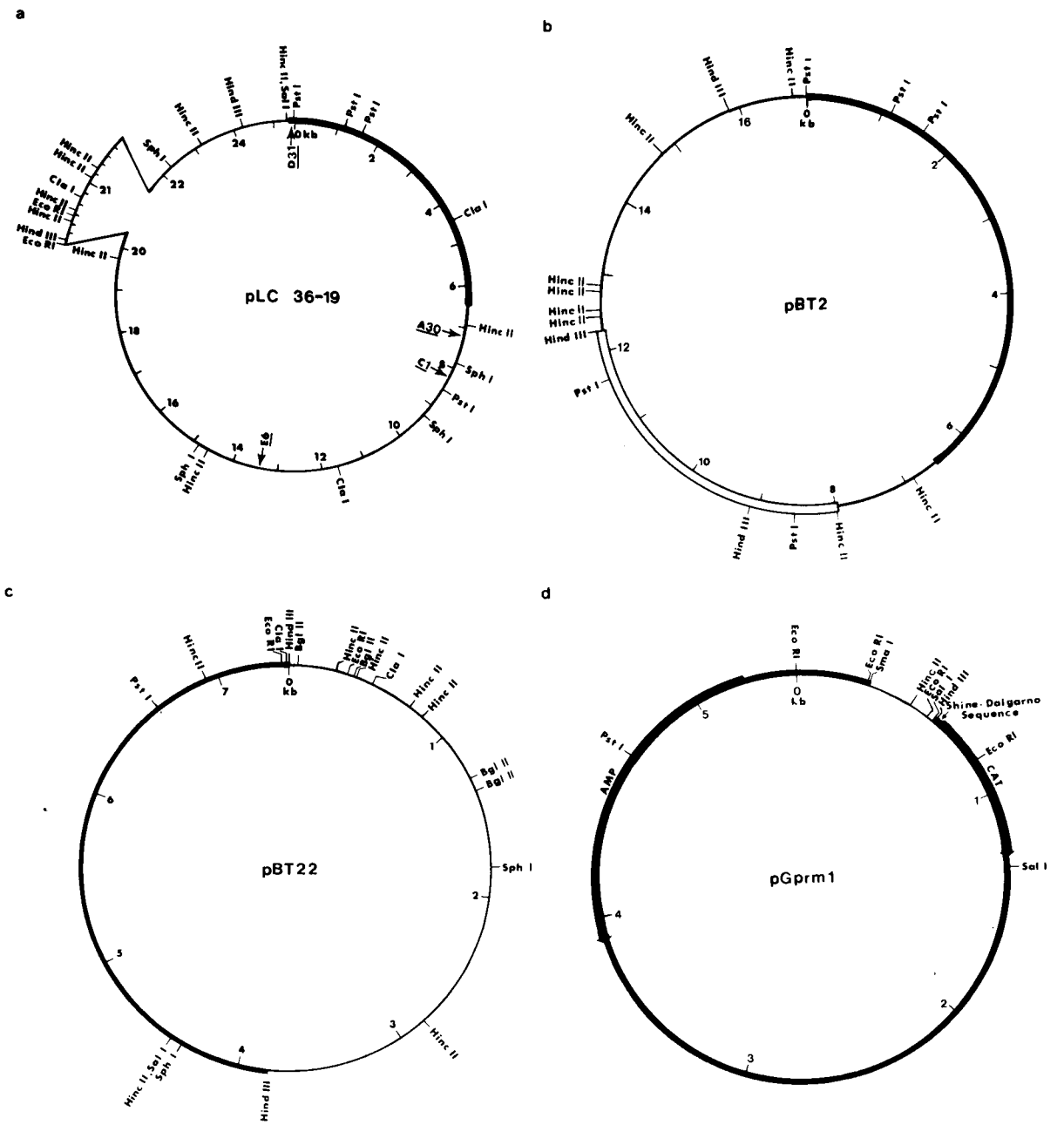

Fig. 1. Restriction maps of plasmids. (**a**) pLC36-19, (**b**) pBT2, (**c**) pBT22 and (**d**) pGprml. The thick lines represent the vector [ColE1 in (**a**) and (**b**), pAT153 in (**c**) and pKK232-8 in (**d**)] and the thin lines represent the cloned segments of *E. coli* genomic DNA. In (**a**), transposon Tn*5* insertions in pLC36-19 are marked with arrows on the inside of the circle. In (**b**), the open box represents the portions of transposon Tn*5*. Plasmid pBT1 differs from pBT2 (**b**) only in having the 3.8-kb genomic fragment from 12.1 to 15.9 kb deleted. AMP and CAT [very thick lines in (**d**)] indicate the Ap^R and Cm^R genes.

cally from pBT2 only in lacking the 3.8-kb fragment but it also differed functionally in not complementing the catalase deficiency suggesting that all or part of the catalase gene was contained on this fragment. The remainder of pBT1 and pBT2 was composed of ColE1 vector and portions of the transposon.

(b) Subcloning of the catalase gene

The 3.8-kb *Hin*dIII fragment was cloned from pLC36-19 into the *Hin*dIII site of pAT153. Two Ap^RTc^S, *kat*$^+$ transformants of catalase-deficient UM228 were selected and found to contain plas-

TABLE II

Catalase activity of cultures of transformed UM53 in mid-logarithmic growth phase

Plasmid	Catalase activity (units/mg dry cell weight)[a]
None	0.24
pLC36-19	31.9
pE6	26.4
pA30	20.6
pD31	3.3
pC1	21.0
pBT1	N.D.
pBT2	29.8
pBT5	N.D.
pBT22	91.6
pBT54	80.0
pBT28	109.3
pBT29	N.D.
pBT30	N.D.
pBT24	N.D.

[a] Refer to MATERIALS AND METHODS, section **b** for definition of units.
N.D., not detectable

mids, both of which yielded two fragments from *Hin*dIII digestion, 3.6 kb (pAT153) and 3.8 kb (encoding catalase). Restriction nuclease analysis revealed that the two plasmids differed in having the fragments joined in opposite orientation and they were labelled pBT22 (Fig. 1c) and pBT54. Both plasmids conferred a high level of catalase activity to the host (Table II). The fact that both these plasmids encoded catalase confirmed that expression of the HPI gene was occurring independent of a vector promoter.

(c) Length determination of the catalase gene

The minimum length required for expression of the catalase gene was determined by the analysis of deletion mutants of pBT22. For reference the two ends of the insert in pBT22 will be referred to as the 0-kb and the 3.8-kb ends. The 340-bp *Eco*RI fragment at the 0-kb end was deleted by nuclease cleavage and ligase treatment to produce pBT24 (Fig. 2) which did not complement catalase deficiency. This indicated that one end of the catalase gene was located within 300 bp of the 0 kb end.

An 8-kb *Sph*I fragment from pLC36-19 extending from the 1.85-kb site of pBT22 to beyond the 0-kb end was cloned into pAT153 giving rise to pBT5. This plasmid did not complement catalase deficiency indicating that the catalase gene extended beyond the 1.85-kb site (Fig. 2). Deletions starting from the 3.8-kb end of pBT22 were generated by BAL 31 nuclease giving rise to pBT28, pBT29 and pBT30 (Fig. 2) of which only pBT28, with approx. 2.5 kb of insert DNA remaining, complemented catalase deficiency. Both pBT29 and pBT30 which did not complement the catalase deficiency contained slightly less insert DNA but still contained the *Sph*I site. Based on these results the catalase gene extends from between 0 and 300 bp at the 0-kb end to approx. 2.5 kb which is in good agreement with the minimum gene size of 2.4 kb based on the published amino acid analysis and 84-kDa subunit size. pBT22 and pBT28 like pLC36-19 encoded both isoenzyme variants of HPI.

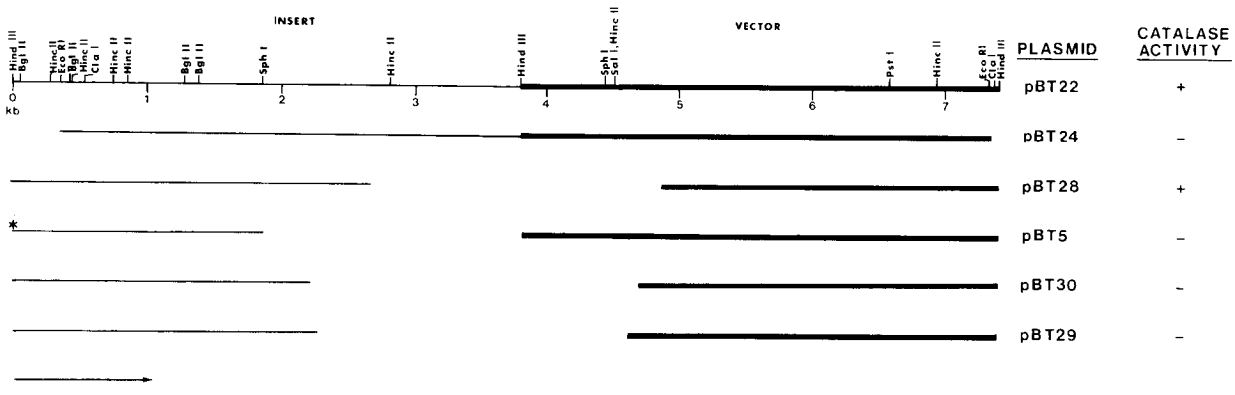

Fig. 2. Localization of *katG* by deletion mapping. The arrow indicates the direction of transcription of the gene. Thin and thick lines are as in Fig. 1. The asterisk indicates that the insert extends 5.7 kb beyond the *Hin*dIII site.

(d) Promoter location and direction of transcription

Proteins encoded by various plasmids were expressed in a maxicell strain and analyzed on SDS polyacrylamide gels. As shown in Fig. 3, the plasmids that complemented catalase, pBT22, pBT54 and pBT28, all produced an 84-kDa protein corresponding in size to the HPI subunit. The noncomplementing plasmids, pBT29 and pBT30 produced much smaller amounts of a similar size protein suggesting either impaired transcription or decreased stability had resulted from a portion of the protein being missing. The other noncomplementing plasmid, pBT24, which lacked 340 bp at the 0-kb end, produced a 45-kDa polypeptide (Fig. 3), a much smaller protein than would be expected if the DNA deletion were simply affecting the carboxy terminus. These data suggested that normal transcription of the catalase gene started near the 0-kb end and progressed towards the 3.8-kb end (Fig. 2).

Further confirmation regarding the location of the promoter was obtained using the promoter-cloning

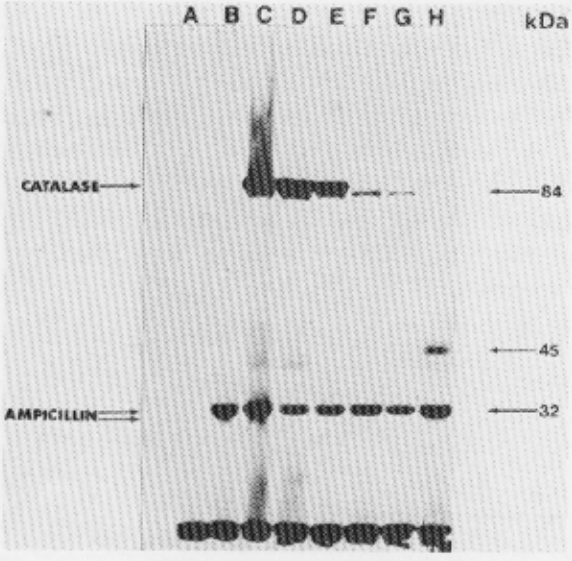

Fig. 3. Maxicell analysis of plasmid-encoded proteins. ^{14}C-labelled polypeptides were analyzed by electrophoresis on a 0.1% SDS–8% polyacrylamide gel at 40 mA for 4 h. Irradiation and fluorography were as described in MATERIALS AND METHODS, section **f**. Lanes: (A) CSR603; (B) CSR603[pAT153]; (C) CSR603[pBT22] (*katG*$^+$); (D) CSR603[pBT54] (*katG*$^+$); (E) CSR603[pBT28] (*katG*$^+$); (F) CSR603[pBT29] (*katG*-23); (G) CSR603[pBT30] (*katG*-24); (H) CSR603(pBT24] (*katG*-22). The 84-kDa band is the HPI subunit. The origin of the 45-kDa band is unknown. The bands at 32 kDa are the two bands of β-lactamase.

vector pKK232-8 (Brosius, 1984) which contains a Cm^R (*cat*) gene preceded by a Shine–Dalgarno sequence and a multiple cloning site but no promoter. If an appropriate promoter is inserted into the multiple cloning site, it will direct the transcription of the Cm^R gene conferring Cm resistance to the host. A *Bgl*II digest of pBT22 was mixed with *Bam*HI-cut pKK232-8 and treated with ligase. The mixture was used to transform UM228 and the resulting Ap^R colonies were streaked onto plates with increasing concentrations of Cm. One clone, resistant to more than 60 μg Cm/ml was chosen and the plasmid, labelled pGprml, was isolated (Fig. 1d). Cleavage of pGprml with *Sma*I + *Sal*I in the multiple cloning site on either side of the insert produced a fragment of approx. 330 bp. This is slightly larger than the original *Bgl*II fragment but because the hybrid *Bam*HI/*Bgl*II site could not be cut directly, the *Sma*I + *Sal*I cleavage resulted in small segments of the multiple cloning site being attached. Retransformation of UM228 with pGprml produced clones resistant to more than 60 μg Cm/ml indicating that the 320-bp fragment contained a promoter that efficiently directed the transcription of the Cm^R gene. Restriction nuclease mapping of pGprml confirmed that the *Bgl*II fragment was inserted in the same orientation relative to the Cm^R gene as to the catalase gene (Fig. 1d).

(e) Gene *katG* encodes HPI

The evidence suggesting that HPI was encoded by *katG* was limited to the observation that mutations or transposon insertions in *katG* affected HPI synthesis. Other interpretations including *katG* as a regulatory element affecting HPI synthesis were also possible and it was desirable to confirm that *katG* was the structural gene for HPI. The strain UM202 was selected as a catalase-deficient strain following transposon Tn*10* mutagenesis and subsequent mapping localized the Tn*10* insert in *katG* (Loewen et al., 1985). Genomic DNAs from UM202 and its *kat*$^+$ parent MP180 were digested with *Hinc*II and the mixtures were separated by electrophoresis on 1% agarose gel. A Southern blot of the gel was hybridized with ^{32}P-labelled pBT22 and, as shown in Fig. 4, there was a significant change in the hybridization pattern caused by the Tn*10* insertion. The large 2.1-kb fragment in the MP180 digest was re-

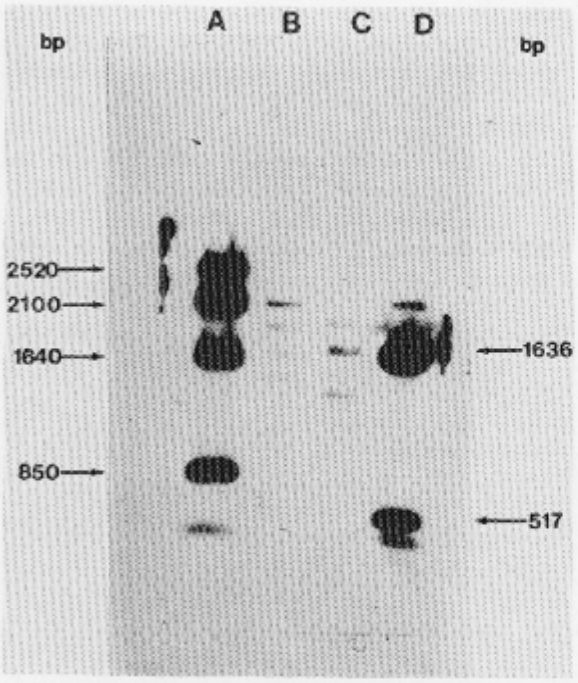

Fig. 4. Autoradiogram of the change in hybridization pattern when genomic DNA with or without a transposon Tn*10* insertion in *katG* was digested with *Hin*cII and probed with ^{32}P-labelled pBT22. The following samples were electrophoresed on a 0.8% agarose gel at 80 V for 3 h: A, pBT22 digested with *Hin*cII; B, MP180 genomic DNA digested with *Hin*cII; C, UM202 (*katG*::Tn*10*) genomic DNA digested with *Hin*cII; D, M_r standard containing pBR322 fragments created by *Hin*fI digestion which hybridized with the vector portion of pBT22. The numbers along the sides indicate the sizes in bp of the known fragments.

placed in the UM202 digest by 1.6-kb and 1.1-kb fragments composed of portions of the original fragment and approx. 390 bp (in each new fragment) of transposon DNA. This change in hybridization pattern confirmed that the Tn*10*, previously mapped in *katG*, was in the gene encoding HPI leading to the conclusion that *katG* encodes HPI. The change in pattern also allowed a definition of the location of the Tn*10* as being between 1.6 and 2.1 kb on the pBT22 map.

(f) Conclusions

(1) The *E. coli* gene encoding catalase HPI was located on a 3.8-kb fragment in the plasmid pLC36-19 and cloned into pAT153.

(2) The length of DNA required to encode active catalase was shown to be approx. 2.5 kb corresponding closely to the length predicted as being necessary to encode an 84-kDa protein.

(3) The promoter was shown to be at the 0-kb end of pBT22 and was isolated on a 320-bp fragment in a promoter expression vector.

(4) The equivalence of the catalase gene on pBT22 and *katG* was confirmed.

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