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

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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 H2O2 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 repair from oxidative damage.

A variety of external stresses exert deleterious effects on bacterial and eukaryotic cells through the generation of intracellular reactive 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 (H2O2) (2). H2O2 has been shown to be a photoprotective of NUV irradiation, as well as being a product of superoxide dismutase-mediated catalysis of the superoxide anion (O2-) (3). However, accumulation of H2O2 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 H2O2 inducible and has a low Km for H2O2 (4, 5). Synthesis of HP-I has been shown to be under the control of the oxyR regulon (6). In contrast, HP-II is a hexameric monofunctional catalase, possessing a high Km for H2O2. HP-II synthesis is controlled by the phase of growth and is maximal during late exponential phase. Expression of HP-II includes 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 H2O2 (10). Thus, HP-I 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 biblical 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 H2O2, 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 (Tn10) 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-35S]thiophosphate (dATP[α-35S])/(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 PI-mediated transduction of the transposon Tn10 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 exclusion that 35S-labeled M13 DNA was used in place of 3H-labeled T7 DNA. In principle, the

Abbreviations: NUV, near-ultraviolet; HP-I and -II, hydroperoxidasises I and II; exo III, exonuclease III; dATP[α-35S], adenosine-5'-[α-35S]thiophosphate.

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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 35S-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-35S] (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 OD600 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 A 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 ApR double mutant showed no detectable exonuclease activity (Fig. 1).

Absence of Exo III Activity in katF Mutants. E. coli katF mutants containing a Tn10 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::Tn10 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 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source or ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0797</td>
<td>MP180, wild type</td>
<td>P. Loewen</td>
</tr>
<tr>
<td>0568</td>
<td>as 0797, but katF::Tn10</td>
<td>8</td>
</tr>
<tr>
<td>0797</td>
<td>as 0797, but katF::Tn10</td>
<td>8</td>
</tr>
<tr>
<td>0798</td>
<td>as 0797, but katF::Tn10</td>
<td>11</td>
</tr>
<tr>
<td>pMMkatF3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0799</td>
<td>as 0797 + pMMkatF3</td>
<td>11</td>
</tr>
<tr>
<td>0735</td>
<td>BW295, xthA</td>
<td>B. Weiss</td>
</tr>
<tr>
<td>0427</td>
<td>BW9101, ΔxthA-panA</td>
<td>21</td>
</tr>
<tr>
<td>0786</td>
<td>as 0735, but katF::Tn10</td>
<td>P(0568) × 0735 → TcR</td>
</tr>
<tr>
<td>0439</td>
<td>pxthA (pSGR3)</td>
<td>12</td>
</tr>
<tr>
<td>0818</td>
<td>as 0735 + pMMkatF3</td>
<td>p(0799) × 0735 → ApR</td>
</tr>
<tr>
<td>0820</td>
<td>as 0568 + pSGR3</td>
<td>p(0439) × 0568 → ApR</td>
</tr>
<tr>
<td>0838</td>
<td>AB3027, xthA, polA</td>
<td>Y. Kow</td>
</tr>
</tbody>
</table>

TcR, tetracycline resistance; ApR, ampicillin resistance.

Table 2. Specificity of exo III assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Exo III activity, units/μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0427</td>
<td>ΔxthA-panA</td>
<td>5.6</td>
</tr>
<tr>
<td>0439</td>
<td>pSGR3 (pxthA)</td>
<td>52.4</td>
</tr>
</tbody>
</table>

Strains 0427 (ΔxthA-panA) 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.
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 exonuclease assays of katF plasmid-bearing strains, we noted high levels of exonuclease activity. To preclude the possibility that the katF+ plasmid itself was contributing the exonuclease activity, we introduced the katF+ plasmid into a xthA background. The resulting katF plasmid strain showed a slight increase in exonuclease 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 exonuclease activity, but rather influenced a chromosomal gene to increase exonuclease synthesis.

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 exonuclease 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 H2O2 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::Tn10 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 H2O2 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 H2O2 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

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.
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 H2O2, 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 H2O2-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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