

# Proline Metabolism Increases *katG* Expression and Oxidative Stress Resistance in *Escherichia coli*

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The oxidation of L-proline to glutamate in Gram-negative bacteria is catalyzed by the proline utilization A (PutA) flavoenzyme, which contains proline dehydrogenase (PRODH) and  $\Delta^1$ -pyrroline-5-carboxylate (P5C) dehydrogenase domains in a single polypeptide. Previous studies have suggested that aside from providing energy, proline metabolism influences oxidative stress resistance in different organisms. To explore this potential role and the mechanism, we characterized the oxidative stress resistance of wild-type and *putA* mutant strains of *Escherichia coli*. Initial stress assays revealed that the *putA* mutant strain was significantly more sensitive to oxidative stress than the parental wild-type strain. Expression of PutA in the *putA* mutant strain restored oxidative stress resistance, confirming that depletion of PutA was responsible for the oxidative stress phenotype. Treatment of wild-type cells with proline significantly increased hydroperoxidase I (encoded by *katG*) expression and activity. Furthermore, the  $\Delta katG$  strain failed to respond to proline, indicating a critical role for hydroperoxidase I in the mechanism of proline protection. The global regulator OxyR activates the expression of *katG* along with several other genes involved in oxidative stress defense. In addition to *katG*, proline increased the expression of *grxA* (glutaredoxin 1) and *trxC* (thioredoxin 2) of the OxyR regulon, implicating OxyR in proline protection. Proline oxidative metabolism was shown to generate hydrogen peroxide, indicating that proline increases oxidative stress tolerance in *E. coli* via a preadaptive effect involving endogenous hydrogen peroxide production and enhanced catalase-peroxidase activity.

The conversion of L-proline to glutamate is a four-electron oxidation process that is coordinated in two successive steps by the enzymes proline dehydrogenase (PRODH) and  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase (P5CDH) (Fig. 1) (1). In eukaryotes, PRODH and P5CDH are separately encoded enzymes localized in the mitochondrion. In Gram-negative bacteria, PRODH and P5CDH are combined into a bifunctional enzyme known as proline utilization A (PutA) (1, 2). The PRODH domain contains a noncovalently bound flavin adenine dinucleotide (FAD) cofactor and couples the two-electron oxidation of proline to the reduction of ubiquinone in the cytoplasmic membrane (3). The product of the PRODH reaction,  $\Delta^1$ -pyrroline-5-carboxylate (P5C), is subsequently hydrolyzed to glutamate- $\gamma$ -semialdehyde (GSA), which is then oxidized to glutamate by the NAD<sup>+</sup>-dependent P5CDH domain (2). In certain Gram-negative bacteria such as *Escherichia coli*, PutA also has an N-terminal ribbon-helix-helix (RHH) DNA-binding domain (residues 1 to 47) (4). The RHH domain enables PutA to act as an autogenous transcriptional regulator of the *putA* and *putP* (high-affinity Na<sup>+</sup>-proline transporter) genes (4). PutA represses *put* gene expression by binding to five operator sites in the *put* regulatory region (5). Transcription of the *put* genes is activated by proline, which causes a reduction of the PutA flavin cofactor and subsequent localization of PutA on the membrane (5–9).

Proline has been shown to be an important carbon and nitrogen source supporting growth under various nutrient conditions for *Escherichia coli*, *Pseudomonas putida*, *Bradyrhizobium japonicum*, and *Helicobacter pylori* (10–14). In *H. pylori*, L-proline is a preferred respiratory substrate in the gut, with proline levels 10-fold higher in the gastric juice of patients infected with *H. pylori* than in noninfected individuals (10, 15). An *H. pylori putA* mutant strain was shown to be less efficient in the colonization of mice than the wild-type strain (16). A *putA* mutant strain of the closely related mouse pathogen *Helicobacter hepaticus* also exhibited less

pathogenicity in mice than the wild-type strain (17). Thus, in certain ecological niches, PutA and the proline catabolic pathway have a critical role in bacterial pathogenesis.

Besides being an important energy source, proline also provides protective benefits against abiotic and biotic stresses in a broad range of organisms (18–23). Proline is a well-known osmoprotectant (24, 25), and in *E. coli*, proline has been described as being a thermoprotectant by diminishing protein aggregation during heat stress (26). Proline has also been found to help combat oxidative stress, a property which has been explored in eukaryotes such as fungi, plants, and animals (19, 20, 27, 28). The mechanism by which proline protects against oxidative stress appears to involve protection of intracellular redox homeostasis and, in the fungal pathogen *Colletotrichum trifolii*, upregulation of catalase (27). In mammalian cells, proline protection was shown to require PRODH activity and activation of signaling cascades that promote cell survival (28).

To uncover fundamental mechanisms of proline-mediated stress protection, we explored whether proline metabolism has a role in oxidative stress resistance in *E. coli*. We provide evidence that proline enhances oxidative stress tolerance in *E. coli* and that

Received 15 September 2014 Accepted 6 November 2014

Accepted manuscript posted online 10 November 2014

Citation Zhang L, Alfano JR, Becker DF. 2015. Proline metabolism increases *katG* expression and oxidative stress resistance in *Escherichia coli*. J Bacteriol 197:431–440. doi:10.1128/JB.02282-14.

Editor: P. de Boer

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doi:10.1128/JB.02282-14

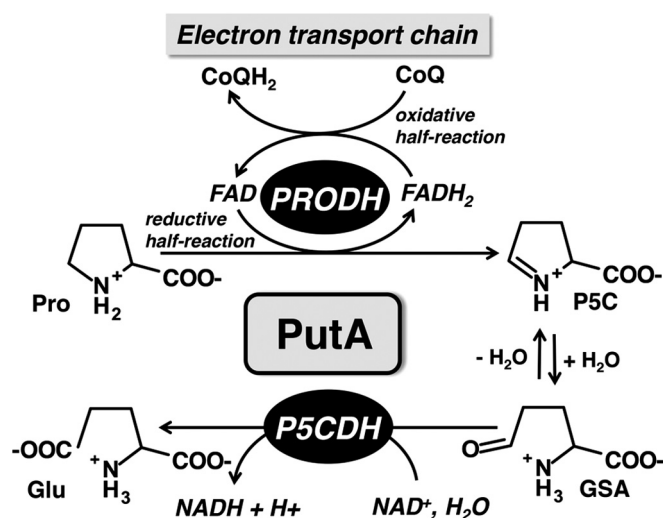


FIG 1 Reactions catalyzed by the PRODH and P5CDH domains of PutA. Reduction of ubiquinone (CoQ) in the electron transport chain is coupled to proline oxidation.

protection is dependent on the catalytic activity of PutA. Evidence supporting the involvement of hydroperoxidase I (encoded by *katG*) and the OxyR regulon, which have critical roles in oxidative stress defense, is also shown. The results suggest that proline metabolism can promote oxidative stress adaption, a feature which may facilitate pathogenesis in certain biological environments.

## MATERIALS AND METHODS

**Reagents, bacterial strains, and culture conditions.**  $\beta$ -Mercaptoethanol, *o*-aminobenzaldehyde (*o*-AB), *o*-nitrophenyl- $\beta$ -D-galactopyranoside, L-proline, and L-tetrahydro-2-furoic acid (L-THFA) were purchased from Sigma. All other chemicals were purchased from Thermo Fisher unless noted otherwise. The PutA-pUC18 plasmid was described previously (5). *E. coli* strains used in this study are listed in Table 1. The MG1655  $\Delta$ putA strain was generated in this work by P1 transduction of the MG1655 wild-type strain (29). *E. coli* cultures were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) or glucose minimal medium (0.5 g glucose, 0.1 g thiamine, 1 mM MgSO<sub>4</sub>, 0.5 g NaCl, 1 g NH<sub>4</sub>Cl, 3 g KH<sub>2</sub>PO<sub>4</sub>, and 6 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O per liter). Ampicillin and kanamycin were used as needed at 50  $\mu$ g/ml. Cultures were grown at 37°C with shaking at 225 rpm. To perform measurements upon exponentially growing cells, cultures grown overnight were diluted 1,000-fold with fresh medium and grown to mid-logarithmic phase, which corresponds to an optical density at 600 nm (OD<sub>600</sub>) of 0.3.

**Disk assay for oxidative stress sensitivity.** Cells were grown in LB broth to an OD<sub>600</sub> of 0.3. Aliquots (0.5 ml) from each culture were then mixed with 4.5 ml cooled-down soft agar (glucose minimal medium, 0.8% agar, and no antibiotics) and then poured immediately onto LB plates (no antibiotics). After the soft agar solidified, a round filter paper (0.8-cm diameter) saturated with 10  $\mu$ l of 6.6 M H<sub>2</sub>O<sub>2</sub> was placed in the center of the plate. Plates were then incubated at 37°C overnight. The inhibitory zone diameter was measured from three different directions to calculate a mean value for the diameter of the inhibition zone.

**Cell counting assay.** Cells were grown in glucose minimal medium to an OD<sub>600</sub> of 0.3 with (or without) 10 mM L-proline. Cells were then collected and diluted to an OD<sub>600</sub> of 0.1 with fresh medium prior to a 30-min treatment with 5 mM H<sub>2</sub>O<sub>2</sub>. After serial dilution, cells were spread onto LB plates and allowed to grow overnight at 37°C. Cell survival rates were calculated as CFU of H<sub>2</sub>O<sub>2</sub>-treated cells divided by that of untreated cells.

**$\beta$ -Galactosidase activity.** To measure the effect of proline oxidation on *katG* expression, AL441 cells were grown in glucose minimal medium to an OD<sub>600</sub> of 0.3 before treatment with 10 mM L-proline and L-THFA. Samples were collected at designated time points, and  $\beta$ -galactosidase activities were measured as described previously (30). To determine the effect of H<sub>2</sub>O<sub>2</sub> on *katG* expression, cells were cultured as described above and then treated with different concentrations of H<sub>2</sub>O<sub>2</sub> for 30 min, followed by measurement of  $\beta$ -galactosidase activity.  $\beta$ -Galactosidase activity assays were performed as previously described (30) and are reported in Miller units (29).

**Catalase activity.** MG1655 wild-type and  $\Delta$ putA cells were grown in glucose minimal medium to an OD<sub>600</sub> of 0.3 with (or without) 10 mM L-proline. Cells were then collected, centrifuged, and lysed with bacterial extraction reagent (Pierce). Cell debris was removed by centrifugation, and catalase activity in the supernatant was measured with 20  $\mu$ M H<sub>2</sub>O<sub>2</sub> by an Amplex Red catalase assay kit (Life Technology), using a newly prepared H<sub>2</sub>O<sub>2</sub> standard curve according to the manufacturer's protocols, and measuring the absorbance at 568 nm with a Powerwave XS microplate reader. Protein concentrations were determined with a 660-nm protein assay (Pierce). One unit of catalase activity is defined as the decomposition of 1.0  $\mu$ mol H<sub>2</sub>O<sub>2</sub> per min at pH 7.0 at 25°C.

**H<sub>2</sub>O<sub>2</sub> clearance assay.** To determine the effect of proline oxidation on H<sub>2</sub>O<sub>2</sub> scavenging, MG1655 wild-type and  $\Delta$ putA cells were grown in glucose minimal medium to an OD<sub>600</sub> of 0.3 with (or without) 10 mM L-proline. Cells were then pelleted, washed, and resuspended in phosphate-buffered saline (PBS) to an OD<sub>600</sub> of 0.1. After the addition of 5  $\mu$ M H<sub>2</sub>O<sub>2</sub>, 0.45-ml aliquots were removed at different time intervals, and H<sub>2</sub>O<sub>2</sub> was measured immediately by an Amplex Red hydrogen peroxide-peroxidase assay kit (Life Technology) at room temperature, as previously described (31). Fluorescence measurements were made with an Agilent (Varian) Cary Eclipse fluorescence spectrophotometer with excitation at 545 nm and fluorescence emission monitoring at 590 nm.

**Real-time PCR.** MG1655 wild-type and  $\Delta$ putA cells were grown in glucose minimal medium to an OD<sub>600</sub> of 0.3. At time zero (0 min), 10 mM L-proline was added to the cultures. Immediately afterwards, a sample of 0.5 ml was taken and mixed with 1 ml of RNeasy Protect Bacteria reagent (Qiagen) for the time zero point. Additional samples were then withdrawn from the cultures at different time points. RNA was extracted by using an RNeasy minikit (Qiagen) according to the manufacturer's protocol. Genomic DNA was removed from the RNA preparations with RNase-free DNase I (Fermentas). The cDNA was synthesized by using the RevertAid First Strand cDNA synthesis kit (Fermentas) with 100 ng of template RNA and random hexamer primers. The following primers were used to analyze the expression of *katG*, *grxA*, *trxC*, and 16S rRNA: *katG*

TABLE 1 Strains used in this study

<i>E. coli</i> K-12 strain	Relevant genotype	Reference
MG1655	Wild type (F <sup>-</sup> $\Delta$ $\lambda$ <i>rph-1</i> )	65
MG1655 $\Delta$ putA	MG1655 plus $\Delta$ putA758::kan	This work
AL441	MG1655 plus $\Delta$ ( <i>lacZ1::cat</i> )1 att $\lambda$ ::[pSJ501::katG <sup>+</sup> - <i>lacZ</i> <sup>+</sup> cat <sup>+</sup> ]	66
CSH4	Wild type [F <sup>-</sup> <i>lacZ</i> 1125 $\lambda$ <i>trpA</i> 49(Am) <i>relA1</i> <i>rpsL</i> 150(Str <sup>r</sup> ) <i>spoT</i> 1]	14
JT31	CSH4 plus <i>putA1</i> ::Tn5	14
JT34	CSH4 plus <i>putP3</i> ::Tn5	14
BW25113	Wild type [F <sup>-</sup> $\Delta$ ( <i>araD</i> - <i>araB</i> )567 $\Delta$ <i>lacZ</i> 4787(::rrnB-3) $\lambda$ <i>rph-1</i> $\Delta$ ( <i>rhaD</i> - <i>rhaB</i> )568 <i>hsdR</i> 514]	42
JW0999	BW25113 plus $\Delta$ putA758::kan	42
JW1721	BW25113 plus $\Delta$ katE731::kan	42
JW3914	BW25113 plus $\Delta$ katG729::kan	42
JW3933	BW25113 plus $\Delta$ oxyR749::kan	42
JW4024	BW25113 plus $\Delta$ soxR757::kan	42

forward primer 5'-AATCCAGTTCGAAGCGGTAG-3', *katG* reverse primer 5'-CACCAGCATTGT CGGTTTAC-3', *grxA* forward primer 5'-GATCTGGCTGAGAAATTGAG-3', *grxA* reverse primer 5'-GTTTACCT GCCTTTTGTGT-3', *trxC* forward primer 5'-AATACCGTTTGATC CCAT TG-3', *trxC* reverse primer 5'-GCTTCGGTATTCACCTTCAC-3', 16S rRNA forward primer 5'-GGATGATCAGCCACACTGGA-3', and 16S rRNA reverse primer 5'-CCAAT ATTCCTCACTGCTGCC-3'. The 20- $\mu$ l real-time PCR mixture contained 10  $\mu$ l SsoFast EvaGreen Supermix (Bio-Rad), 300 nM primers, and 50 ng cDNA. Thermal cycling was performed by using an iCycler iQ instrument (Bio-Rad) for 40 cycles in 3 steps: 95°C for 15 s, 58°C for 30 s, and 65°C for 60 s. Relative mRNA levels were calculated by using the  $2^{-\Delta\Delta CT}$  method and by using 16S rRNA as the internal control. PCR products were also analyzed by agarose gel electrophoresis to confirm product size and specificity.

**PutA Western blotting.** The expression of PutA was confirmed by Western blot analysis, as described previously, using an antibody against a polypeptide containing PutA residues 1 to 47 (5).

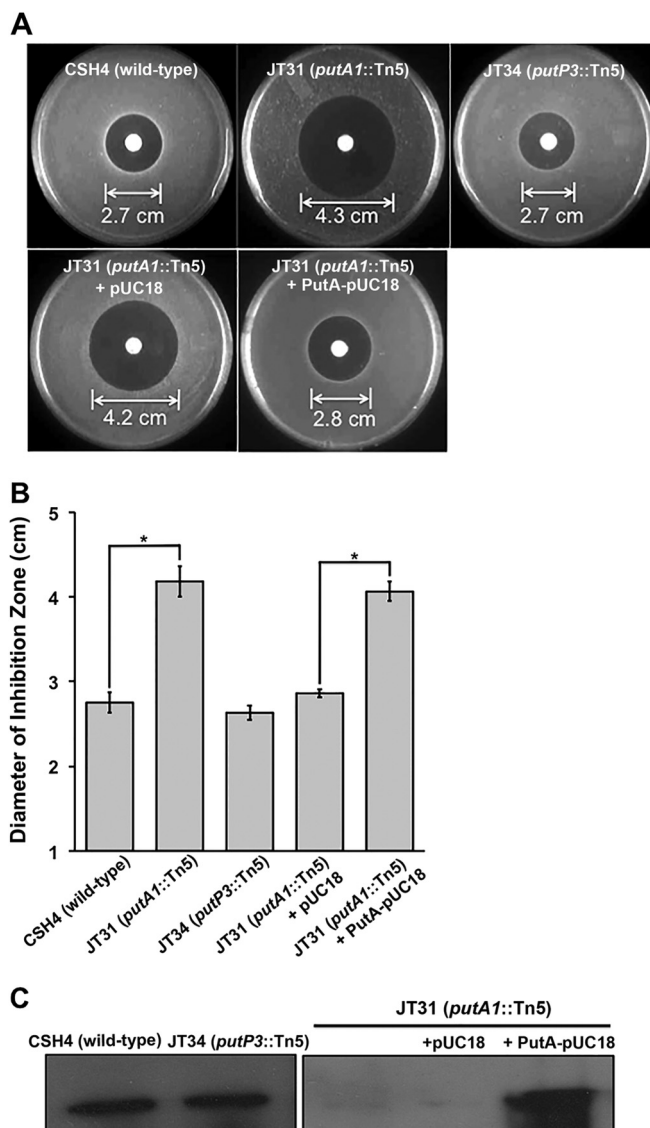
**H<sub>2</sub>O<sub>2</sub> production assays.** H<sub>2</sub>O<sub>2</sub> produced and accumulated within cells passes through membranes and equilibrates with the culture medium (32). To determine the effect of L-proline treatment on H<sub>2</sub>O<sub>2</sub> production *in vivo*, BW25113 (Keio strain collection) wild-type and  $\Delta$ *katG* (strain JW3914) cells were grown to an OD<sub>600</sub> of 0.3 in glucose minimal medium with (or without) 10 mM L-proline. Cells were then pelleted, and the H<sub>2</sub>O<sub>2</sub> content in the supernatant was measured immediately by using the Amplex Red hydrogen peroxide-peroxidase assay kit with excitation at 545 nm and fluorescence emission monitoring at 590 nm.

The kinetics of H<sub>2</sub>O<sub>2</sub> formation from proline were determined by using inverted membrane vesicles from the  $\Delta$ *katG* strain (JW3914). Inverted membrane vesicles were prepared, as described previously (33), from JW3914 ( $\Delta$ *katG*) cells grown in minimal A medium [33 mM KH<sub>2</sub>PO<sub>4</sub>, 51 mM K<sub>2</sub>HPO<sub>4</sub>, 8 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 mM MgSO<sub>4</sub>, 0.5 mM tryptophan, 10 mM L-proline, 8% glycerol, and 0.05% glucose] to an OD<sub>600</sub> of 0.7. Assays were performed at room temperature with 50  $\mu$ g/ml of membrane vesicles (or membrane protein) and 10 mM L-proline to estimate the rate of H<sub>2</sub>O<sub>2</sub> production. The assay buffer (pH 7.2) included 40 U/ml superoxide dismutase, 125 mM KCl, 4 mM KH<sub>2</sub>PO<sub>4</sub>, 14 mM NaCl, 20 mM HEPES-NaOH, 1 mM MgCl<sub>2</sub>, 0.2% bovine serum albumin (BSA), and 0.02 mM EDTA (34). H<sub>2</sub>O<sub>2</sub> was quantified by using the Amplex Red hydrogen peroxide-peroxidase assay kit with excitation at 555 nm and fluorescence emission monitoring at 581 nm, as described previously (34), with the rate defined as pmol of H<sub>2</sub>O<sub>2</sub> formed min<sup>-1</sup> mg<sup>-1</sup> of membrane protein. To determine the rate of intracellular H<sub>2</sub>O<sub>2</sub> formation, we used the relationship that 1 ml of bacteria at 1.0 OD unit comprises ~0.47  $\mu$ l of cytosolic volume (35). For these assays, 20 mg of membrane protein vesicles was isolated from 500-ml cultures grown to 0.8 OD units, which corresponds to a total cytosolic volume of ~188  $\mu$ l. The rate of intracellular H<sub>2</sub>O<sub>2</sub> formation ( $\mu$ M min<sup>-1</sup>) was calculated from the measured H<sub>2</sub>O<sub>2</sub> formation rate: (pmol min<sup>-1</sup> mg<sup>-1</sup> of membrane protein)  $\times$  [total membrane protein (20 mg)/total cytosolic volume (188  $\mu$ l)]. The effect of L-THFA on H<sub>2</sub>O<sub>2</sub> production was determined by using the same assay conditions as those described above, with the proline concentration fixed at 10 mM and various L-THFA concentrations (1 to 10 mM). Background formation of H<sub>2</sub>O<sub>2</sub> was determined in control assays without L-proline. PutA activity in the membrane vesicles was confirmed by quantifying P5C production (nmol P5C min<sup>-1</sup> mg<sup>-1</sup> of membrane protein) using o-AB, as previously described (36).

**Statistical analysis.** The reported mean values and standard deviations are from three to five experiments. Data were analyzed by Student's *t* tests, with statistical significance considered to be a *P* value of <0.05.

## RESULTS

***E. coli putA* mutants have increased oxidative stress sensitivity.** Oxidative stress disk assays were performed with wild-type *E. coli* strain CSH4 and isogenic *putA* (JT31) and *putP* (JT34) mutant strains grown in LB broth to exponential phase (Fig. 2A). The



**FIG 2** Depletion of PutA increases oxidative stress sensitivity. (A) Disk assays were performed with CSH4 (parental wild type), JT31 (*putA1::Tn5*), JT34 (*putP3::Tn5*), and JT31 transformed with the empty pUC18 vector or the pUC18 vector bearing wild-type PutA by using filter paper saturated with 10  $\mu$ l of 6.6 M H<sub>2</sub>O<sub>2</sub>. (B) Inhibition zone diameters from five replicates of the disk assays shown in panel A (\*, *P* < 0.05). (C) Western blot analysis of PutA expression in strains used for panels A and B.

inhibition zone found for the JT31 strain is almost twice the size of the zones observed for the CSH4 and JT34 strains (Fig. 2A and B). This finding indicates that JT31 cells have increased sensitivity to H<sub>2</sub>O<sub>2</sub>. The observed phenotype of the JT31 cells can be complemented by transformation with the pUC18 vector bearing wild-type PutA (Fig. 2), confirming that depletion of PutA contributes to the H<sub>2</sub>O<sub>2</sub> sensitivity of JT31 cells. Expression levels of PutA in the different strains were confirmed by Western blot analysis, as shown in Fig. 2C.

The above-described experiments were performed in LB broth, which is abundant in L-proline (9.5 mM) and contains low glucose (<0.1 mM), necessitating *E. coli* to utilize amino acids for growth (37). It was previously reported that L-proline is significantly uti-

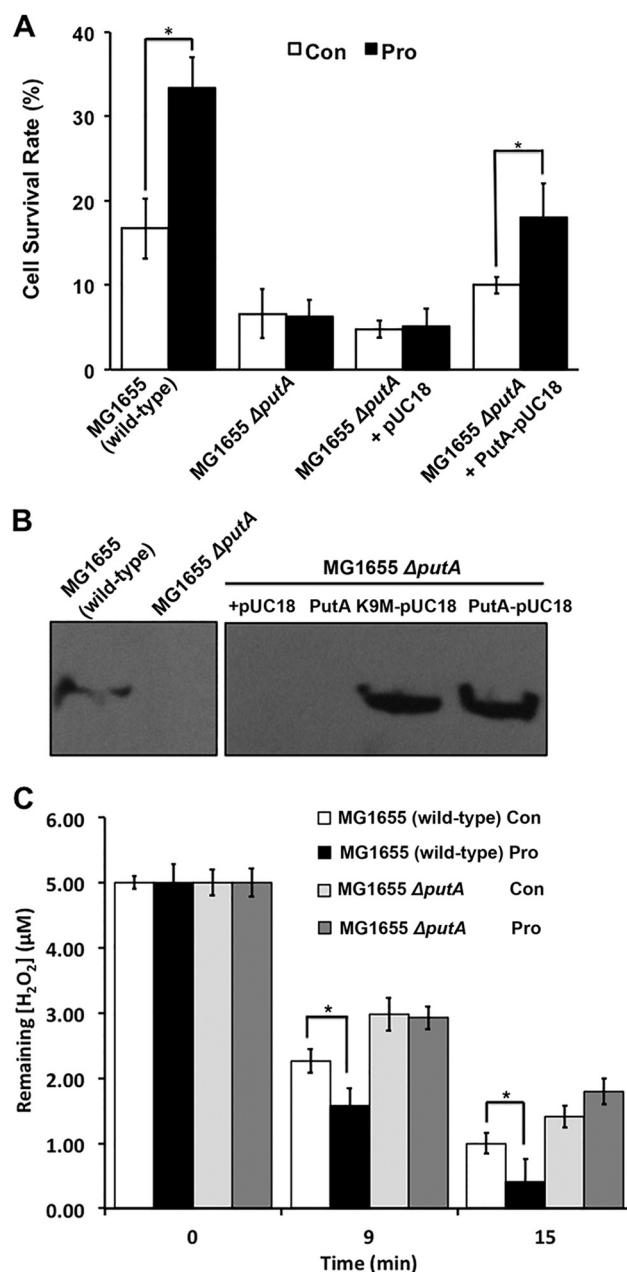


lized by *E. coli* in LB broth (37). Thus, we hypothesized that proline catabolism may account for the differences in oxidative stress resistance observed between strains CSH4 and JT31. Although the CSH4 and JT31 strains are commonly used for proline studies (4, 5, 9), the CSH4 strain contains mutations in *relA* and *spoT*, which regulate (p)ppGpp levels and are important for bacterial survival under nutrient starvation and oxidative and osmotic stress conditions (38, 39). Thus, to further evaluate the effects of proline metabolism on oxidative stress sensitivity, a  $\Delta putA$  strain of MG1655 was generated by P1 transduction (Fig. 3B). MG1655 wild-type and  $\Delta putA$  strains exhibit similar growth profiles, and proline supplementation did not affect the growth of either strain (data not shown). Figure 3A shows that proline supplementation promotes cell survival of the wild-type MG1655 strain by 2-fold after exposure to  $H_2O_2$ , whereas no protection by proline was observed in  $\Delta putA$  cells. To confirm that the lack of proline protection was due to the loss of PutA, MG1655  $\Delta putA$  cells were transformed with the PutA-pUC18 vector. PutA expression in the different strains was confirmed by Western blot analysis, as shown in Fig. 3B. MG1655  $\Delta putA$  cells transformed with the PutA-pUC18 vector showed increased survival with proline (Fig. 3A), while the empty pUC18 vector had no effect. Thus, PutA is required for the improved oxidative stress tolerance with proline. The PutA-pUC18 vector, however, did not completely restore the  $H_2O_2$  resistance of  $\Delta putA$  cells, as survival rates remained lower than those of wild-type cells. Figure 3A also shows that  $\Delta putA$  cells were more sensitive to  $H_2O_2$  in the absence of proline than wild-type cells. One explanation for this finding may be the inability of  $\Delta putA$  cells to utilize endogenous proline, which would lower resistance to oxidative stress. Consistent with this possibility, the survival rate of MG1655  $\Delta putA$  cells increased nearly 2-fold with the PutA-pUC18 vector.

Next, we questioned the mechanism by which proline enhances resistance to  $H_2O_2$ . Scavenging enzymes, like peroxidases and catalases, are a key defense mechanism against  $H_2O_2$  (40). To test whether proline increased the scavenging of  $H_2O_2$ , which is membrane permeable, extracellular  $H_2O_2$  levels (Fig. 3C) were measured in cultures of MG1655 wild-type and  $\Delta putA$  cells grown to exponential phase in medium supplemented with and without proline. In wild-type cells, extracellular  $H_2O_2$  (5  $\mu M$ ) was cleared at a significantly higher rate with proline than without proline (Fig. 3C). In the MG1655  $\Delta putA$  strain, proline had no effect on the  $H_2O_2$  clearance rate, indicating that the faster clearance of  $H_2O_2$  in wild-type cells with proline is dependent on PutA. Proline alone did not decrease  $H_2O_2$  levels during 30 min of incubation of proline (10 mM) and  $H_2O_2$  (5  $\mu M$ ) in medium without cells (data not shown).

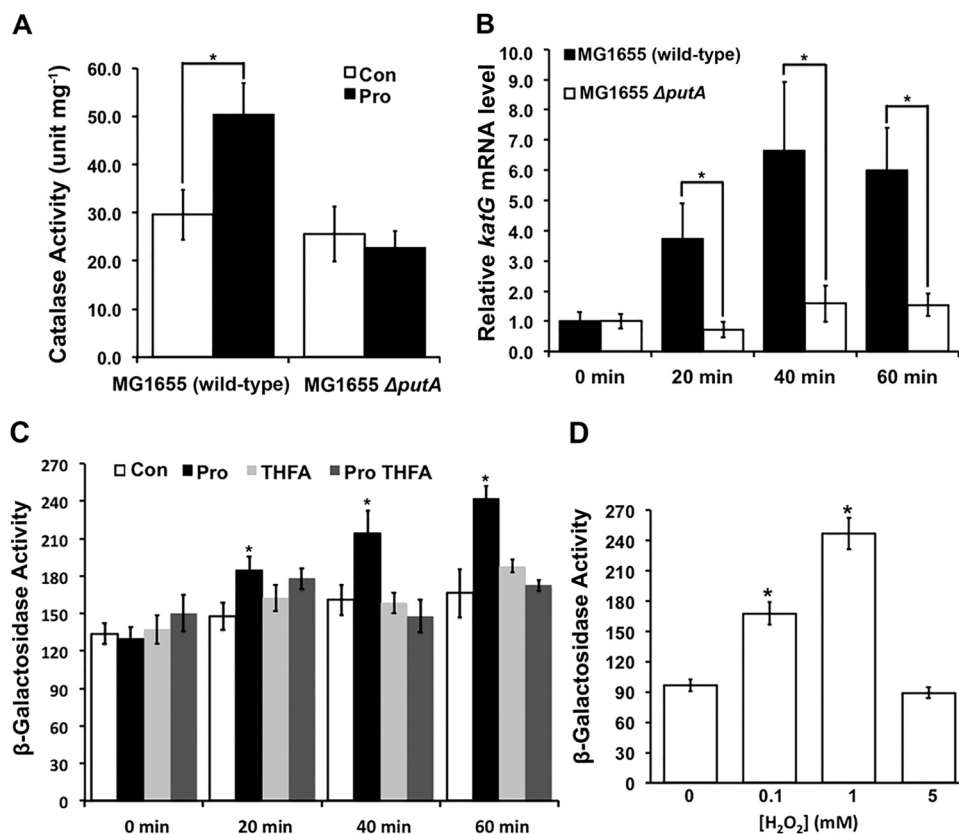
**Proline metabolism upregulates *katG* expression and activity.** The influence of proline on catalase activity was next evaluated as a possible means for the increased oxidative stress resistance and faster clearance of  $H_2O_2$ . Figure 4A shows that MG1655 wild-type cells exhibited 1.7-fold-higher catalase activity with proline than did cells without proline treatment. In MG1655  $\Delta putA$  cells, no significant change in catalase activity was observed with proline.

*E. coli* has two catalases, hydroperoxidase I and hydroperoxidase II, which are encoded by *katG* and *katE*, respectively (41). The expression of *katE* is regulated by RpoS and is upregulated during stationary phase, whereas the expression of *katG* is regulated by OxyR and is induced by  $H_2O_2$  (41). Changes in expression levels of



**FIG 3** Proline enhances cell survival and  $H_2O_2$  clearance. (A) Cell survival rates of MG1655 wild-type (parental) and  $\Delta putA$  strains and the  $\Delta putA$  strain transformed with the empty vector or the PutA-pUC18 vector in the absence (Con) and presence (Pro) of 10 mM proline. Cells grown in minimal medium were treated with 5 mM  $H_2O_2$  (30 min) prior to plating onto LB agar plates (\*,  $P < 0.05$ ). (B) Western blot analysis of PutA expression in strains used for panel A and the MG1655  $\Delta putA$  strain transformed with the PutA Lys9Met-pUC18 vector. (C)  $H_2O_2$  clearance in MG1655 wild-type and  $\Delta putA$  cells grown in minimal medium in the absence (Con) and presence (Pro) of 10 mM proline. After the addition of 5  $\mu M$   $H_2O_2$  to cells suspended in PBS,  $H_2O_2$  was measured at the indicated times by using Amplex Red (\*,  $P < 0.05$ ). The concentration of  $H_2O_2$  remaining is inversely proportional to the amount of  $H_2O_2$  scavenged.

*katG* were quantified by real-time PCR in cells treated with proline for up to 60 min (Fig. 4B). A 3.5-fold increase in the *katG* expression level was observed for wild-type MG1655 cells at 20 min, and a >6-fold increase was observed by 40 min. In  $\Delta putA$  cells, no



**FIG 4** Catalase expression and activity are upregulated by proline. (A) Catalase activity of MG1655 wild-type and  $\Delta putA$  cells grown in minimal medium with (Pro) or without (Con) 10 mM proline (\*,  $P < 0.05$ ). (B) Time course of *katG* expression in MG1655 wild-type and  $\Delta putA$  cells. After the addition of 10 mM proline to cells grown to exponential phase in minimal medium, cells were harvested at the times indicated, and *katG* expression was measured by real-time PCR (\*,  $P < 0.05$ ). 16S rRNA was used as the internal control. (C) Effect of proline on *katG'* promoter activity was determined by monitoring *katG':lacZ* reporter construct activity in AL441 cells grown to exponential phase in minimal medium. After reaching exponential phase, cells were treated with proline (10 mM) and L-THFA (10 mM), as indicated (0 to 60 min), and  $\beta$ -galactosidase activity was then measured (\*,  $P < 0.05$ ). (D) Same as panel C except that AL441 cells were treated with increasing concentrations of H<sub>2</sub>O<sub>2</sub> (30 min) prior to measurement of  $\beta$ -galactosidase activity (\*,  $P < 0.05$ ).  $\beta$ -Galactosidase activity is reported as Miller units (U/OD<sub>600</sub>).

increase in *katG* expression was observed in response to proline. Thus, proline induction of *katG* expression is dependent on PutA. Additional evidence for proline increasing *katG* expression levels was obtained by using a *katG':lacZ* expression reporter construct in MG1655 cells (strain AL441), in which the expression of *katG* is monitored by changes in  $\beta$ -galactosidase activity. The expression level of *katG* was 1.5-fold higher in cells with proline than in control cells without proline at 60 min (Fig. 4C). Incubation of cells with proline and L-THFA, a competitive inhibitor of PutA/PRODH activity ( $K_i = 1.6$  mM) (3), blocked the observed increase in  $\beta$ -galactosidase activity, suggesting that PutA catalytic activity is critical for the effects of proline on *katG* expression (Fig. 4C). The effect of proline metabolism on the *katG':lacZ* reporter was then compared to the effect of the addition of H<sub>2</sub>O<sub>2</sub> to the cell medium. Figure 4D shows that exposure of cells to 0.1 mM and 1 mM H<sub>2</sub>O<sub>2</sub> for 30 min results in 1.7- and 2.5-fold increases in the *katG* expression level, respectively. Thus, the increased level of *katG* expression by proline is similar to that observed with 0.1 to 1 mM H<sub>2</sub>O<sub>2</sub>. Altogether, these results strongly suggest that proline metabolism promotes the expression of *katG* and catalase activity.

**DNA-binding function of PutA is not required for increased oxidative stress resistance.** *E. coli* PutA contains an RHH DNA-

binding domain (residues 1 to 47) that enables PutA to act as transcriptional repressor of the *putA* and *putP* genes (4). Because PutA is a DNA-binding protein, it is feasible that the effect of PutA on *katG* expression may be via direct PutA-DNA interactions. *E. coli* PutA-DNA binding involves a GTTGCA consensus motif (5), which is not found in the promoter region of *katG*. Nevertheless, to rule out the possibility that PutA regulates *katG* expression by DNA binding, we transformed the MG1655  $\Delta putA$  strain with the PutA Lys9Met (K9M) mutant (Fig. 3B). Previously, Lys9 was determined to be critical for PutA-DNA interactions, as the Lys9Met mutation abolished PutA-DNA binding (5). Figure 5 shows that  $\Delta putA$  cells expressing the PutA K9M mutant responded to proline with a 2-fold increase in cell survival, similar to that observed with wild-type PutA (Fig. 3A). Expression of the PutA K9M mutant was confirmed by Western blot analysis (Fig. 3B). Therefore, the DNA-binding function of PutA is not required for the proline-dependent increase in oxidative stress resistance.

**Proline metabolism activates the OxyR regulon.** The effect of proline on cell survival was next tested by using a panel of mutants from the *E. coli* Keio strain collection (42). The cell survival rates of BW25113 wild-type and mutant strains after H<sub>2</sub>O<sub>2</sub> stress treatment were tested in the absence and presence of proline (Fig. 6A).

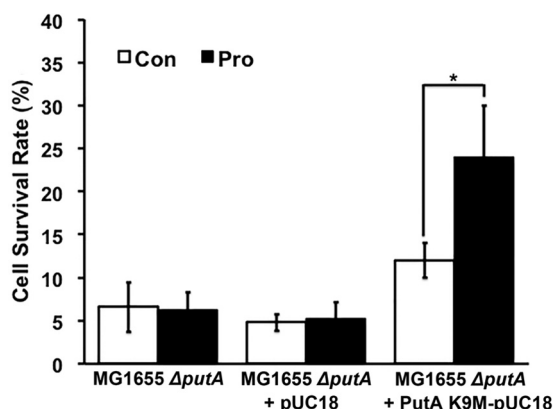


FIG 5 DNA binding is not required for proline-dependent oxidative stress resistance. Shown are survival rates of MG1655  $\Delta putA$  cells and  $\Delta putA$  cells transformed with the empty vector or the PutA K9M-pUC18 vector. PutA K9M is a mutant that does not bind DNA (\*,  $P < 0.05$ ).

As anticipated, proline increased the survival rate of BW25113 wild-type cells but not  $\Delta putA$  cells. Similar to the  $\Delta putA$  strain, the survival rates of the  $\Delta katG$  and  $\Delta oxyR$  cells were not increased by proline, suggesting that OxyR, which regulates *katG*, is involved in the protective mechanism of proline. In contrast, proline increased the survival rates of  $\Delta katE$  and  $\Delta soxR$  cells, indicating that hydroperoxidase II and SoxR, which is a transcription factor activated in response to redox-active metabolites (43), are not essential for the mechanism of proline protection.

Besides  $H_2O_2$  scavenging, activation of OxyR initiates other oxidative stress systems, such as the sequestration of unincorporated iron by Dps and the repair of polypeptide cysteine oxidation by thioredoxins and glutaredoxins (40). Because OxyR appears to have a critical role in proline-promoted oxidative stress resistance, the transcription levels of other antioxidant genes in the OxyR regulon were evaluated. Similar to *katG*, the expression levels of *grxA* (glutaredoxin 1) and *trxC* (thioredoxin 2) in MG1655 wild-type cells increased (~7-fold) in a time-dependent manner upon treatment with proline (Fig. 6B and C). In MG1655  $\Delta putA$  cells, no changes in *grxA* or *trxC* transcription levels were observed.

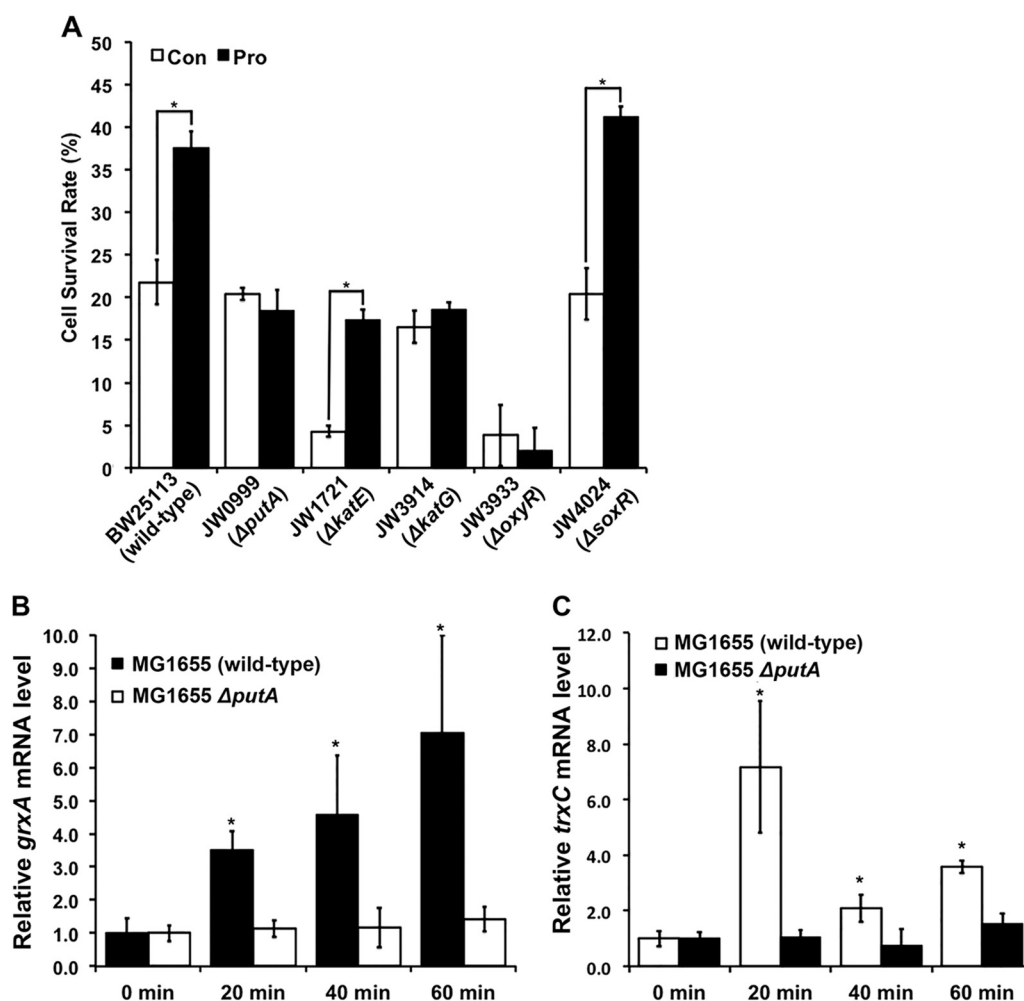
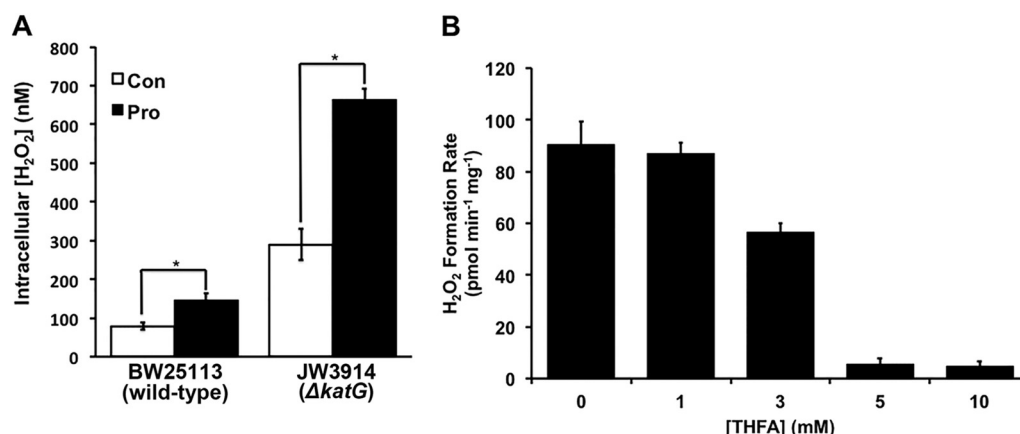


FIG 6 Proline protection involves the OxyR regulon. (A) Cell survival rates of the BW25113 wild-type strain and the BW25113  $\Delta putA$ ,  $\Delta katE$ ,  $\Delta katG$ ,  $\Delta oxyR$ , and  $\Delta soxR$  mutants (\*,  $P < 0.05$ ). (B and C) Time course of *grxA* (B) and *trxC* (C) expression in MG1655 wild-type and  $\Delta putA$  cells. Experiments were performed as described in the legend of Fig. 4B, using 16S rRNA as an internal control (\*,  $P < 0.05$ ).



**FIG 7** Proline metabolism generates  $H_2O_2$ . (A) BW25113 wild-type and  $\Delta katG$  (strain JW3914) cells were grown to exponential phase in minimal medium in the absence (Con) and presence (Pro) of 10 mM L-proline. The  $H_2O_2$  content in the medium was measured by the Amplex Red assay and converted to an estimate of the intracellular  $H_2O_2$  concentration (\*,  $P < 0.05$ ). (B) *In vitro* assays of  $H_2O_2$  production using inverted membrane vesicles from  $\Delta katG$  (strain JW3914) cells. Assays were performed with 50  $\mu\text{g/ml}$  of membrane vesicles and 10 mM proline in the presence of various concentrations of the PutA inhibitor L-THFA, as indicated.  $H_2O_2$  formation was estimated by the Amplex Red assay (\*,  $P < 0.05$ ).

These results are consistent with proline metabolism activating OxyR.

**Proline catabolism generates reactive oxygen species.** Because of the above-described results, we suspected that proline respiration may generate sufficient  $H_2O_2$  to activate OxyR. To test this,  $H_2O_2$  levels in BW25113 wild-type and  $\Delta katG$  strains were measured with and without proline. Without proline, the estimated intracellular concentration of  $H_2O_2$  in  $\Delta katG$  cells was nearly 3-fold higher ( $289 \pm 46$  nM) than that in wild-type cells ( $79 \pm 7$  nM). In both strains,  $H_2O_2$  levels were significantly higher in the presence of proline, with a  $>2$ -fold increase being observed for  $\Delta katG$  cells ( $663 \pm 9$  nM). In wild-type cells, intracellular  $H_2O_2$  levels increased to  $145 \pm 8$  nM with proline.

To further evaluate reactive oxygen species (ROS) production by proline oxidative metabolism, we performed *in vitro* assays using membrane vesicles prepared from BW25113  $\Delta katG$  mutant strain cells. The rate of  $H_2O_2$  formation with 10 mM proline was  $91 \pm 9$  pmol min<sup>-1</sup> mg<sup>-1</sup>, which is equivalent to  $9.7 \pm 0.9$   $\mu\text{M min}^{-1}$  when converted into an intracellular endogenous rate (Fig. 7B). The activity of PutA in membrane vesicles was measured at  $103 \pm 7$  nmol P5C min<sup>-1</sup> mg<sup>-1</sup>, indicating that the  $H_2O_2$  production rate is  $\sim 0.1\%$  of the PutA turnover rate. L-THFA was observed to inhibit  $H_2O_2$  formation in a dose-dependent manner, with 5 mM L-THFA almost completely blocking  $H_2O_2$  production (Fig. 7B). With membrane vesicles as the electron acceptor, PutA has a  $K_m$  value of 1.5 mM proline (3). These results indicate that PutA/PROD activity is required for  $H_2O_2$  formation with proline.

## DISCUSSION

Proline is a multifaceted amino acid with important roles in carbon and nitrogen metabolism; protein synthesis; and protection against various environmental factors such as drought (44), metal toxicity (45, 46), osmotic stress (24, 25), ultraviolet irradiation (47), unfolded protein stress (26, 48), and oxidative stress (19, 23, 27, 28, 49). In this study, we explored the role of proline metabolism in oxidative stress protection by characterizing the oxidative stress response of wild-type and *putA* mutant *E. coli* strains. Wild-

type *E. coli* strains exhibited significantly greater resistance to  $H_2O_2$  stress than did the *putA* mutant strains in medium supplemented with proline. Complementation of the *putA* mutant strains with PutA restored oxidative stress protection to levels near those of the parent wild-type strain. These results indicate that stress protection afforded by proline is a general phenotype in *E. coli* and is dependent on PutA.

The addition of proline to the culture medium increased total catalase activity and led to significantly higher expression levels of *katG* in wild-type cells, whereas no significant increase in catalase activity was observed with proline in  $\Delta putA$  cells. Proline did not protect  $\Delta katG$  cells, indicating that hydroperoxidase I is necessary for proline-enhanced protection against  $H_2O_2$  stress in *E. coli*. The *katG* gene is regulated by the transcription factor OxyR (50), which is a critical regulator of the cellular response to  $H_2O_2$  and thiol redox changes. The OxyR regulon regulates response genes, such as *katG*, *grxA* (glutaredoxin I), *trxC* (thioredoxin 2), and *ahpCF* (peroxiredoxin AhpCF), that provide protection against reactive oxygen species (51). Consistent with hydroperoxidase I having a critical role in proline protection, proline did not improve the oxidative stress survival of the *oxyR* mutant strain. In addition, proline increased the expression levels of other genes in the OxyR regulon, such as *grxA* and *trxC*. Whether proline broadly affects the OxyR regulon will require a more extensive profiling of gene expression changes. Altogether, our results indicate that proline catabolism activates OxyR, leading to increased expression of *katG*. In contrast to the *oxyR* mutant, proline enhanced the oxidative stress resistance of *soxR* mutant cells, indicating that SoxR is not essential for proline protection.

The finding that proline increases the transcription of genes in the OxyR regulon suggests that proline metabolism increases intracellular  $H_2O_2$  levels. OxyR reacts with  $H_2O_2$  to form a disulfide bond between Cys199 and Cys208, which results in transcriptional activation of the OxyR regulon (52, 53). Oxidation of OxyR and activation of the OxyR regulon have been reported to occur with 0.05 to 0.2  $\mu\text{M}$   $H_2O_2$  (32, 54). We observed a significant increase in endogenous  $H_2O_2$  levels in  $\Delta katG$  cells with proline, and in wild-type cells,  $H_2O_2$  levels were found to be  $>0.1$   $\mu\text{M}$  with



proline. Thus, it appears that proline oxidative metabolism can drive  $\text{H}_2\text{O}_2$  concentrations to levels that are sufficient to induce OxyR. The rapid increase of *katG* transcription by proline treatment (20 min) is also consistent with the response time of the OxyR regulon to  $\text{H}_2\text{O}_2$  stress (51).

Previous work has addressed metabolic sources of endogenous ROS in *E. coli* and indicated that the respiratory chain contributes to the majority of endogenous  $\text{H}_2\text{O}_2$  production (55). It was found, however, that  $\text{H}_2\text{O}_2$  can also be significantly generated in *E. coli* by enzymes not associated with the respiratory chain (56). The oxidation of proline by PutA provides reducing equivalents directly to the respiratory pathway via ubiquinone (3). The PRODH domain of PutA contains a FAD cofactor that couples the oxidation of proline (reductive half-reaction) to the reduction of ubiquinone in the membrane (oxidative half-reaction). The rate-limiting step in the proline:ubiquinone oxidoreductase reaction catalyzed by PutA is the oxidative step (reduced FAD [ $\text{FADH}_2$ ] oxidation by ubiquinone) (57). The production of  $\text{H}_2\text{O}_2$  by proline oxidation would conceivably involve increased flux in the respiratory chain or aberrant electron transfer from  $\text{FADH}_2$  to molecular oxygen, generating superoxide anion radicals, a general feature of flavoenzymes. In a previous study, the reactivity of different PutA proteins with molecular oxygen was evaluated, and PutA from *E. coli* was shown to have a turnover rate of  $<0.3 \text{ min}^{-1}$  with oxygen (58). Thus, we propose that endogenous  $\text{H}_2\text{O}_2$  from proline metabolism is not generated directly by PutA but rather by PutA/PRODH mainly passing electrons into the ubiquinone pool and PutA/P5CDH producing NADH, both of which would lead to increased electron flux through the respiratory chain. Consistent with this, we observed that proline increases respiration in wild-type *E. coli* cells by 4-fold using the redox indicator 2,3,5-triphenyl tetrazolium chloride (data not shown). Superoxide that results from proline metabolism would be converted to  $\text{H}_2\text{O}_2$  either non-enzymatically or enzymatically by superoxide dismutase. Our measurement of intracellular  $\text{H}_2\text{O}_2$  in wild-type cells grown without proline is consistent with the physiological concentration of  $\text{H}_2\text{O}_2$  ( $<0.1 \mu\text{M}$ ) reported previously for *E. coli* in the exponential growth phase (59). Twofold increases in  $\text{H}_2\text{O}_2$  production, which we observed with proline, have also been shown to significantly induce *katG* expression with intracellular  $\text{H}_2\text{O}_2$  at 0.1 to 0.2  $\mu\text{M}$  (55). Thus, increases in the endogenous levels of  $\text{H}_2\text{O}_2$  as a by-product of proline metabolism are likely enough to activate OxyR and induce *katG* expression.

The observation that proline metabolism can influence hydroperoxidase I activity indicates that besides serving as an important growth substrate in nutritionally deplete microenvironments, proline may offer a competitive advantage to bacteria in harsh oxidative environments. Bacteria often encounter oxidative stress from the host immune system, such as the respiratory burst associated with phagocytic killing of microbes (60). Previously, pretreatment of *E. coli* cells with small amounts of  $\text{H}_2\text{O}_2$  was shown to have a protective effect by a 10-fold induction of hydroperoxidase I (61, 62). Here, proline metabolism may also provide a preconditioning effect by generating  $\text{H}_2\text{O}_2$  as a by-product and elevating hydroperoxidase I levels, thereby increasing the overall stress tolerance of the cell. Various studies of proline metabolism in eukaryotes have shown that proline oxidation, which in eukaryotes occurs in the mitochondrion, generates ROS (19, 23, 63, 64), which can mediate cell death (63), cell survival against oxidative stress (28), and life span (64). The results from this work

further illustrate the fundamental importance of how  $\text{H}_2\text{O}_2$ , as a metabolic by-product, can enhance oxidative stress tolerance and appears to be an underlying feature of proline metabolism that is conserved between *E. coli* and mammals.

## ACKNOWLEDGMENTS

We thank James Imlay for generously sharing *E. coli* strains MG1655 and AL441 and for insightful discussions. We also thank Melanie Simpson and Paul Black for sharing microplate reader instrumentation.

This research was supported in part by grants GM079393, GM061068, and P30GM103335 from the National Institutes of Health.

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