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## *Escherichia coli* $\Delta fur$ mutant displays low HPII catalase activity in stationary phase

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Iron is among the most important micronutrients used by bacteria. As a partner of the Fenton reaction, however, iron potentiates oxygen toxicity. Strict regulation of iron metabolism, and its coupling with regulation of defenses against oxidative stress, is an essential factor for life in the presence of oxygen. In *Escherichia coli*, iron metabolism is regulated by the Fur protein. A Fur-deficient mutant, in stationary phase, displayed about 30y-fold lower HPII activity than the respective, Fur-proficient parental strain. Deletion of *fur* seems to affect HPII catalase specifically, since the mutant was capable of inducing HPI catalase when challenged with  $H_2O_2$ . Low HPII catalase activity appears to be among the reasons for hydrogen peroxide hypersensitivity of the  $\Delta fur$  mutant.

### INTRODUCTION

Bacteria in nature often encounter nutrient limitation, and their life normally alternates between growth and stationary phases. At the onset of starvation, *Escherichia coli* expresses ~80 genes involved in enhanced general resistance.<sup>1</sup> Among them are genes involved in defense against oxidative stress.<sup>2</sup> Stationary phase cells are more resistant to exogenous  $H_2O_2$  challenge than are growing cells,<sup>3</sup> which is not surprising keeping in mind that catalase is induced in non-growing cultures. *E. coli* produces two catalases, HPI and HPII, which differ in structure and kinetic properties.<sup>4,5</sup> HPI is transcriptionally induced during logarithmic growth in response to low concentrations of hydrogen peroxide. This induction requires the positive activator OxyR.<sup>4</sup> HPII, on the other hand, is induced in stationary phase and is *rpoS*-regulated.<sup>4</sup>

We demonstrate here that a non-growing Fur-deficient mutant displays about 30-fold lower HPII activity than the parental strain. In *E. coli*, the Fur protein regulates iron metabolism. Deletion of *fur* seems to affect HPII

catalase specifically, since the mutant was capable of inducing HPI catalase when challenged with  $H_2O_2$ . The effect also appears not to be due to suppression of *rpoS* because the mutant was able to induce other *rpoS*-regulated enzymes.

### MATERIALS AND METHODS

#### *Strains and media*

LB medium contained 10 g Bacto-tryptone, 5 g yeast extract, and 10 g NaCl per liter and was adjusted to pH 7.0 with ~1.5 g of  $K_2HPO_4$ .

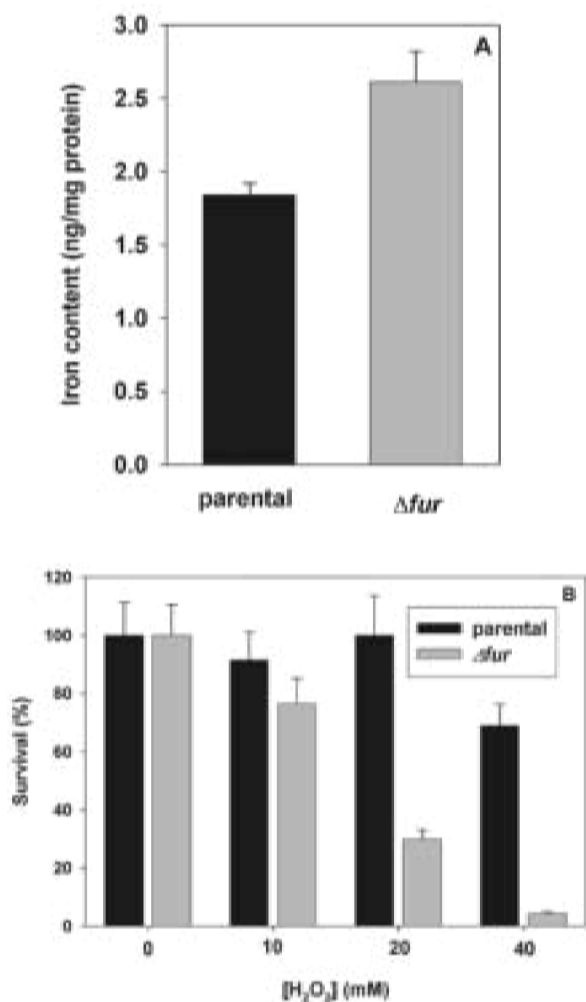
The strains of *E. coli* used were as follows: AB 1157 = parental, and KK204 = AB1157 *fur::kan*, (provided by Dr J. Imlay, University of Illinois at Urbana-Champaign, Urbana, IL, USA). A catalase-deficient mutant UM1 with its parental strain GSH7<sup>6</sup> were obtained from I. Fridovich (Duke University Medical Center, Durham, NC, USA). Strains were grown overnight at 37°C, with shaking in air, in LB medium containing the required antibiotics. The overnight cultures were diluted 200-fold into fresh LB medium, and were grown for 15–18 h for the stationary phase experiments or to a density of  $A_{600}$  ~0.6 if the activities of enzymes in mid-log cultures were to be assayed. For enzyme assays, cells were washed 3 times in the buffer of the assay and lysed by a French press. Debris was removed by centrifugation.

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**Fig. 1.** Iron content and killing by hydrogen peroxide of stationary phase cells. Cultures were grown for 16 h in LB medium and washed 3 times with Chelex-treated potassium phosphate buffer, pH 7.5. Half of the cells were resuspended in the same buffer to a density of  $A_{600} = 0.1$  and were incubated with  $H_2O_2$  for exactly 30 min. The other half was lysed by a French press and cell-free extracts were assayed for iron. (A) Iron content; (B) killing by  $H_2O_2$ . Bars represent mean  $\pm$  SEM ( $n = 5$ ).

### Killing by $H_2O_2$

$H_2O_2$  killing was performed essentially as described by Carlioz and Touati.<sup>7</sup> Cultures were diluted to  $A_{600} = 0.1$  with M9 salts and were incubated with  $H_2O_2$  for exactly 30 min. The concentration of the  $H_2O_2$  stock solution was adjusted using an extinction coefficient at 240 nm of  $43.6 \text{ M}^{-1}\text{cm}^{-1}$ .<sup>8</sup>

### Catalase activity determination

Cell-free extracts were assayed for HPI and HPII catalase activities as described by Visick and Clarke.<sup>9</sup> In

brief, after measuring the total catalase activity, the crude extracts were heated in a  $55^\circ\text{C}$  water bath for 15 min and the assay was repeated. This gives the activity of the heat-stable HPII. HPI activity was estimated by subtracting the HPII activity from the total activity.

### Glutathione reductase assay

Cell-free extracts from mid-log and stationary phase cultures were assayed for glutathione reductase activity as described by Davis *et al.*<sup>10</sup> In brief, a 1.0 ml reaction mixture contained 1.2 mM oxidized glutathione, 0.35 mM NADPH, and 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 50 mM potassium phosphate buffer, pH 8.0. The reaction was followed spectrophotometrically at 412 nm.

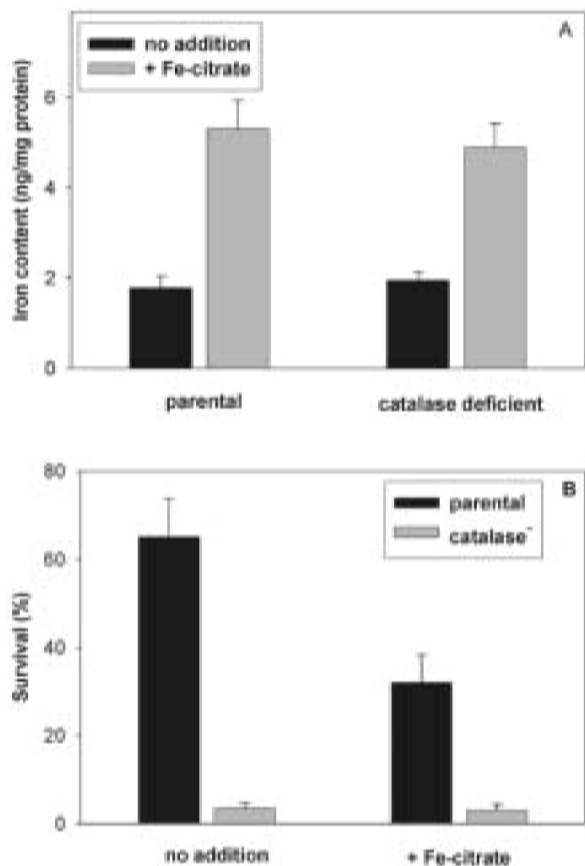
### Iron assay

Cells were harvested, washed 3 times in 50 mM Chelex-treated potassium phosphate, pH 7.5, resuspended in the same buffer and lysed in a French press. The iron content of cell-free extracts was measured using a flame atomic absorption spectrophotometer (Varian Spectra AA 400, Australia) with a deuterium background corrector. Samples were diluted 1:5 with de-ionized water. Seronorm 103 serum standard (Nycomed, Oslo, Norway) was used for standardization of element analysis and the mean ( $n = 7$ ) concentration of iron deviated  $-3\%$ , from the certified value. As reference material, bovine liver standard 1577a (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used for validation of analytical methods. The analytical value ( $n = 7$ ) for iron deviated  $-1.3\%$  from the certified value.

All experiments were repeated at least three times with 3–5 replicates. Bars on figures represent SEM.

## RESULTS

It has been reported that exponentially growing  $\Delta fur$  mutants are more sensitive to  $H_2O_2$  than the corresponding wild-type cells.<sup>11</sup> This  $H_2O_2$  sensitivity was attributed to the iron-overload, leading to increased production of the extremely reactive hydroxyl radical by the Fenton reaction.<sup>11</sup> Similarly to what was found for growing cultures, in stationary phase the  $\Delta fur$  mutant accumulated about 30% more iron, and was much more sensitive, especially to high concentrations of  $H_2O_2$ , than was the parental strain (Fig. 1). This hypersensitivity to hydrogen peroxide might not only reflect iron-overload, but also low catalase activity.<sup>12</sup> The importance of catalase is

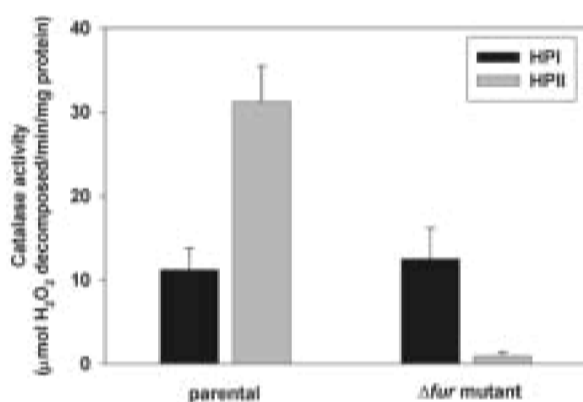


**Fig. 2.** Iron content and killing by hydrogen peroxide of parental and catalase-deficient cells. All conditions are as in the caption to Figure 1 except that the strains were grown with or without the addition of 0.5 mM  $\text{Fe}^{2+}$ -citrate. (A) Iron content; (B) killing by 20 mM  $\text{H}_2\text{O}_2$ . Bars represent mean  $\pm$  SEM ( $n = 3$ ).

illustrated in Figure 2, which shows that lack of catalase, without iron overload, dramatically increases the sensitivity to  $\text{H}_2\text{O}_2$ .

To check if deletion of *fur* affects the  $\text{H}_2\text{O}_2$  detoxifying capacity of the cells, we assayed the activities of the HPI and HP11 catalases. Figure 3 demonstrates that the mutant and the parent display similar HPI catalase activities, while HP11 catalase was about 30 times lower in the mutant. The effect of Fur on HP11 catalase might be either specific or might be a general response to the altered iron uptake, and thus would affect other iron-containing enzymes as well. In an attempt to distinguish between these two possibilities, we compared the induction of HPI by  $\text{H}_2\text{O}_2$  in the parental and in the  $\Delta fur$  strain. Figure 4 shows that 60 mM  $\text{H}_2\text{O}_2$  increased HPI activity about 4-fold in both the mutant and the parent. It thus appears that deletion of *fur* specifically affects HP11 catalase activity.

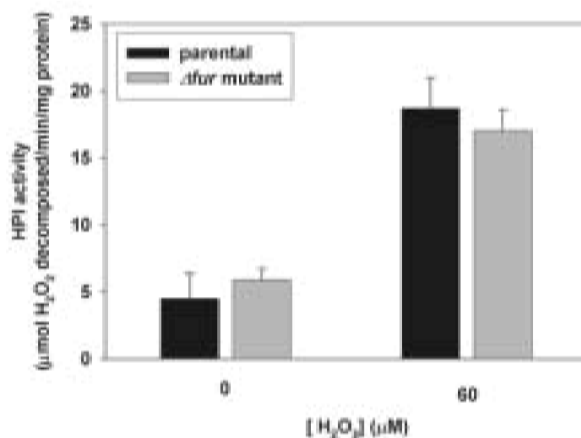
It might be supposed that deletion of *fur* prevents the induction of HP11 in stationary phase. The gene coding for HP11, *katE*, is a member of the *rpoS* regulon.<sup>4</sup>



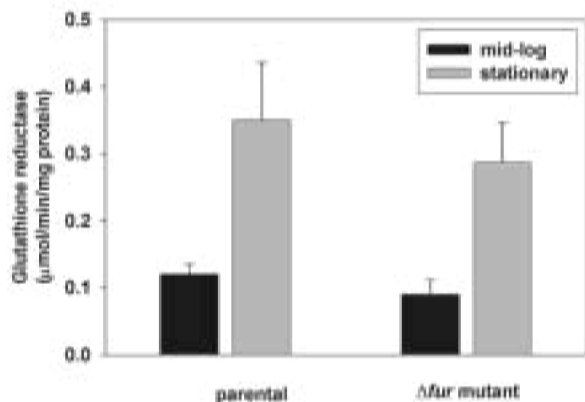
**Fig. 3.** HPI and HP11 activities in stationary phase. Parental and  $\Delta fur$  mutant strains were grown for 17 h, and cell-free extracts were assayed for HPI and HP11 catalase activities. Bars represent mean  $\pm$  SEM ( $n = 3$ ).

Deletion of *fur* might prevent the activation of the *rpoS* regulon, which would affect the induction in stationary phase of all *rpoS*-dependent genes. If so, other *rpoS*-dependent enzymes would also have low activity in non-growing  $\Delta fur$  cultures. This idea, however, was rejected when other *rpoS*-controlled enzymes were assayed. For example, glutathione reductase is *rpoS*-controlled,<sup>13</sup> and as shown in Figure 5, deletion of *fur* did not affect its activity in stationary phase.

It now appears that two factors might contribute to hydrogen peroxide sensitivity of the  $\Delta fur$  mutant – iron overload and low HP11 catalase. In an attempt to assess the relative importance of each of these factors, we used  $\text{CN}^-$  to block the catalase activities, and compared the survival of the two strains when challenged with  $\text{H}_2\text{O}_2$ . Cyanide, at a concentration 150 μM, completely blocked catalase activity without affecting the plating efficacy. Figure 6 shows that pretreatment with  $\text{CN}^-$  decreased the



**Fig. 4.** Induction of HPI by hydrogen peroxide. Cells were grown to a density of  $A_{600} \sim 0.6$ . Hydrogen peroxide (60 μM) was added to half of the flasks and cultures were kept on the shaker for an additional 30 min. After that time, the cells were washed and HPI activity was assayed in cell-free extracts. Bars represent mean  $\pm$  SEM ( $n = 3$ ).



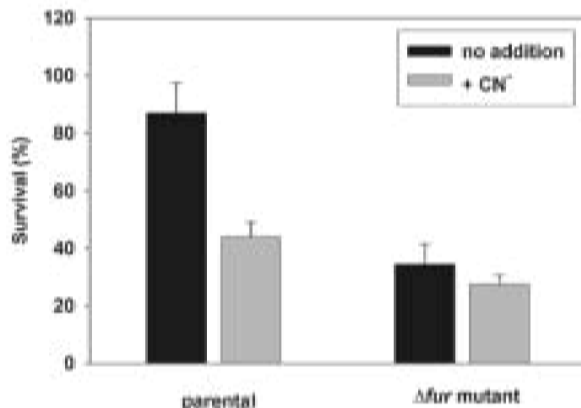
**Fig. 5.** Glutathione reductase activity. Parental and  $\Delta fur$  mutant strains were grown to a mid-log ( $A_{600} \sim 0.6$ ) and stationary phase (17 h). Cells were harvested and cell-free extracts were assayed for glutathione reductase. Bars represent mean  $\pm$  SEM ( $n = 3$ ).

survival of the peroxide-treated parental strain by about 40%, but had negligible effect on the mutant. As both strains had practically equal HPI activity before the  $CN^-$  treatment, it might be concluded that the difference between the two strains is due to the absence of HPII in the  $\Delta fur$  mutant. Even when catalases were completely blocked by  $CN^-$ , the mutant remained more sensitive to  $H_2O_2$ , which can be attributed to the iron overload.

To assess the impact of the iron overload, we assayed  $H_2O_2$  killing in highly diluted bacterial suspensions, where the amount of  $H_2O_2$  catabolised by bacteria would be negligible. In such dilute suspensions,  $H_2O_2$  concentration remains practically constant for the time of incubation; therefore, differences in survival would reflect the availability of intracellular reactive iron. In this experiment, the initial suspensions with a density of  $A_{600} = 0.1$  were diluted  $10^4$ -fold and then challenged with 5 mM  $H_2O_2$ . After 30 min of incubation, the survival was  $83 \pm 7\%$  for the parental strain versus  $57 \pm 5\%$  for the mutant.

## DISCUSSION

Iron is an indispensable element for living cells, since many enzymes have iron as a cofactor in their active sites. On the other hand, through the Fenton reaction, iron also promotes the formation of hydroxyl radicals, which indiscriminately damage all cellular components. Cells have evolved regulatory systems to ensure sufficient uptake of iron to meet their physiological requirements yet at the same time minimize iron toxicity. In *E. coli*, iron metabolism is regulated by the Fur protein.<sup>14</sup> In recent years, an increasing number of genes have been shown also to be Fur regulated, although they have no apparent involvement in iron supply. Among



**Fig. 6.** Effect of  $CN^-$  on killing by  $H_2O_2$ . The experiment was performed as described in the caption to Figure 1, except that after cell suspensions were diluted to  $A_{600} = 0.1$ , freshly prepared KCN solution was added to a final concentration of 150  $\mu M$ . After 15 min, 20 mM hydrogen peroxide was added, and 30 min later suspensions were diluted and plated for enumeration of colonies. Bars represent mean  $\pm$  SEM ( $n = 5$ ).

them are the genes for superoxide dismutases, which together with catalases, play a major role in the defense against oxygen toxicity.<sup>15,16</sup>

HPII hydroperoxidase is the major catalase produced in aerobically growing cultures of *E. coli*.<sup>4</sup> Its activity is much higher in stationary phase cells than in exponentially growing cultures. This is due to *rpoS*-dependent induction, and is important for long-term survival in stationary phase.<sup>4,5</sup> Our results show that in a  $\Delta fur$  mutant, HPII has very low activity. The effect of *fur* on HPII seems to be specific, since *fur* deletion did not affect HPI catalase activity.

Our study does not provide enough data about the mechanism by which *fur* affects HPII catalase. It might appear that as with the genes for superoxide dismutases, the gene coding for HPII catalase is also Fur-regulated. Further, more detailed studies are under way in our laboratory to check this possibility.

## CONCLUSIONS

It now appears that the hypersensitivity of non-growing  $\Delta fur$  cultures to hydrogen peroxide is due not only to impaired iron acquisition, but also to low HPII catalase activity, and, as a consequence, to inability to remove  $H_2O_2$  efficiently from the environment.

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