Involvement of the \textit{ytfK} gene from the PhoB regulon in stationary-phase H$_2$O$_2$ stress tolerance in \textit{Escherichia coli}

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\textbf{Abstract}

The \textit{Escherichia coli} PhoB-PhoR two-component system responds to phosphate starvation and induces the expression of many genes. Previous studies suggested that phosphate starvation induces oxidative stress, but the involvement of the PhoB regulon in oxidative stress tolerance has not been clarified. Here, we showed that \textit{ytfK}, one of the PhoB regulon genes, is involved in cell tolerance to a redox-cycling drug, menadione, and H$_2$O$_2$ in stationary-phase cells. A \textit{ytfK} deletion mutant was sensitive to H$_2$O$_2$ when the cells were grown anaerobically or micro-aerobically in the presence of nitrate. Genetic analysis suggested that the \textit{ytfK} gene has a functional relationship with the \textit{oxyR} and \textit{fur} genes, among the \textit{oxyR} regulon, at least, a catalase-encoding \textit{katG} gene and peroxidase-encoding \textit{ahpCF} genes. Overproduction of \textit{YtfK} resulted in a KatG-dependent decrease of H$_2$O$_2$ concentration in the cell suspension, suggesting that \textit{katG} is one of the targets of \textit{YtfK}. Using a \textit{katG}–\textit{lacZ} reporter fusion, we showed that \textit{YtfK} enhances the transcription of \textit{katG} although it was not clarified whether \textit{YtfK} functions directly or not. We also showed that \textit{ytfK} disruption results in reduced viability of stationary-phase cells under phosphate starvation. These results indicated that \textit{YtfK} is involved in H$_2$O$_2$ tolerance by stimulating directly or indirectly the transcription of at least the catalase gene, and that this system plays an important role in cellular survival during phosphate starvation.

\textbf{INTRODUCTION}

Phosphorus is one of the essential elements. Living organisms use it in the form of phosphate (PO$_4^{3-}$). Phosphate is a constituent of ATP, DNA, RNA, phospholipids etc., and plays important roles in carbon metabolism and signal transduction [1]. \textit{Escherichia coli} preferably utilizes inorganic phosphate, but its quantity is not always sufficient to support optimal microbial growth [2, 3].

To survive in phosphate-limited environments, \textit{E. coli} cells sense its concentration and respond to phosphate starvation by utilizing a PhoB-PhoR two-component system. PhoR is a histidine kinase that lacks a sensory domain, and requires five additional proteins – PstS, PstC, PstA, PstB and PhoU, for sensing external phosphate concentration [2]. PstS, PstC, PstA and PstB are components of a phosphate ATP-binding cassette transporter, and PhoU is a chaperone-like protein [2]. PhoR is not active in the presence of more than 4 µM phosphate; at phosphate concentrations below 4 µM, PhoR is activated and, in turn, activates PhoB, leading to the induction of more than 41 genes of the PhoB regulon [4–8]. In addition to the phosphate transporter described above, the PhoB regulon includes genes involved in phosphate recycling: \textit{phoA}, encoding an alkaline phosphatase, and \textit{ann}, encoding an AMP nucleosidase [9–11]. PhoB also induces the genes of metal ion homeostasis: \textit{cusC}, \textit{cusF}, \textit{cusB} and \textit{cusA} of a cation (copper/silver) efflux system [5, 12]; and \textit{waaH}, encoding a lipopolysaccharide glycosyltransferase, which protects cells from zinc stress [13].

Phosphate starvation reduces the viability of hydrogen peroxide (H$_2$O$_2$) scavenger deletion mutants \textit{ΔahpCF} and \textit{ΔahpCF ΔkatG} [14]. During phosphate starvation under aerobic conditions, glucose metabolism leads to oxidative stress in stationary-phase cells. Furthermore, levels of thio-barbituric acid-reactive substances, byproducts of lipid peroxidation, are increased by phosphate starvation [15]. These studies indicate that oxidative stress is enhanced by phosphate starvation, although it was not clarified whether phosphate starvation accelerates cellular H$_2$O$_2$ production or diminishes H$_2$O$_2$ tolerance.

Oxidative stress is caused by reactive oxygen species (ROS), such as superoxide (O$_2^-$) and H$_2$O$_2$. These are produced within cells by adventitious autoxidation of redox enzymes [16]. One-electron reduction of O$_2$ results in O$_2^-$ formation, and two-electron reduction of O$_2$ results in H$_2$O$_2$ formation.
In *E. coli*, NADH dehydrogenase II, fumarate reductase and sulfite reductase are significant sources of O$_2^•$ and H$_2$O$_2$ [17, 18]. External sources of ROS also exist; for example, cells are exposed to high levels of oxidative stress by the host immune system upon phagocytosis. The host defence system produces not only O$_2^•$ and H$_2$O$_2$ but also peroxynitrite (ONOO$^−$) and hypochlorous acid (HOCl), which are synthesized from O$_2$ and H$_2$O$_2$[19]. In some pathogenic bac teria, PhoB functions to increase toxin production in response to such oxidative stress [20, 21]. In enterohemorrhagic *E. coli* (EHEC) in response to phosphate starvation, PhoB-independent regulation for expression of the genes for oxidative stress tolerance (*ahpCF* etc.) is also suggested [22].

Although phosphate starvation potentiates oxidative stress, induction of the defence system by phosphate starvation has not been clarified. Two genes of the PhoB regulon are markedly induced by H$_2$O$_2$ treatment. One is the *phoH* gene, which codes for an ATP-binding protein, and the other is the *ytfK* gene [23]. Specifically, the *ytfK* gene is induced by phosphate starvation in a PhoB-dependent manner [24]; PhoB binds to its promoter region [25]. The *ytfK* gene is also induced in stationary-phase cells grown with LB medium, and its transcription depends on RpoS, a master regulator of stationary-phase gene expression [26]. Although its expression has been studied, the function of *ytfK* has not yet been clarified.

In the current study, we constructed a *ytfK* deletion mutant and investigated its phenotype. We showed that the *ytfK* gene is involved in H$_2$O$_2$ stress tolerance. Furthermore, we showed that the gene is involved in the expression of *katG*, which encodes a catalase, one of the H$_2$O$_2$ scavengers. In addition, the viability of the *ytfK* deletion mutant was reduced during phosphate starvation. These results indicate that *E. coli* indeed possesses a defence system against oxidative stress induced by phosphate starvation.

**METHODS**

**Bacterial strains and plasmids**

All *E. coli* strains used in this study are derivatives of MG1655, and their genotypes are presented in Table S1 (available in the online version of this article). Plasmids pBAD/Myc His A (Invitrogen, USA), pACYC184 [27] and mk4 [28] were used in the study. Primers used for the construction of deletion mutations are listed in Table S2.

**Menadione sensitivity assay**

Overnight cultures in LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) were diluted (1/500) in fresh LB medium, or LB medium supplemented with 48 mM NaNO$_2$ or 48 mM NaNO$_3$. Micro-aerobic incubation was achieved by culturing (3 ml/tube) in test tubes; aerobic incubation was performed by culturing (5 ml/flask) in 100 ml flasks; and anaerobic incubation was performed by culturing (30 ml/tube) in 30 ml glass tubes topped by a butyl stopper. The cultures were incubated for 24 h at 37°C with shaking (130 r.p.m.). Stationary-phase cultures were washed twice with the same volume of 0.85% NaCl, and suspended in the same volume of ice-cold MOPS buffer (20 mM MOPS, pH 6.7 and 0.85% NaCl); 48 mM NaNO$_3$ or 48 mM NaNO$_2$ was added to obtain NO$_3^−$ or NO$_2^−$-supplemented cultures, respectively. Cellular suspensions (750 µl) in 1.5 ml sampling tubes were mixed with the appropriate amount of H$_2$O$_2$, and incubated for 24 h at 4°C with vertical rotation. After serial dilution, the cultures were plated on LB agar plates supplemented with 0.1% glucose and incubated overnight at 37°C. c.f.u.s were determined by colony counting. The antibiotics were added as required, at 50 µg/ml ampicillin (Ap), 50 µg/ml Km and 15 µg ml$^{-1}$ chloramphenicol (Cm).

**H$_2$O$_2$ decomposition assay**

Overnight cultures in LB medium were diluted (1/500) in fresh LB medium supplemented with 48 mM NaNO$_3$. The cultures (3 ml/tube) were incubated in test tubes (micro-aerobic conditions) for 24 h at 37°C with shaking (130 rpm). The stationary-phase cells were washed twice with the same volume of 0.85% NaCl, and suspended in an equivalent volume of ice-cold MOPS buffer. For whole-cell assays, the cells were suspended to OD$_{600}$=1.0 (deletion mutants) or OD$_{600}$=0.5 (over-producers). The assays were carried out on ice. The reactions were started by the addition of H$_2$O$_2$ (0.15% final concentration) to 1 ml of the cell suspension; the residual H$_2$O$_2$ was then measured at the indicated time points by the FOX-1 method [29]. Briefly, after serial dilution, the cell suspensions were mixed with 190 µl of colouring agent containing 100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 100 mM sorbitol and 25 mM H$_2$SO$_4$. After completion of the colour development step, the cell suspensions were centrifuged and the OD$_{560}$ of the supernatants was measured. For the cell-free extract assays, the stationary-phase cells were suspended in ice-cold MOPS buffer and sonicated. Cell debris was removed by centrifugation (27 173 g, 5 min), and the supernatant was used as the cell-free extract. The assay was carried out with the cell-free extracts (40 µg protein/ml) as described above.
Promoter activity assay
Overnight cultures in LB medium were diluted (1/500) in fresh LB medium supplemented with 48 mM NaN3. The cultures (3 ml/tube) were incubated in test tubes (micro-aerobic conditions) for 14 h at 37 °C with shaking (130 r.p.m.). The cells were suspended to OD600=0.13 in pre-warmed LB medium containing 48 mM NaN3. After incubation for 15 min in the presence or absence of H2O2 at 37 °C, the cells were harvested and suspended in Z buffer. β-galactosidase activity was measured as described previously [30]. The final concentrations of H2O2 (%) were 2.64×10^-3 (MG1655), 1.17×10^-3 (ΔkatG), 4.69×10^-3 (ΔyfK/pBAD) and 0.68×10^-3 (ΔyfKΔkatG/pBAD).

Phosphate starvation viability assay
Overnight cultures in LB medium were diluted (1/500) in MOPS minimal medium [31] supplemented with 0.4% glucose and 1 mM K2HPO4. The cultures (3 ml/tube) were incubated in test tubes for 12 h at 37 °C with shaking (130 r.p.m.). The cells were harvested, washed with 0.85% NaN3 and suspended in the same volume of 0.85% NaN3. The suspensions were diluted (1/500) in MOPS minimal medium containing 0.4% glucose and K2HPO4 and 48 mM NaN3. The cultures (3 ml/tube) were incubated in test tubes at 37 °C with shaking (130 r.p.m.). After serial dilution, the cultures were plated on LB agar plates supplemented with 0.1% glucose and incubated overnight at 37 °C. c.f.u.s were determined by colony counting.

RESULTS
Menadione sensitivity of the ytfK mutant
Previous studies demonstrated that the expression of ytfK is induced by H2O2 stress [23], by paraquat treatment [32], during biofilm formation [33] and under iron-rich conditions [34]. These results suggested that the ytfK gene is involved in oxidative stress tolerance. To clarify its role, we constructed a ytfK deletion mutant and studied its sensitivity to oxidative stress. First, we examined the strain’s sensitivity to menadione, a redox-cycling drug, which accepts electron(s) from diaphorases and subsequently reduces oxygen, leading to the constitutive production of O2 and H2O2 [35]. Because the expression of ytfK is increased upon transition to stationary phase not only in phosphate-limited medium [24] but also in LB medium [26], we examined menadione tolerance using LB medium. When the ytfK deletion mutant was grown in LB medium, menadione sensitivity was similar to that of the wild-type strain (Fig. 1a).

Several genes (e.g. ytfE and msrA) involved in tolerance to nitric oxide (NO) stress [36–38] are located in the vicinity of the ytfK gene. NO is produced during nitrate and nitrite metabolism [39], and reactive nitrogen species (RNS), including NO, increase ROS toxicity [19, 40]. We therefore changed the medium to one supplemented with nitrate or nitrate. The ytfK deletion mutant was sensitive to menadione in that medium (Fig. 1b, c). To confirm that this phenotype was caused by ytfK deletion, a single-copy mini-F plasmid harbouring the ytfK gene was introduced into the ytfK deletion mutant. The resultant strain became menadione-tolerant, indicating that the loss of ytfK function was indeed responsible for decreased menadione tolerance (Fig. 1d). These results indicated that ytfK is involved in menadione sensitivity in stationary-phase cells in the presence of nitrite or nitrate. The ytfK deletion mutant was also sensitive to another redox-cycling drug, phenazine methosulfate (PMS) (Fig. S1).

H2O2 sensitivity of the ytfK mutant
Two possible functions of YtfK may explain the reduced menadione tolerance linked to ytfK deletion: either YtfK repressed ROS production from menadione or it stimulated ROS degradation. To clarify the YtfK function, we examined the H2O2 sensitivity of the ytfK deletion mutant. If YtfK repressed ROS production from menadione, ytfK deletion would not have resulted in decreased H2O2 tolerance; however, the effect would have been opposite if YtfK had stimulated ROS decomposition. Some difficulties are associated with the examination of H2O2 tolerance at 37 °C. First, the cells rapidly decompose H2O2. Second, the reduced cellular viability is cancelled out by re-growth of the survivors. Third, the pH of the culture markedly affects H2O2 decomposition, and therefore many indirect factors, e.g. the concentration of fermentation acids, could affect H2O2 tolerance. We therefore developed a simple method for the examination of H2O2 tolerance that circumvented these problems. Briefly, the stationary-phase cells were harvested, washed twice with 0.85% NaN3 and suspended in the MOPS buffer. After the addition of the approximate amount of H2O2, the cells were incubated at 4 °C and the colony-forming units (c.f.u.s) were determined after 24 h.

When the ytfK deletion mutant was grown in LB medium, the H2O2 sensitivity of the mutant was similar to that of the wild-type (Fig. 2a). When it was grown in LB medium containing nitrate, the deletion mutant was sensitive to H2O2 similarly to the parental strain (Fig. 2b). However, when that deletion mutant was grown in LB medium containing nitrite, the survival of the ytfK deletion mutant in the presence of 2.1% H2O2 equalled that of the wild-type strain in the presence of 3% H2O2 (Fig. 2c), i.e. the deletion mutant was about 30% more sensitive to H2O2. This phenotype was complemented by a single-copy mini-F plasmid harbouring the ytfK gene, which confirmed that the loss of the ytfK function was responsible for the reduction of H2O2 tolerance (Fig. 2d). These results indicated that YtfK is involved in H2O2 tolerance. When the cells were grown in MOPS minimal medium containing 0.2 mM phosphate, the ytfK deletion mutant became more sensitive to H2O2 than the wild-type strain even in the absence of nitrate, but the increased sensitivity was less than that for the cells grown in the LB medium with nitrate (Fig. S2a).

Next, we examined the effect of O2 availability on H2O2 sensitivity. Oxygen is required for endogenous production of ROS; the balance between the cellular oxidation-reduction state and O2 levels determines ROS production [16, 41, 42]. A micro-aerobic environment reduced redox enzymes,
which are easily autoxidized, resulting in O$_2^-$ production [43]; hence the transition from anaerobic to aerobic conditions results in the production of ROS. Furthermore, the expression of certain genes involved in oxidative stress tolerance, such as yhjA [44] and yfgF [45], is regulated by O$_2^-$ availability, but these genes are expressed even under anaerobic conditions. In the current work, the cells were typically grown micro-aerobically to the stationary phase; on the other hand, H$_2$O$_2$ tolerance was examined in cells grown aerobically or anaerobically. When the ytfK deletion mutant was grown aerobically, its H$_2$O$_2$ sensitivity was similar to that of the wild-type strain (Fig. 2e). In contrast, when the ytfK deletion mutant was grown anaerobically, it was about 15% more sensitive to H$_2$O$_2$ (Fig. 2f). These results show that ytfK is responsible for H$_2$O$_2$ stress tolerance under both anaerobic and micro-aerobic conditions.

**H$_2$O$_2$ sensitivity of the ytfK oxyR and ytfK fur double mutants**

To clarify the functional relationship between the ytfK gene from the PhoB regulon and genes from other regulons, we examined the effects of deletion of two transcriptional regulators on the H$_2$O$_2$ sensitivity of the ytfK deletion mutant. The first regulator was OxyR, which is activated directly by H$_2$O$_2$ [46, 47], regulating the expression of genes involved in H$_2$O$_2$ scavenging, e.g., katG and ahpCF, and genes involved in iron homeostasis, e.g., dps, hemH and yaaA [23, 48]. OxyR plays a central role in protecting the cell from endogenous and exogenous H$_2$O$_2$ [49, 50]. The other regulator was Fur, which senses intracellular free iron levels and regulates the expression of genes involved in iron homeostasis [51]. Under H$_2$O$_2$ stress, ferrous iron reduces H$_2$O$_2$ by the Fenton reaction, resulting in the formation of the hydroxyl radical (HO•), which oxidizes all types of cellular component. The transcription of fur is induced by H$_2$O$_2$ stress, perhaps because the regulation of intracellular free iron levels is critical for decreasing H$_2$O$_2$ toxicity [52].

First, we examined the effect of oxyR deletion on the H$_2$O$_2$ sensitivity of the ytfK deletion mutant. Because the oxyR deletion mutant will not grow aerobically on a plate, the plates were incubated anaerobically. The oxyR deletion
resulted in an increase in the $\text{H}_2\text{O}_2$ sensitivity of the wild-type strain. The c.f.u. ratio ($\text{H}_2\text{O}_2$ treatment/control) of the $\text{oxyR}$ deletion mutant was 2.5-fold lower than that of the wild-type. If $\text{ytfK}$ and $\text{oxyR}$ function independently in $\text{H}_2\text{O}_2$ tolerance, $\text{ytfK}$ deletion in the $\text{oxyR}$ deletion mutant would cause a further increase in $\text{H}_2\text{O}_2$ sensitivity. As shown in Fig. 3(a, c), the $\text{ytfK}$ deletion did not result in an increase in the $\text{H}_2\text{O}_2$ sensitivity of the $\text{oxyR}$ deletion mutant. These results suggest that the positive effect of $\text{ytfK}$ on $\text{H}_2\text{O}_2$ tolerance requires the $\text{oxyR}$ function.

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**Fig. 2.** $\text{ΔytfK}$ strain is $\text{H}_2\text{O}_2$-sensitive in the presence of nitrate under anaerobic or micro-aerobic conditions. $\text{H}_2\text{O}_2$ sensitivity of the wild-type (squares) and $\text{ΔytfK}$ (circles) strains (a) in LB medium, (b) in LB medium with nitrite and (c) in LB medium with nitrate. (d) The introduction of $\text{mk4}$ plasmid harbouring the $\text{ytfK}$ gene into the $\text{ΔytfK}$ strain restored its tolerance to $\text{H}_2\text{O}_2$ in LB medium supplemented with nitrate. $\text{H}_2\text{O}_2$ sensitivity (e) during aerobic growth in LB medium with nitrate, and (f) during anaerobic growth in LB medium with nitrate.
Next, we examined the effect of fur deletion on the H$_2$O$_2$ sensitivity of the ytfK deletion mutant. Since the fur deletion caused a pronounced increase in the H$_2$O$_2$ sensitivity of the wild-type strain, the fur deletion mutant was thereafter treated with a lower concentration of H$_2$O$_2$. If ytfK and fur function independently in H$_2$O$_2$ tolerance, ytfK deletion in the fur deletion mutant would result in a further increase in H$_2$O$_2$ sensitivity. As shown in Fig. 3(a, c), the ytfK deletion did not result in an increase in the H$_2$O$_2$ sensitivity of the fur deletion mutant. These results suggest that the positive effect of ytfK on H$_2$O$_2$ tolerance requires the fur function.

H$_2$O$_2$ sensitivity of the ytfK, katG, and/or ahpCF double or triple mutants

To clarify the functions of oxyR and fur required for the function of ytfK in H$_2$O$_2$ tolerance, we examined involvement of the genes for H$_2$O$_2$ scavengers in the H$_2$O$_2$ sensitivity of the ytfK deletion mutant. Three enzymes have been identified as the major H$_2$O$_2$ scavengers in E. coli, and the expression of their encoding genes is induced by OxyR and/or Fur proteins: the expression of the katG gene, encoding a bifunctional catalase (hydroperoxidase I, HPI) [53], is induced by OxyR and Fur [23, 54]; that of the katE gene, encoding a monofunctional catalase (HPII), is induced by Fur [54, 55]; and that of the ahpCF genes, encoding subunits of an alkyl hydroperoxide reductase, is induced by OxyR [23, 56]. It is not known whether Fur directly regulates katG and katE expression. Because another group reported that the expression of katE and katG was not affected by the deletion of fur [57], fur-dependent induction of katG and katE might occur only under specific conditions.

Single, double and triple mutants of katG, katE and ahpCF were constructed, and their H$_2$O$_2$ sensitivity was examined. As described above, we reasoned that if YtfK function depended on a scavenger function, ytfK deletion in a scavenger mutant would not cause a further increase in H$_2$O$_2$ sensitivity. There are at least two reasons why evaluation of the results was not simple. First, different concentrations of H$_2$O$_2$ had to be used for the mutants. This could be ascribed to the difference of H$_2$O$_2$-decomposing activity of the scavengers under the assay conditions. Second, more than one scavenger may have depended on the YtfK function, to varying degrees. We identified mutants whose H$_2$O$_2$ sensitivity was not markedly increased by ytfK deletion. When the ytfK gene was deleted in the katG ahpCF mutant, the c.f.u. ratio was decreased only 2.1-fold (Fig. 3b, d); when the ytfK gene was deleted in the ahpCF mutant, the c.f.u. ratio was decreased only 4-fold (Fig. 3b, d); when it was deleted in the katE katG ahpCF mutant, the c.f.u. ratio was decreased only 7.3-fold (Fig. 3b, d). When the ytfK gene was deleted in other mutants, the H$_2$O$_2$ sensitivity was increased to a greater degree (Fig. 3b, d). These results suggest that the effect of ytfK deletion on H$_2$O$_2$ decomposition activity requires the function of AhpC-AhpF or KatG.

H$_2$O$_2$-decomposing catalase activity of whole cells and cell-free extracts

To confirm the functional interaction of YtfK with the H$_2$O$_2$ scavenger KatG, we examined the cellular decomposition of H$_2$O$_2$ in the culture media. First, we examined the wild-type, the katG, katE and katE katG deletion mutants, and these mutants with an additional ytfK deletion. In this assay system, the katE katG double deletion diminished the H$_2$O$_2$-consuming activity of whole cells (Fig. 4d, e). The experiment revealed that ytfK deletion did not cause a significant decrease in the H$_2$O$_2$-decomposing activity of the wild-type strain and the katG deletion mutant (Fig. 4a, c and e). In contrast, ytfK deletion in the katE deletion mutant resulted in a decrease in the H$_2$O$_2$-decomposing activity (Fig. 4b, e). In the absence of the marked H$_2$O$_2$-decomposing activity of KatE, a subtle reduction in KatG activities was observed. These results suggest that YtfK enhanced the KatG-dependent H$_2$O$_2$-decomposing activity of whole cells.

To confirm the enhancement of KatG-dependent H$_2$O$_2$-decomposing activity by YtfK, we next assayed H$_2$O$_2$-decomposing activity using cell lysates of the over-producing strains, because whole-cell activity does not always correspond to enzymatic activity. For example, during the exponential phase, H$_2$O$_2$ diffusion through the cell membrane is limited and equivalent amounts of whole cells and cell extracts exhibit different H$_2$O$_2$-decomposing activities [58]. The ytfK gene was cloned into the ColE1-related pBAD plasmid and introduced into the wild type, the katG, katE and katE katG deletion mutants, with an additional ytfK deletion.

The experiment revealed that the activity of the cell lysate of the ytfK and katE ytfK deletion mutants carrying the pBAD-ytfK plasmid was much higher than that of cells bearing the pBAD vector (Fig. 4f, g and j). In contrast, the cell lysate activity of the katG ytfK and katE katG ytfK deletion mutants carrying pBAD-ytfK was similar to that of cells bearing the pBAD vector (Fig. 4h–j). In this assay system, no cell lysate activity of the katE katG ytfK deletion mutant was detected (Fig. 4i, j). These results indicate that the H$_2$O$_2$-decomposing activity of KatG increased in a strain carrying the pBAD-ytfK plasmid.

Interestingly, when the cell lysate of a strain carrying pBAD-ytfK was mixed with the cell lysate of a strain harbouring a multi-copy plasmid with the katG gene, pACYC184-katG, H$_2$O$_2$-consuming activity was similar to the activity of the cell lysate of a pACYC184-katG-bearing strain but not to that of a strain bearing both pBAD-ytfK and pACYC184-katG (Fig. S3). These results demonstrate that the high level of KatG activity could not be reconstituted by mixing the two separate lysates prepared from a KatG over-producer and a YtfK over-producer. The results indicate that it is unlikely that YtfK stimulated KatG activity simply as an accessory protein.
Effects of the deletion or multiple copies of \textit{ytfK} on \textit{katG} promoter activity

To clarify the mechanism of the enhancement of KatG activity by YtfK, we next investigated the activation of expression of \textit{katG} by YtfK. We constructed a strain harbouring a chromosomal \textit{katG}\textsuperscript{-lacZ} transcriptional fusion by inserting the promoter region of the \textit{katG} gene into the \textit{lacZ} upstream region. When the pBAD-\textit{ytfK} plasmid was introduced into the strain with the chromosomal \textit{katG}\textsuperscript{-lacZ} fusion, \textit{b}-galactosidase activity was increased 1.7-fold. When the same plasmid was introduced into the \textit{katG} deletion mutant, higher activity was detected (Fig. 5a). When the \textit{ytfK} gene was deleted in the wild-type and the \textit{katG} deletion mutant strains bearing the chromosomal \textit{katG}\textsuperscript{-lacZ} fusion, the \textit{b}-galactosidase activity was decreased (Fig. 5b). Transcription of the \textit{katG} gene is induced by OxyR following activation of the protein’s DNA binding by \textit{H}_{2}\textit{O}_{2} (47). We examined the YtfK activation of \textit{katG} expression following \textit{H}_{2}\textit{O}_{2} treatment. As shown in Fig. 5c, \textit{b}-galactosidase activity of the chromosomal \textit{katG}\textsuperscript{-lacZ} fusion increased after the introduction of the \textit{ytfK} plasmid, and \textit{b}-galactosidase activity of the chromosomal \textit{katG}\textsuperscript{-lacZ} fusion decreased after \textit{ytfK} was deleted (Fig. 5D). In the absence of chromosomal \textit{katG}, we observed a similar increase in \textit{b}-galactosidase activity in the presence of the \textit{ytfK} plasmid and a similar decrease in \textit{b}-galactosidase activity after \textit{ytfK} deletion (Fig. 5). These results indicate that YtfK stimulated \textit{katG} transcription.
Effects of ytfK deletion on stationary-phase cell viability during phosphate starvation

To clarify the biological function of ytfK, we examined the viability of the ytfK deletion mutant in the stationary phase, in a phosphate-limited MOPS medium. When the ytfK deletion mutant was incubated in the MOPS medium containing 0.2 mM phosphate, the viability of the mutant was similar to that of the wild-type strain (Fig. 6a). However, when this strain was incubated in the presence of nitrate, its viability was decreased more rapidly than that of the parental strain (Fig. 6b).

In contrast, when these strains were incubated in the MOPS medium containing 2 mM phosphate and nitrate, their viability was drastically reduced even after 24 h (Fig. 6c). Furthermore, when these strains were incubated in a medium containing 20 mM phosphate and nitrate, their viability had recovered after 42 h (Fig. 6d). The reduced viability of the ytfK deletion mutant in the medium containing 0.2 mM phosphate and nitrate was complemented by the mini-F plasmid (mk4) carrying ytfK (Fig. 6e). These results indicate that ytfK plays an important role in cellular survival during phosphate starvation.

The viability of the katG deletion mutant was lower than that of the wild-type strain, but the viability of the katG ytfK deletion mutant was similar to that of the ytfK deletion mutant (Fig. 5b). This result indicates that the YtfK function for cellular survival under this culture condition is dependent on KatG function.

DISCUSSION

Previous work suggested that phosphate starvation induces oxidative stress. Phosphate co-precipitates with divalent metal ions, such as Zn$^{2+}$, Fe$^{2+}$, Cd$^{2+}$ and Cu$^{+}$, and phosphate depletion increases the levels of free metal ions in a medium [59]. Metal ion toxicities depend on the concentration of phosphate, and high levels of active metal ions cause oxidative stress [60, 61]. Metal ions abiotically catalyse the formation of H$_2$O$_2$ on the surface of aerobic and anaerobic environments [48]. Furthermore, Fe$^{2+}$ and Cu$^{+}$ catalyse the reduction of H$_2$O$_2$, resulting in the production of a more toxic hydroxyl radical by Fenton reaction. Hence, during phosphate starvation, cells are exposed to high oxidative stress. Nevertheless, the oxidative stress response that operates during phosphate starvation had not been clarified.

In the current study, we identified the ytfK gene from the E. coli PhoB regulon as a key factor of the oxidative stress response during phosphate starvation. ytfK is a member of the PhoB regulon, which is induced by phosphate starvation [24]; ytfK is also induced by oxidative stress [23]. We first showed that ytfK is involved in the cellular resistance to a redox-recycling drug, menadione, which produces ROS, and consequently leads to oxidative stress. Second, we showed that ytfK is involved in the H$_2$O$_2$ resistance of cells. Third, our results suggest that the ytfK function is dependent on both OxyR and Fur. Fourth, we showed that the reduction of H$_2$O$_2$ concentration by KatG, whose encoding gene is a member of both OxyR and Fur regulons, was affected by ytfK. Fifth, we showed that the expression of Fig. 4. H$_2$O$_2$-decomposing catalase activity of KatG depends on YtfK. H$_2$O$_2$ decomposition by whole cells: (a) the wild-type and ΔytfK, (b) ΔkatG and ΔkatG ytfK, (c) ΔkatE and ΔkatE ytfK, and (d) ΔkatE katG and ΔkatE katG ytfK. (e) H$_2$O$_2$-decomposing activities of whole cells. H$_2$O$_2$ decomposition by cell-free extracts of cells harbouring the vectors pBAD-ytfK or pBAD: (f) ΔytfK, (g) ΔkatE ytfK, (h) ΔkatG ytfK and (i) ΔkatE katG ytfK. (j) H$_2$O$_2$-decomposing activities of cell-free extracts. A specified concentration of H$_2$O$_2$, 0.15 %, was defined as 100. Symbols: circles, ytfK$^-$ cells; squares, ytfK$^+$ cells (a–i).
katG was affected by the cellular ytfK status. These results suggest that the regulation of the expression of katG constitutes the biochemical function of ytfK. We also showed that ytfK plays a role in cellular viability during phosphate starvation, which suggests that cellular resistance to phosphate starvation comprises the biological function of ytfK.

We showed that the ytfK katG double-deletion mutant was more sensitive to H$_2$O$_2$ than the katG deletion mutant (Fig. 3). These results suggest that the ytfK function depends not only on katG but also on ahpCF genes. However, we showed that ytfK is involved in the reduction of H$_2$O$_2$ concentration by KatG, but not by AhpC-AhpF (Fig. 4). These two proteins constitute an alkyl hydroperoxide reductase, which reduces H$_2$O$_2$ to H$_2$O using NADH as an electron donor. During our experiments, the cells were suspended in a nutrient-free buffer and hence sufficient NADH may not have been supplied to AhpC-AhpF, and AhpC-AhpF-dependent H$_2$O$_2$ decomposition may not have been detected. Because ytfK is involved in the regulation of katG expression, ytfK may also participate in the regulation of the ahpCF operon. The ytfK gene may be involved in the regulation of the expression of other genes, as suggested by our observations that the H$_2$O$_2$ stress tolerance of the katG ahpCF double mutant and the katG ahpCF katE triple mutant was somewhat reduced after ytfK deletion. Further microarray or RNA-seq analyses will be necessary to delineate the overall function of ytfK in the regulation of gene expression.

On the other hand, previous studies showed that ytfK is induced by H$_2$O$_2$ stress, but the regulatory mechanism of ytfK has not been clarified [23]. The ytfK gene was induced in the oxyR mutant; therefore, ytfK is not a member of the OxyR regulon. It was not clear whether Fur was involved in the regulation of ytfK expression. Fur regulates a set of genes different to those regulated by OxyR and Fur, because Fe$^{3+}$ binds Fur as a co-factor [57]. Further analysis is necessary for a full understanding of the regulation of ytfK gene expression.

The mechanism by which YtfK stimulates katG transcription remains to be uncovered. YtfK, a small basic
protein (theoretical pI of 10.14), may function as a DNA-binding protein that binds to a promoter region of katG and stimulates its activity. Similarly to the nucleoid proteins IHF and HU, which are small basic proteins [62], YtfK may bind and bend DNA to facilitate DNA binding of transcriptional regulators. Alternatively, YtfK may function as an accessory protein of a transcription factor; YtfK may interact with a transcriptional factor and stimulate its activity. The small protein of a transcription factor; YtfK may interact with a specific role together with other PhoB regulon members that connecting phosphate metabolism to the glucose-phosphate stress response.

In the current study, we showed that the sensitivity of the ytfK mutant to menadione and H2O2 was increased only in the presence of nitrate. The viability of the ytfK mutant during phosphate starvation also decreased only in the presence of nitrate. Nitrate metabolism produces RNS [39]. Because highly reactive byproducts, such as peroxynitrite, are synthesized through the interaction of ROS with RNS, the addition of nitrate would exaggerate the toxic effect of ROS, allowing the detection of the ytfK deletion phenotype. McLean and his colleagues revealed that some members of the PhoB regulon other than ytfK were induced by exposure to peroxynitrite [64], formed by an interaction between NO and superoxide, or between nitrite and H2O2. Their statistical analysis of transcriptomic data indicated that PhoB is the active transcriptional regulator stimulated by peroxynitrite treatment [64]. The ytfK gene product may play a specific role together with other PhoB regulon members that are induced in the presence of both ROS and RNS. The requirement of nitrate for the ytfK function may provide a clue for a more precise understanding of the ytfK function.

**Fig. 6.** The ΔytfK strain prematurely loses viability in the stationary phase in phosphate-limiting medium. (a) Viability in MOPS minimal medium containing 0.4 % glucose, 0.2 mM K2HPO4 without nitrate. (b) Viability in MOPS minimal medium containing 0.4 % glucose, 0.2 mM K2HPO4 with nitrate. (c) Viability in MOPS minimal medium containing 0.4 % glucose, 2 mM K2HPO4, with nitrate. (d) Viability in MOPS minimal medium containing 0.4 % glucose, 20 mM K2HPO4, with nitrate. Symbols: circles, ytfK+ cells; squares, ytfK– cells. (e) The introduction of mk4 plasmid harboring the ytfK gene into the ΔytfK strain restored premature loss of viability in MOPS minimal medium containing 0.4 % glucose, 0.2 mM K2HPO4, with nitrate.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

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