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Genetics

Exonuclease III and the catalase hydroperoxidase II in *Escherichia coli* are both regulated by the *katF* gene product

(near-ultraviolet radiation/hydrogen peroxide/oxidative stress/superoxide dismutase)

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ABSTRACT **The levels of both exonuclease III (exo III, product of *xthA*) and hydroperoxidase II (HP-II, product of *katE*) activity in *Escherichia coli* were influenced by a functional *katF* gene. The *katF* gene product is also necessary for synthesis of HP-II. Mutations in either *katF* or *xthA*, but not *katE*, result in sensitivity to H_2O_2 and near-UV (300–400 nm) radiation. Exo III, encoded by the *xthA* locus, recognizes and removes nucleoside 5′-monophosphates near apurinic and apyrimidinic sites in damaged DNA. Extracts of *katF* mutant strains had little detectable exo III activity. When a *katF*$^+$ plasmid was introduced into the *katF* mutant, exo III activity exceeded wild-type levels. We propose that the *katF* gene is a trans-acting positive regulator of exo III and HP-II enzymes, both of which are involved in cellular recovery from oxidative damage.**

A variety of external stresses exert deleterious effects on bacterial and eukaryotic cells through the generation of intracellular active oxygen species. Oxidative stress via near-ultraviolet radiation (NUV) damages cells, at least in part, by single-strand breaks in DNA (1). In *Escherichia coli* these single-strand breaks result from direct damage by hydrogen peroxide (H_2O_2) (2). H_2O_2 has been shown to be a photoproduct of NUV irradiation, as well as being a product of superoxide dismutase-mediated catalysis of the superoxide anion (O_2^-) (3). However, accumulation of H_2O_2 is prevented from reaching high levels in the cell by the action of catalase.

The two species of hydroperoxidase (HP-I and HP-II) in *E. coli* differ in activity and induction; HP-I, the product of the *katG* gene, is a tetrameric bifunctional catalase and *o*-dianiside peroxidase with a molecular mass of 337 kDa. The *katG* locus is H_2O_2 inducible and has a low K_m for H_2O_2 (4, 5). Synthesis of HP-I has been shown to be under the positive control of the *oxyR* regulon (6). In contrast, HP-II is a hexameric monofunctional catalase, possessing a high K_m for H_2O_2. HP-II synthesis is controlled by the phase of growth and is maximal during late exponential phase. Expression of HP-II requires both functional *katE* and *katF* genes (7, 8) and is expressed both aerobically and anaerobically (9).

E. coli catalase mutants differ with respect to their sensitivities to NUV. Lesions in the *katF* gene, but not the *katE* gene, result in sensitivity to broad spectrum NUV as well as to H_2O_2 (10). Thus, HP-II is not essential for a cell to recover from NUV damage. The *katE* and *katF* genes have been cloned and their products were characterized (11). *katE* encodes a 93-kDa protein, the same size as the subunit of HP-II, and thus is implicated as the HP-II structural gene. *katF* encodes a 44-kDa protein that is required for the production of HP-II and must also enable the synthesis of some other protein that confers protection from NUV-mediated killing.

Another locus involved in repair of NUV damage is the *xthA* gene, which encodes exonuclease III (exo III). Mutations in *xthA* also result in significantly enhanced sensitivity to NUV-mediated inactivation. Exo III recognizes apurinic and apyrimidinic sites in damaged DNA and catalyzes the endonucleolytic hydrolysis of 3′-terminal phosphomonoesters and also releases 5′-mononucleotides from the 3′ ends of DNA strands at such sites (12). Exo III is also a 3′ to 5′ exonuclease specific for bihelical DNA and removes any remaining blocking groups from the DNA 3′ termini to activate the DNA for subsequent synthesis by DNA polymerase I (*polA*) (13, 14).

Because of the similar sensitivity of *katF* and *xthA* mutants to NUV and to H_2O_2, we questioned whether there was any relationship between these two genes; in particular, we studied the influence of the *katF* genetic locus on the level of exo III activity in cells, as assayed in extracts with radiolabeled DNA as a substrate. In experiments presented in this report, we show that a transposon insertion (Tn*10*) into the *katF* gene eliminates exo III activity and that increasing the gene dosage of *katF* by plasmid transformation results in an increase in exo III activity.

Furthermore, a functional *katF* gene is necessary to promote synthesis of active exo III, since the enzyme is not expressed when a plasmid carrying *xthA*$^+$ is introduced into a *katF*$^-$ cell.

MATERIALS AND METHODS

Enzymes and Chemicals. Modified T7 DNA polymerase deficient in 3′ to 5′ exonuclease activity, M13 universal primer, and deoxyribonucleoside triphosphates were obtained from United States Biochemical; adenosine-5′-[α-[^{35}S]thio]triphosphate (dATP[α-^{35}S])/(1000 Ci/mmol; 1 Ci = 37 GBq) was from NEN. Salmon DNA, tetracycline, and ampicillin were from Sigma. LB contained (per liter) 10 g of tryptone (Difco), 5 g of yeast extract, and 5 g of NaCl (15). When present, ampicillin was used at 50 μg/ml and tetracycline was used at 15 μg/ml.

Strains and Plasmids. The *E. coli* strains used are described in Table 1. Extraction of plasmid DNA, plasmid transformation, and P1-mediated transduction of the transposon Tn*10* have been described (15–17). Selection in each case was based on antibiotic resistance, and resistant colonies were further screened for specific enzyme activities.

Exo III Assays. The assay for exonuclease activity in whole-cell extracts of *E. coli* was that described by Rogers and Weiss (12), with the exception that ^{35}S-labeled M13 DNA was used in place of ^{3}H-labeled T7 DNA. In principle, the

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Abbreviations: NUV, near-ultraviolet; HP-I and -II, hydroperoxidases I and II; exo III, exonuclease III; dATP[α-^{35}S], adenosine-5′-[α-[^{35}S]thio]triphosphate.

Table 1. Strains used in this study

Strain	Relevant genotype	Source or ref.
0797	MP180, wild type	P. Loewen
0568	as 0797, but *katF*::Tn*10*	8
0567	as 0797, but *katE*::Tn*10*	8
0798	as 0797, but *katF*::Tn*10* pMM*katF3*	11
0799	as 0797 + pMM*katF3*	11
0735	BW295, *xthA*	B. Weiss
0427	BW9101, Δ*xthA-pncA*	21
0786	as 0735, but *katF*::Tn*10*	P1(0568) × 0735 → Tc^R
0439	p*xthA* (pSGR3)	12
0818	as 0735 + pMM*katF3*	p(0799) × 0735 → Ap^R
0820	as 0568 + pSGR3	p(0439) × 0568 → Ap^R
0838	AB3027, *xthA*, *polA*	Y. Kow

Tc^R, tetracycline resistance; Ap^R, ampicillin resistance.

assay measures the release of acid-soluble radioactive material (mononucleotides) from filamentous phage M13mp8 DNA made double stranded with DNA polymerase I. Labeled DNA was mixed with unlabeled salmon DNA in each assay. The substrate DNA was sheared with a cell disruptor (model W-375, Heat Systems, Plainview, NY) to increase the concentration of 3′-hydroxyl termini. Crude *E. coli* extracts were incubated with the substrate solutions for 30 min at 37°C and then the tube was placed in an ice bath. To stop the reaction, chilled sheared salmon sperm DNA and chilled 10% (vol/vol) trichloroacetic acid were added in succession and then microcentrifuged at 17,000 × *g* for 5 min. A 0.5-ml sample of the supernatant fluid was removed and added to Aquasol (NEN) liquid scintillation fluid for the determination of radioactivity. When no cell extract was added to the reaction mixture <6% of the counts were acid soluble; presumably these are due to incomplete removal of unincorporated free nucleotides or radiolysis. Trichloroacetic acid-insoluble radioactivity in the absence of added cell extract was subtracted from total counts in the calculation of enzyme activity. One unit of exonuclease activity was defined as the amount causing the release of 1 nmol of acid-soluble ^{35}S-labeled nucleotides in 30 min at 37°C. The assay has been described as being 85–90% specific for DNase activity due to exo III. Assays are linear up to ≈40% hydrolysis (12).

Preparation of Labeled M13 DNA. The M13 oligonucleotide universal primer was hybridized to single-stranded M13mp8 DNA by slow cooling from 65°C. Synthesis of the complementary strand was carried out in the presence of Sequenase DNA polymerase and dATP[α-^{35}S] (NEN) such that one of the strands is uniformly radioactive at adenine residues. The DNA was purified by extractions with phenol and chloroform followed by precipitation with ethanol. Free nucleotides were then separated from DNA with four successive washings of the DNA pellet with 85% ethanol. The specific activity of the DNA was ≈75,000 cpm/nmol.

Growth Conditions. Overnight cultures were grown in LB medium and subcultured 1:50 the next day into the same medium in culture flasks at 200 rpm at 37°C. Growth was continued to late exponential phase to an OD_{600} of 1.1–1.3, and cultures were stored on ice before use.

Preparation of Cell Extracts. Culture samples were harvested by centrifugation, resuspended in 50 mM Tris·HCl (pH 8.0), and washed twice in this buffer. The cells were ruptured by subjecting the suspensions to sonication with the cell disruptor (4 times for 15 s each at 150 W) and centrifuged at 17,000 × *g* to remove cell debris. Cell extracts were stored on ice. Protein concentration in cell extracts was measured as described by Bradford (18) with bovine serum albumin used as the standard.

Visualization of Catalase on Nondenaturing Polyacrylamide Gels. Catalase isozymes were resolved and identified by applying crude extract containing 15 μg of protein on 9.5% polyacrylamide minigels (model Mini Protean II, Bio-Rad) as described (19), except that the resolving buffer was pH 8.1. The activity stain to visualize catalase was that of Harris and Hopkinson (20).

NUV Survival Curves. For inactivation studies, exponential-phase cells were prepared and exposed to NUV as described (1).

RESULTS

Exonuclease Assays. To test the specificity of the assay for exo III, we compared an *xthA* mutant strain and a strain carrying a plasmid with a 3-kilobase fragment containing $xthA^+$ under partial repression by the *cI857* gene of bacteriophage λ encoded on the same plasmid (12). The results demonstrated that the exonuclease activity of the plasmid-bearing strain 0439, when induced to overproduction of plasmid-borne exo III by growth at 42°C, was 9-fold higher than in the *xthA* mutants (both strains 0427 and 0735) (Table 2). Remaining exonuclease activity in the *xthA* mutant strains was determined by dividing acid-soluble radioactivity remaining after incubation with *xthA* mutant cell extracts by acid-soluble radioactivity remaining after incubation with buffer containing no added enzyme, and it was found to be ≈14%. This was determined to be due to the exonuclease activity of DNA polymerase I, as an *xthA polA* double mutant showed no detectable exonuclease activity (Fig. 1).

Absence of Exo III Activity in *katF* Mutants. *E. coli katF* mutants containing a Tn*10* insertion demonstrated a level of exonuclease activity that was comparable to the *xthA* mutant. The data shown represent the average of 10 separate experiments, and in no instance did the *katF* mutant show exonuclease activity >10% above that of the *xthA* mutant (Fig. 1). We questioned whether a plasmid containing the $xthA^+$ gene could express exonuclease activity in a cell in which the *katF* gene product was absent. To answer this question, we introduced the $xthA^+$ plasmid from strain 0439 into the *katF*::Tn*10* insertion mutant strain (0568) and assayed exo III activity. No exonuclease activity was detected in our assays. Indeed, levels of exonuclease activity in this strain (0820) were slightly less than that of the *xthA* mutant (Fig. 1).

Effect of *katF* Plasmid. A plasmid containing the $katF^+$ gene was introduced into the *katF* mutant strain to test whether the plasmid could restore the exonuclease activity of the *katF* mutant. The success of the transformation was confirmed by the acquisition of ampicillin resistance as well as the restoration of catalase HP-II activity, as shown on activity-stained gels (Fig. 2, lane 4). The levels of exonuclease activity in the *katF* mutant containing the $katF^+$ plasmid were found to be 3-fold higher than that of the *katF* mutant and approximated those of the wild-type strain. Significantly, further increases in exonuclease activity resulted when the same plasmid was transformed into the wild-type parent strain of the *katF* mutant (MP180). The exonuclease activity of this strain was 6-fold higher than the isogenic wild-type strain (Fig. 1). Parallel results were obtained with HP-II activity in which

Table 2. Specificity of exo III assay

Strain	Relevant genotype	Exo III activity, units/mg
0427	Δ*xthA-pncA*	5.6
0439	pSGR3 (p*xthA*)	52.4

Strains 0427 (Δ*xthA-pncA*) and 0439 (pSGR3). Strain 0439 was grown at 32°C, and then the temperature-sensitive repressor encoded by the plasmid was thermally induced by growth at 42°C for 15 min prior to harvesting, resulting in high expression of plasmid-encoded exo III.

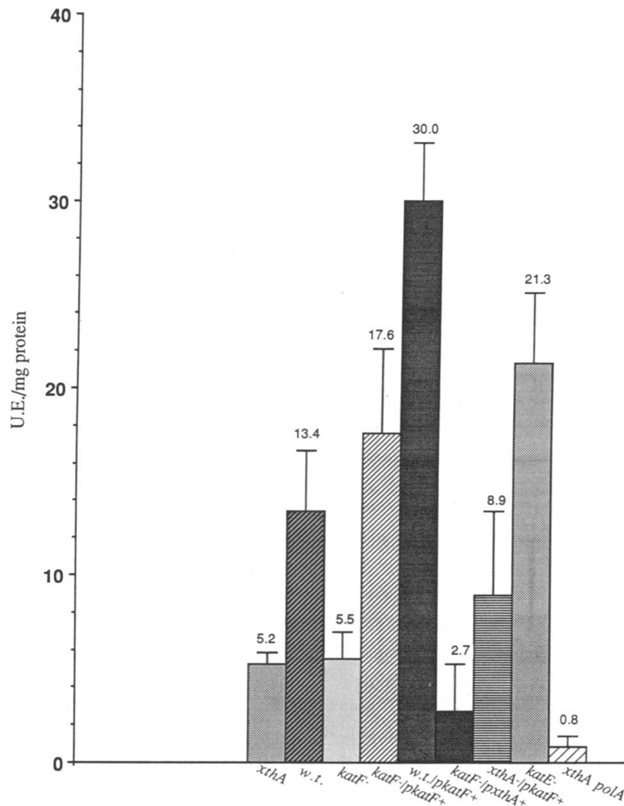

FIG. 1. Influence of *katF* on exo III activity. Double-stranded dATP[^{35}S]-labeled M13 phage DNA (10 nmol) was incubated at 37°C with 100 μg of *E. coli* sonic lysates. Shown are units of exonuclease per mg of extract. One unit of exonuclease activity (U.E.) is defined as the amount causing release of 1 nmol of acid-soluble nucleotides in 30 min at 37°C. The strains are, from left to right: 0735, 0797, 0568, 0798, 0799, 0820, 0818, 0567, and 0838.

slight increases in HP-II activity were found to occur in a wild-type strain transformed with a $katF^+$ plasmid (Fig. 2, lane 5).

Absence of Exonuclease Activity Encoded by the *katF* Plasmid. In each of our exo III assays of *katF* plasmid-bearing strains, we noted high levels of exo III activity. To preclude the possibility that the $katF^+$ plasmid itself was contributing the exo III activity, we introduced the $katF^+$ plasmid into a *xthA* background. The resulting *katF* plasmid strain showed a slight increase in exo III activity over the parent *xthA* strain (Fig. 1). However, the increase did not approach the levels of the *katF* plasmid strains with a wild-type background. We conclude that the *katF* plasmid itself was not responsible for the increases in exo III activity, but rather influenced a chromosomal gene to increase exonuclease synthesis.

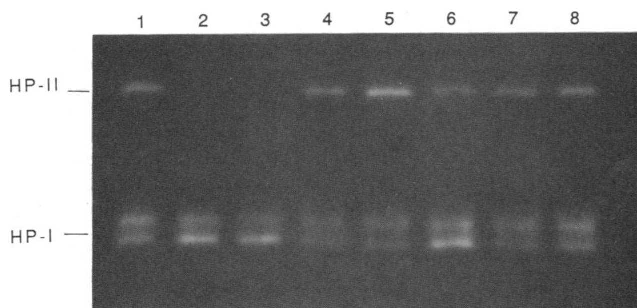

FIG. 2. Native polyacrylamide gel stained for catalase activity. Lanes: 1, 0797; 2, 0567; 3, 0568; 4, 0798; 5, 0799; 6, 0735; 7, 0439; 8, 0797.

Effect of Growth Phase on Exo III Activity. It has been shown that *katE* expression (9) and HP-II activity (22, 23) change with phase of growth and reach a maximum in late exponential phase. We examined exonuclease activity at different phases of growth to compare with HP-II. Interestingly, we found that exo III activity increased throughout the exponential phase and then declined in early stationary phase (Fig. 3). The observation that the activities of *katE* and *xthA* gene products change in parallel throughout growth phase lends support to coregulation by the *katF* gene product.

NUV Sensitivity of *katF* and *xthA* Mutants. Since it has been shown that both *xthA* and *katF* mutants are sensitive to H_2O_2 and to broad-spectrum NUV (refs. 24–26; this study), we compared the kinetics of inactivation to NUV of these two mutants, as well as the sensitivity of a strain possessing both mutations. Interestingly, all three strains were inactivated at the same rate. Furthermore, we found that the $katF^+$ plasmid in a wild-type background was able to confer increased NUV tolerance (Fig. 4). The *katF xthA* double mutant strain is no more sensitive than either of the single mutants *xthA* or *katF* alone.

DISCUSSION

Prior to this study, the function of the *katF* locus was known only to be associated with HP-II production. A regulatory role of *katF* in HP-II synthesis has been proposed (7, 10) and is supported by the *katE*::*lacZ* fusion study of Schellhorn and Hassan (9), which shows that *katE* expression is eliminated by a *katF*::Tn*10* insertion. The finding that *katE* expression parallels HP-II synthesis and that the gene product is the size of a HP-II subunit supports a structural role for *katE* (11). An alternative possibility is that each locus could encode a single subunit, which must associate together to form an active tetrameric catalase; however, no evidence has been presented to support this view. The striking sensitivity of the *katF* mutant to NUV (Fig. 4) and H_2O_2 inactivation (26), while the *katE* mutant is not sensitive, points to the conclusion that HP-II is not required for recovery from NUV damage and suggested to us an additional role for *katF*. Our finding in this study that *katF* is required for *xthA* expression fits our hypothesis, since *xthA* mutants are also sensitive to NUV and H_2O_2 inactivation. Furthermore, our observations that a *katF xthA* double mutant shows the same sensitivity to NUV as either mutant alone, and also that a *katF* plasmid introduced into a $katF^-$ strain complemented the *katF*

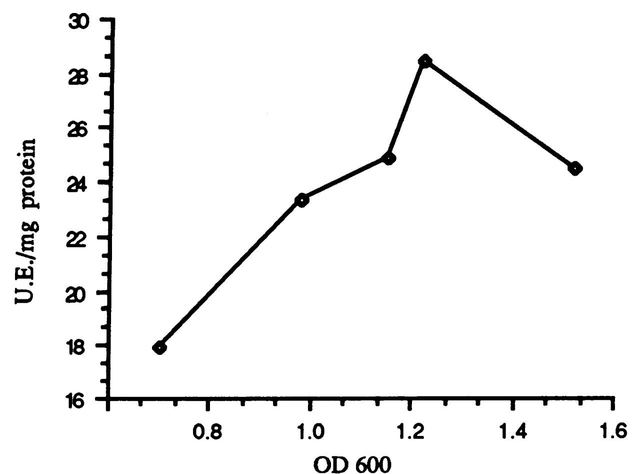

FIG. 3. Effect of growth phase on exo III activity. Sonic lysates (0.1 mg) from 0797 (wild type) were assayed for exo III activity at points throughout exponential growth. U.E., units of exonuclease activity.

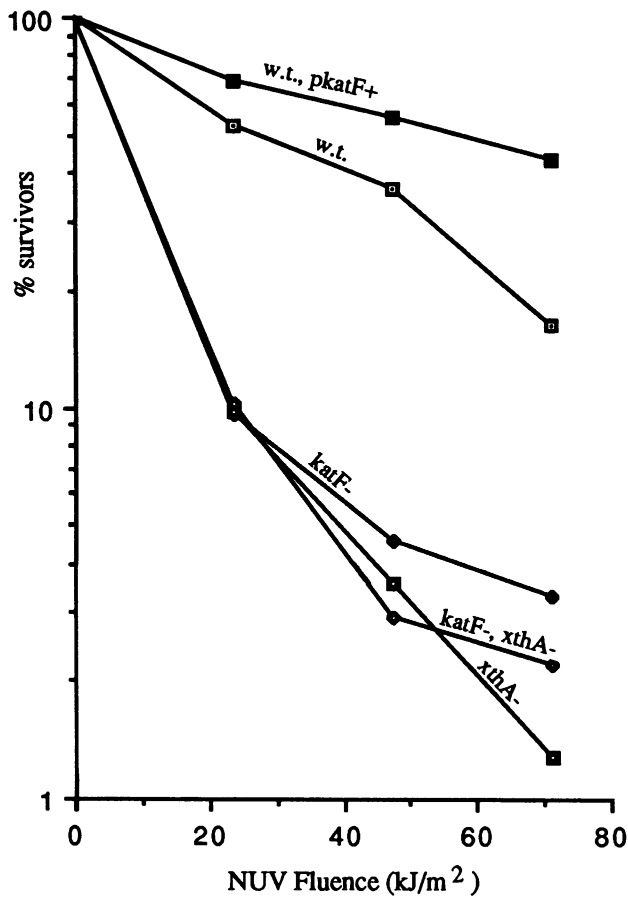

FIG. 4. NUV irradiation survival curves. Each of the strains was grown to OD_{600} = 0.3 in LB, diluted 1:100 in M9, and irradiated. Appropriate dilutions were made into 3 ml of soft agar and incubated 24 hr at 37°C, and then survivors were counted.

mutation both in terms of HP-II and exo III activities, all suggest that both *katE* and *xthA* are regulated by *katF*. This finding offers an explanation for the NUV and H_2O_2 sensitivities of the *katF* mutant in terms of its lack of exo III-mediated repair of damage to DNA.

The proximity of the *xthA* and *katE* genes on the bacterial chromosome (Fig. 5; refs. 8 and 21) as well as their regulation by the same gene product raises the question of a common transcriptional regulation. Indeed, some observations could support the view that these genes belong to the same operon. Transcriptional regulation of the *katE* gene has been described (9), and we found in this study that exo III activity changes in parallel with the HP-II catalase activity throughout the growth cycle. Furthermore, the *xthA* point mutant (0735) shows both a loss of exo III and a slight decrease in HP-II activities, whereas a *katE* mutation (Tn*10* insertion) retained exo III activity (Figs. 1 and 2). Although it is possible that 0735 could be a double *xthA katE* mutant, it is more likely that such phenotypes could be explained by a polar mutation in an operon transcribed from *xthA* to *katE*. Recently, Saporito *et al.* (27) have published the nucleotide sequence of the *xthA* gene. The direction of transcription of *xthA* deduced

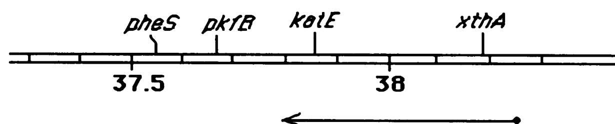

FIG. 5. Genetic map of *E. coli* chromosome region in proximity of minute 38 and possible direction of transcription [redrawn from Loewen (8) and White *et al.* (21)].

from their data, together with the observation that the pSGR3 (exo III) plasmid (12) also overproduces catalase, HP-II (Fig. 2) allows determination of *xthA* transcription direction on the *E. coli* chromosome map in relation to *katE* position (Fig. 5). This again is compatible with an operon containing both *xthA* and *katE*. From the sequence analysis and the mapping data, it is possible that the *xthA* gene might be controlled by an activator protein. This is consistent with a transcriptional activation of the *xthA* gene by the *katF* gene product. Saporito *et al.* (27) have proposed that exo III is expressed monocistronically, since they observed beyond the coding region a potential transcription terminator structure and, farther downstream, a promoter structure with a −35 and a −10 site, a Shine–Dalgarno site, and the beginning of an open reading frame. However, they also remark that the potential terminator structure would not predict a strong terminator (27). Further studies are therefore necessary to determine whether *xthA* and *katE* are transcriptionally coregulated by the *katF* gene product.

Although exo III and catalase HP-II have different cellular functions, it is likely that they are both implied in protection against oxidative stress: HP-II to eliminate the potentially damaging H_2O_2, and exo III to help repair damage brought on, in part, by reactive oxygen species-mediated DNA damage. The study of Heimberger and Eisenstark (28), which localizes HP-II exclusively to the cytoplasm, is consistent with a role of HP-II as a defense against H_2O_2-induced DNA breaks.

The *sodB* locus involved in cellular recovery from oxidative stress by producing iron-containing superoxide dismutase (FeSOD), maps near minute 37 on the *E. coli* chromosome (29); thus, it was possible that it might be part of the same operon. We tested whether mutations in *katF* affected production of FeSOD. No differences were detected between *katF* and wild-type strains in terms of their FeSOD activities (data not shown). Thus, it was evident that *katF* did not regulate *sodB*. Finally, the finding that the *xthA* mutation in a strain lacking both superoxide dismutases (*sodA sodB*) reduces the oxygen radical-mediated mutagenesis frequency below that found with a functional *xthA* in a *sodA sodB* background (30) makes it relevant to further explore the role of *katF* in mutagenesis.

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