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Growth of *Escherichia coli* in Iron-enriched Medium Increases HPI Catalase Activity

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***Escherichia coli* has two catalases, HPI and HPII. HPI is induced during logarithmic growth in response to low concentrations of hydrogen peroxide. This induction is OxyR-dependent. On the other hand, HPII is not peroxide-inducible but is induced in entry to the stationary phase. We demonstrate here that *E. coli* displayed higher HPI catalase activity when compared to the cultures that were grown in a normal medium, if grown in a medium supplemented with iron-citrate. Iron supplementation had no effect on HPII catalase. This increase of HPI activity was OxyR-independent and not observed in a Δ*fur* mutant. The physiological significance of the increase of HPI activity is unclear, but it appears that the *katG* gene that codes for HPI catalase is among the genes that are regulated by Fur.**

Keywords: Catalase activity, Fur, Hydroperoxidase I, Iron

Introduction

Iron is among the most important micronutrients. As a partner of the Fenton reaction, however, iron potentates oxygen toxicity (Benov, 2001). Strict regulation of iron metabolism, and its coupling with the regulation of defenses against oxidative stress, is an essential factor for life in the presence of oxygen (Touati, 2000). In *E. coli* as in almost all other aerobic cells, the first line of defense against oxygen-derived free radicals includes superoxide dismutases and catalases. Because of its role in Fenton chemistry, one might anticipate that iron availability would affect the expression of these enzymes. Indeed, the iron-dependent regulation of SODs has been observed in several bacteria (Touati, 2000). Here we demonstrate that the growth of *Escherichia coli* in a medium containing Fe(II)-citrate rises HPI catalase activity. This increase of HPI catalase was not affected by the deletion of the s*oxRS*, *oxyR* or *rpoS* regulons, but was abolished by the deletion of *fur*.

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Materials and Methods

Strains and media The LB medium contained 10 g Bacto-tryptone, 5 g yeast extract, and 10 g NaCl per l and was adjusted to pH 7.0 with ~1.5 g of K_2HPO_4. The M9CA medium consisted of minimal A salts (Maniatis *et al.*, 1982), 0.2% casamino acids, 0.2% glucose, 3 mg pantothenate, and 5 mg of thiamine per l.

The *E. coli* strains that were used are as follows: GC4468 = parent and DJ 901 = GC4468 D(soxR-Zjc2204) Zjc2205::Tn10Km (Touati *et al.*, 1995), (D. Touati, Institute Jacques Monod, CNRS, Universite Paris, France); AB 1157 = parental, KK204 = AB1157 *fur*::kan, AS430 = GC4468 Δ*oxyR*::Spec, ZK126 = parental, and ZK1000 *rpoS*::kan. These were provided by Dr. J. Imlay (University of Illinois at Urbana-Champaign, Urbana, Illinois, USA). The strains were grown overnight at 37°C (with shaking in the air) in a LB medium containing the required antibiotics. The overnight cultures were diluted 200-fold into a M9CA medium and grown to a density of A_{600} = 0.6 – 0.8.

Measurement of H_2O_2 consumption H_2O_2 degradation by the cells was monitored at 240 nm (Al-Maghrebi and Benov, 2001). Five hundred μl of cell suspension (diluted with the same buffer to exactly A_{600} = 0.1) and 40 μl of 250 mM H_2O_2 were added to 460 μl of 100 mM phosphate buffer, pH 6.5. The decrease of the H_2O_2 concentration was followed on a recording spectrophotometer. To avoid cell sedimentation and fluctuations in H_2O_2 concentration, the samples were stirred every 60 s. H_2O_2 consumption was expressed in mmoles of H_2O_2 that was consumed per 10 min per 10^3 cells.

Catalase/peroxidase activity determination The cells were harvested, washed twice in 50 mM potassium phosphate, pH 7.5, resuspended in the same buffer, and lysed in a French press. The crude extracts were assayed for HPI and HPII catalases, as described by Visick and Clarke (1997). Briefly, after measuring the total catalase activity, the crude extracts were heated in a 55°C water

bath for 15 min, then the assay was repeated. This gave the activity of the heat stable HPII. The HPI activity was estimated by subtracting HPII from the total activity.

Iron assay The iron content of the cell-free extracts was measured using a flame atomic absorption spectrophotometer (Varian Spectra AA 400, Minasco Australia Pty Ltd., Sunbury, Australia) with a deuterium background corrector. The samples were diluted 1 : 5 with de-ionized water. Seronorm 103 serum standard (Nycomed, Oslo, Norway) was used for the standardization of the element analysis and the mean (n = 7) concentration of iron deviated −3% from the certified value. As a reference material, bovine liver standard 1577a (National Institute of Standards and Technology, Gaithersburg, USA) was used for validation of the analytical methods. The analytical value (n = 7) for iron deviated −1.3% from the certified value.

All of the experiments were repeated at least three times with 3-5 replicates.

Results

E. coli, when grown in an iron-enriched medium, contained about four fold more iron (6.1 ± 0.6 ng iron/mg protein) compared to the cells that were grown in a normal M9CA medium (1.5 ± 0.3 ng iron/mg protein). Possibly that part of this iron is in a "free" form that is capable of decomposing hydrogen peroxide (Keyer and Imlay, 1996). To check if the high iron content correlates with the increased decomposition of hydrogen peroxide, we measured the H_2O_2 consumption by native cells. The cells that were grown with iron-citrate showed a higher rate of H_2O_2 consumption (0.27 ± 0.05 mmole/10 min/10^3 cells) when compared to the cells that were grown in a normal medium (0.17 ± 0.03 mmole/10 min/10^3 cells). Washing the cells with a buffer containing 1 mM EDTA had no effect on the H_2O_2 decomposition (not shown).

H_2O_2 may be decomposed either enzymatically or by intracellular iron complexes. In an attempt to distinguish between these two possibilities, we used CN^- to inhibit the enzymatic decomposition of H_2O_2. Preincubation with 150 μM CN^- for 15 min lowered the H_2O_2 consumption by both the normal (0.04 ± 0.02 mmole/10 min/10^3 cells) and iron-loaded (0.05 ± 0.03 mmole/10 min/10^3 cells) cells to the same level.

E. coli has two catalase enzymes, HP I and HP II, which catalyze the decomposition of H_2O_2 to water and oxygen (Visick and Clarke, 1997). Iron supplementation more than doubled the HPI catalase activity, from 6 ± 1 to 13 ± 2 μmole H_2O_2/min/mg protein. However, it had no effect on the HPII catalase activity, 6 ± 2 and 7 ± 2 μmole H_2O_2/min/mg protein in the normal and iron-supplemented cells, respectively.

SoxRS, *oxyR*, and *rpoS* regulons control the expression of a large number of genes that are involved in cellular response to a diverse number of stresses, including oxidative stress and starvation (Storz *et al.*, 1990; Demple, 1991; Loewen *et al.*, 1998). Fur has been identified as a regulatory protein that controls genes that are involved in iron acquisition (Touati, 2000). To confirm if the increase of HPI activity depended on one of these genes, we used Δ*fur*, *soxRS*$^-$, *oxyR*$^-$, and *rpoS*$^-$ mutants. The deletion of the *soxR*, *oxyR*, and *rpoS* genes had no effect on the increase in HPI activity levels in response to iron-supplementation with increases of 94 ± 9%, 100 ± 24%, and 123 ± 19% upon iron-supplementation for the *soxRS*$^-$, *oxyR*$^-$, and *rpoS*$^-$ mutants. Deletion of the *fur* gene limited the increase in HPI catalase activity to 19 ± 3% of its level in the absence of iron supplementation. Deletion of the *soxR*, *oxyR*, *rpoS* and *fur* genes had no effect on the HPII catalase activity levels

Discussion

It is generally accepted that the Fenton reaction:

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\cdot} + HO^-$$

is responsible for both hydrogen peroxide and superoxide toxicity (Liochev, 1999). Because iron plays a key role in this reaction, strict regulation of iron transport, storage, and metabolism has been considered an important part of the antioxidant defense (Touati, 2000). In *E. coli*, iron metabolism is regulated by the Fur protein (Flint *et al.*, 1993; Gruer and Guest, 1994). Fur usually functions as a transcriptional regulator, repressing the expression of target genes (Touati, 2000). There is increasing evidence of coordination between the regulation of iron homeostasis and defense against oxidative stress (Keyer and Imlay, 1996). *E. coli* has two cytoplasmic SODs, MnSOD and FeSOD, and both are regulated by iron (Dubrac and Touati, 2000). We found that *E. coli* that is grown in a medium containing 0.5 mM Fe(II)-citrate had a higher capacity for decomposing hydrogen peroxide than the cells that were grown in a normal medium. The cells were thoroughly washed before the incubation with hydrogen peroxide, which excluded the possibility that the effect was due to the extracellular decomposition of H_2O_2 by the excess of iron. The ability of the cells to decompose H_2O_2 was inhibited by CN^-, indicating that it was enzymatic. An assay of the catalases in the cell-free extracts revealed that iron overloading induced hydroperoxidase I (HPI catalase) without affecting HPII. HPI is transcriptionally induced during logarithmic growth in response to low concentrations of hydrogen peroxide. This induction requires the positive activator OxyR (Visick and Clarke, 1997). However, an o*xyR*- and *rpoS*-independent induction of this enzyme was observed in the stationary phase (Visick and Clarke, 1997). The increase of HPI activity by iron was also *oxyR*$^-$ and *rpoS*$^-$ independent, but was not observed in a Δ*fur* mutant. Under iron-rich conditions, Fur binds Fe(II) and inhibits transcription from virtually all of the genes and operons that are repressed by the metal (Escolar *et al.*, 1999). However, a Fur positive effect on several *E. coli* genes has also been observed (Niederhoffer *et al.*, 1990; Gruer and Guest, 1994; Dubrac and

Touati, 2000; Dubrac and Touati, 2002). It was recently reported that these positive effects of Fur are in fact indirect, resulting from the negative post-transcriptional effect of a small RNA, RyhB, which in turn is under Fur-negative control (Masse and Gottesman, 2002). There is no reason to believe that the six genes that have currently been identified as regulated by RyhB (Masse and Gottesman, 2002) are its only targets. It seams reasonable to expect that other genes that encode iron-containing proteins are also RyhB-dependent, and thus, indirectly regulated by Fur and iron availability.

We demonstrated here that iron overload increases the activity of the HPI catalase, but not of the HPII catalase, via a Fur-dependent way. Whether or not Fur indeed acts positively on the *katG* gene remains to be elucidated.

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