

Requirement of Fur for the Full Induction of *dps* Expression in *Salmonella enterica* Serovar Typhimurium

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Abstract The Dps protein, which is overexpressed in harsh environments, is known to play a critical role in the protection of DNA against oxidative stresses. In this study, the roles of Fur in the expression of the *dps* gene in *Salmonella* and the protection mechanisms against oxidative stress in *Salmonella* cells preexposed to iron-stress were investigated. Two putative Fur boxes were predicted within the promoter region of the *S. typhimurium dps* gene. The profile of *dps* expression performed by the LacZ reporter assay revealed growth-phase dependency regardless of iron-status under the culture conditions. The *fur* mutant, χ 4659, evidenced a reduced level of β -galactosidase as compared to the wild-type strain. The results observed after the measurement of the Dps protein in various *Salmonella* regulatory mutants were consistent with the results acquired in the reporter assay. This evidence suggested that Fur performs a function as a subsidiary regulator in the expression of *dps*. The survival ability of *Salmonella* strains after exposure to oxidative stress demonstrated that the Dps protein performs a pivotal function in the survival of stationary-phase *S. typhimurium* against oxidative stress. *Salmonella* cells grown in iron-restricted condition required Dps for full protection against oxidative stress. The CK24 (Δ *dps*) cells grown in iron-replete condition survived at a rate similar to that observed in the wild-type strain, thereby suggesting the induction of an unknown protection mechanism(s) other than Dps in this condition.

Keywords: Dps, *S. typhimurium*, iron metabolism, Fur, oxidative stress

The ability of *Salmonella* to survive and replicate in host phagocytes is a crucial factor with regard to its virulence [8, 16]. A number of *Salmonella* mutants evidencing increased susceptibility to oxidative stress also demonstrate

a reduced capacity for survival in macrophages [4, 11, 20, 21, 31]. *Salmonella* is able to augment its resistance to phagocyte-derived ROS via the induction of several pathways that are controlled by a number of regulatory systems, including OxyRS [15], SoxRS [15], and RpoS [19].

In cases in which *Salmonella* was exposed to multiple stresses, including oxidative stress, DNA binding protein in stationary phase (Dps) was identified as one of the principal overexpressed proteins. Dps enables binding to chromosomal DNA via nonspecific mechanisms, and also physically protects the DNA against multiple stresses [1, 18, 24]. The Dps protein has been suggested to prevent the formation of ROS via interactions with iron, and also to physically protect DNA against certain dangerous materials, a consequence of its structural properties [34].

Although many elegant studies of Dps have been conducted with *Escherichia coli*, the function of Dps in *Salmonella* pathogenesis had, until now, remained poorly understood. The expression of the *dps* gene was previously determined to be induced after ingestion by macrophages [32], and a *dps* gene-deficient mutant was shown to lower survival ratios in macrophages [26].

In recent analyses of the structure of Dps, it has been determined that Dps evidences a significant degree of structural homology with the ferritins, which exhibit the ability to sequester and store iron [34]. Oxidative stress has been associated closely with iron metabolism [29]. Iron is a crucial mineral for the vast majority of organisms, including *Salmonella*, as it functions as a cofactor in several metabolism-associated enzymes [22, 33]. However, iron is also potentially toxic, owing to the low solubility of the stable oxidation state, Fe(III), in addition to its tendency to generate ROS [13]. Therefore, *Salmonella* has developed a number of systems that allow it to acquire and store iron, simultaneously minimizing its danger (perhaps by storing it in a nontoxic form) [2]. The Ferric uptake regulator (Fur) protein is the primary regulator of a number

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of genes that are involved in the iron metabolism [14]. The Fur protein functions either as an activator or as a repressor, depending on the genes to be expressed [3].

This study attempted to address our current dearth of knowledge regarding the mechanisms operant upon the exposure of *Salmonella* to complex stresses, including both extreme oxidative stress and iron stress. In this study, we have characterized the function of Fur in the expression of the *dps* gene, as well as the roles of Dps in response to iron stress.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids employed in this study are listed in Table 1. *Salmonella typhimurium* and *E. coli* were grown at 37°C in Luria-Bertani (LB) broth or LB agar. Antibiotics, when required, were added to the culture

medium at the concentrations described previously in Kang *et al.* [17]. Diaminopimelic acid (DAP) was added (50 µg/ml) for the *asd* strains. LB agar containing 5% sucrose was employed for *sacB* gene-based counter-selection in the allelic exchange experiment [9]. In order to create iron-depleted or iron-replete growth conditions, 2,2'-dipyridyl (200 µM) or FeCl₃ (100 µM) was added to the appropriate media, respectively.

General DNA Manipulations

DNA manipulations were conducted as described by Sambrook and Russel [28]. Transformation into *E. coli* or *Salmonella* was conducted *via* either rubidium chloride heat shock or electroporation (Bio-Rad). The recombinant suicide plasmid was transferred conjugally into *Salmonella* using *E. coli* χ 7213 (*asd*^r) as a plasmid donor. PCR amplification was employed in order to acquire DNA fragments for the cloning and verification of the mutated genes on the chromosome.

Table 1. Bacterial strains, plasmids, and primers used for this study.

Strain, Plasmid, Primer	Relevant characteristics	Reference or Source
Strain		
<i>Escherichia coli</i>		
<i>E. coli</i> DH5 α	Transformation host for cloning vector, F ⁻ , Φ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (r _k ⁻ ,m _k ⁺) <i>phoA supE44</i> l ⁻ <i>thi</i> ⁻¹ <i>gyrA96 relA1</i>	Promega
<i>E. coli</i> Top10	Transformation host for cloning vector, F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) F80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 ara</i> Δ 139 Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^r) <i>endA1 nupG</i>	Invitrogen
<i>E. coli</i> χ 7213	<i>E. coli</i> DH5 α derivative (Δ <i>asd</i>), Km ^r , DAP required	[27]
<i>Salmonella</i>		
<i>S. typhimurium</i> χ 3339	SL1344, <i>hisG</i> , Str ^r , wild type	[12]
<i>S. typhimurium</i> χ 4659	χ 3339 derivative, <i>hisG fur</i> ::Tn10, Tet ^r	Lab collection
<i>S. typhimurium</i> CK24	χ 3339 derivative, <i>hisG</i> Δ <i>dps</i>	This study
<i>S. typhimurium</i> CK32	χ 3339 derivative, <i>hisG</i> Δ <i>rpoS</i>	Lab collection
<i>S. typhimurium</i> CK59	χ 4659 derivative, <i>hisG fur</i> ::Tn10 Δ <i>rpoS</i> , Tet ^r Km ^r	Lab collection
Plasmid		
pDMS197	Suicide vector, R6K <i>ori</i> , Tet ^r	[7]
pGEM-T vector	Cloning vector for PCR product, ColE1 <i>ori</i> , Ap ^r	Promega
pBP37	6.3 kb EcoRI-PstI <i>lacZYA</i> DNA in pWSK29, Ap ^r	Lab collection
pBP130	2.0 kb DNA containing 5'-flanking and 3'-flanking regions of <i>dps</i> in pGEM-T vector, Ap ^r	This study
pBP132	Derivative of pDMS197, recombinant suicide plasmid for Δ <i>dps</i> , Tet ^r	This study
pBP195	1.0 kb KpnI-HindIII DNA from pBP152 in pBP37, Ap ^r	This study
Primer		
DLN (XbaI)	5'-gctctagatgcgcttttccaaag-3'	
DLC (BamHI)	5'-gcggatccgtcattatttagcggactcat-3'	
DRN (BamHI/SalI)	5'-gcggatccgggctcgactcacgtcgtgaaaa-3'	
DRC (KpnI)	5'-gcggtaccggcgtctgcgcggtt-3'	
RLC (XbaI)	5'-gaattctgaacttcagcgtattctg-3'	
RLN (EcoRI)	5'-gaattcgcaggggctgaatatcgaag-3'	
RRC (EcoRI)	5'-gaattcgcaggggctgaatatcgaag-3'	
RRN (XbaI)	5'-tctagatggacggaatcgactacc-3'	

SDS-PAGE and Immunoblot Analysis

The protein samples were solubilized in 2× digestion buffer [28]. Protein samples suspended in digestion buffer were boiled for 5 min and separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [28]. The separated proteins were stained with Coomassie Brilliant Blue G-250 (Sigma), and then destained using destaining solution.

For immunoblotting, the SDS-PAGE-separated proteins were transferred to nitrocellulose membranes (Bio-Rad) through a modified version of the procedures described by Towbin *et al.* [30]. The membranes were blocked in 5% skim milk and then incubated for 2 h with suitably diluted antisera solution, followed by treatment with a 1:1,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) solution. Immunoreactive bands were detected by the addition of 4-chloro-1-naphthol (Sigma) in the presence of H₂O₂ (Sigma). The reaction was halted by washing with several changes of large volumes of deionized water.

Construction of a *dps::lacZYA* Fusion Plasmid

In order to determine *dps* expression at the transcriptional level, the *dps::lacZYA* fusion plasmid was constructed in the plasmid pBP37 backbone. The pBP37 plasmid harbors pSC101 *ori* and the *lacZYA* reporter gene, which is localized in opposite orientation to the *bla* gene. The reporter gene can be transcribed by the promoter element cloned into the multicloning site, which is located upstream of the reporter gene. The 1.0 kb *dps* promoter region encompassing the putative promoter and the N-terminus of the Dps was PCR-amplified from *S. typhimurium* χ 3339 chromosomal DNA as a template, using a pair of the primers DLN (XbaI) and DLC (BamHI) (Table 1). The 1.0 kb amplified DNA fragment restricted with the XbaI and BamHI enzymes was cloned into pBP37, resulting in the formation of pBP195.

β -Galactosidase Assays

β -Galactosidase assays were conducted in accordance with the methods previously described by Miller [25]. *S. typhimurium* cells grown under various conditions were permeabilized with chloroform and 1% SDS. Enzyme activity was spectrophotometrically measured at 420 nm by measuring the rate of the cleavage of the chromogenic substrate, *o*-nitrophenyl- β -D-galactopyranoside (ONPG). The activity was expressed in Miller units.

Constructions of a *S. typhimurium dps* Deletion Mutant

The 1.0 kb 5'-flanking and 1.0 kb 3'-flanking regions of the *dps* gene were amplified by PCR from *S. typhimurium* χ 3339 chromosomal DNA as a template, using the primer pairs DLN (XbaI) and DLC (BamHI), and DRN (BamHI/SalI) and DRC (KpnI), respectively (Table 1). Each of the

PCR products was ligated within the pGEM-T vector, resulting in the formation of pBP130. The 2.0 kb recombinant DNA in pBP130 deletes the complete *dps* ORF. The 2.0 kb XbaI-KpnI DNA fragment isolated from pBP130 was subcloned into the suicide vector pDMS197, resulting in the formation of the recombinant suicide plasmid pBP132 (Table 1).

The Δ *dps* mutation was introduced in *S. typhimurium* χ 3339 by allelic exchange using the suicide vector pBP132, yielding *S. typhimurium* CK24. The presence of the 0.5 kb deletion was confirmed by PCR with the primer set DLN (XbaI) and DRC (KpnI).

Oxidative-Stress Assays

Salmonella cells were cultured in 30 ml of iron-replete or iron-depleted LB broth until late stationary phase (for 18 h) at 37°C with shaking. In order to create the H₂O₂-induced oxidative stress conditions, various concentrations of H₂O₂ were added to 1-ml aliquots of stationary-phase cultures, and incubation was continued for 1 h at 37°C with aeration. Viable-cell counting was conducted in order to assess the sensitivity of *Salmonella* against H₂O₂-induced oxidative stress. Cell numbers counted at the point at which H₂O₂ was added were considered as initial numbers of the cells. In order to determine the H₂O₂ sensitivity for cells grown to log phase, overnight cultures were inoculated into fresh LB broth at a ratio of 1:100 (v:v), and incubation was continued for 2 h at 37°C with aeration. The viable cell counts were determined after 1 h of H₂O₂ treatment to the culture. All assays were conducted in at least three independent experiments.

RESULTS

Analyses of *Salmonella dps* Promoter Region

The Dps protein is composed of 167 amino acid residues and has a molecular mass of 18.7 kDa. The Dps protein in *S. typhimurium* evidences up to 95% homology with those detected in other enteric Gram-negative bacteria, including *E. coli*, *Shigella*, and *Yersinia*. The nucleotide sequences of the promoter region of *Salmonella dps* share 95% identity with that of the promoter region of *E. coli dps* (data not shown), thereby suggesting that the *Salmonella dps* promoter may behave similarly to the *E. coli dps* promoter. With the existence of OxyR, IHF, and CRP binding motifs, two putative Fur boxes were predicted within the promoter region of the *S. typhimurium dps* gene (Fig. 1). Box 1, which is located 146 nucleotides upstream from the *dps* transcription start site, shared 74% (14/19 nucleotides) identity with known Fur-binding consensus sequences. It was determined to overlap with the CRP binding motif. Another one, box 2, was positioned 95 nucleotides upstream from the *dps* transcription start site. Based on

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acaagttgacgtaaaatagtttcttattctgcatgatgtgcataatcatgcagaata
tcacctcgttattcaccttttattatttggcgtatttagttcgtcattttttgctcatat
ttttttcatttttttaccataaatgagaattattaccpaccaaacagc aaactcgt
                Putative CRP binding site
                Putative Fur box 1
ctaaccaattgattttaatcaaaataaaatattaaatcttgcctgactattaaagt
IHF binding site Putative Fur box 2
gataggtaaatcaaaaaaagcaaaagttaaaaaccggtgctatacttattttcgttaa
Putative OxyR binding site           $\sigma^s$ (RpoS)
ttacctgggacacaaacatcaagggatagagattatgagtagcggtaaacctggta
                RBS                m s t a k l v
aaaacaaaagcgtctaatacgtctttatacccgtaacgatgtatcagagagcgataaa
k t k a s n l l l y t r d v s e s d k

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Fig. 1. Analyses of the *Salmonella dps* promoter region.

The nucleotide sequence of the putative promoter region of the *Salmonella dps* gene is shown. Position +1 represents the transcription start site. The -10 region recognized by σ^s (RpoS) is underlined with indication. The ATG translation start site and the ribosome binding sites (RBS) are indicated in bold face. The IHF, OxyR, and CRP binding sites are indicated with dotted lines. Two open boxes indicate the regions of the putative Fur binding sites.

these analyses, we hypothesized that the Fur regulator may contribute to the regulation of *dps* gene expression in *Salmonella*.

Iron-independent and Fur-dependent Expression of the *dps* Gene

In order to measure *dps* expression at the transcriptional level, a *dps::lacZYA* transcriptional fusion plasmid, named plasmid pBP195 (pSC101 *ori*), was constructed as described in the Materials and Methods section. *S. typhimurium* χ 3339 harboring pBP195 grown in LB broth evidenced β -galactosidase activity. Little or no β -galactosidase activity was detected in *S. typhimurium* χ 3339 harboring pBP37 (a control plasmid carrying promoterless *lacZYA*). The β -galactosidase activity profile was proportional to the cell's growth phase; specific activity increased along with culture time (data not shown). The maximum β -galactosidase activity in cells at the stationary phase is consistent with the growth phase dependency of *dps* expression observed in previous studies [23], thereby indicating that the *dps* promoter region cloned in pBP195 operates normally.

In order to assess *dps* expression in response to iron-stress conditions, β -galactosidase activity was measured along with culture of *S. typhimurium* χ 3339 harboring pBP195 in iron-replete or -depleted conditions. Although we expected to observe different levels of β -galactosidase activity in cells grown under iron-replete or -depleted conditions, the β -galactosidase activity profile revealed growth-phase dependency regardless of iron status in culture conditions (Fig. 2). Interestingly, the β -galactosidase activity in *S. typhimurium* χ 4659 (null mutant for *fur*) harboring

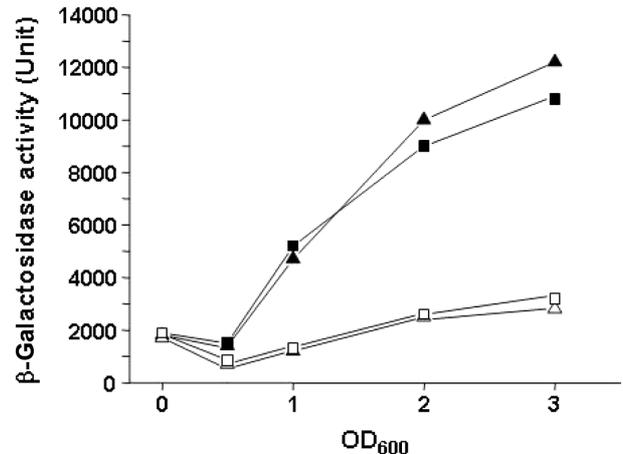


Fig. 2. Fur-dependent expression of the *dps* gene.

Salmonella cells were cultured in iron-replete (triangles) or iron-depleted (squares) LB broth. The β -galactosidase activities in wild-type *S. typhimurium* χ 3339 (solid symbols) and *fur* mutant *S. typhimurium* χ 4659 (open symbols) harboring pBP195 were measured along with the culture. The activities expressed as mean values of triplicate measurements are expressed in Miller units.

pBP195 was dramatically reduced as compared with that of *S. typhimurium* χ 3339 harboring pBP195. The χ 4659 mutant harboring pBP195 expressed a similar level of β -galactosidase regardless of iron conditions, as was also observed in *Salmonella* χ 3339 harboring pBP195. These results indicate that the iron status in culture conditions does not contribute significantly to *dps* expression. Additionally, the association of the Fur regulator with *dps* expression was determined.

Requirement of Fur for the Full Induction of *dps* Gene Expression

RpoS, an alternative sigma factor implicated in the response to stress-induced gene expression, is known to be a typical positive regulator of *dps* expression in *S. typhimurium* [19]. Evidence for the association of Fur with *dps* expression (Fig. 2) enforced the investigation of roles of Fur in RpoS-mediated *dps* expression. *S. typhimurium* strains, χ 3339 (wild-type), χ 4659 (*fur*⁻), CK32 (Δ rpoS), or CK59 (*fur*⁻, Δ rpoS) harboring pBP195 were grown until the stationary phase in LB broth in order to measure the levels of β -galactosidase. The *fur* mutant χ 4659 evidenced a reduced level of β -galactosidase as compared with the wild-type strain (Fig. 3A). As had been expected, the CK32 and CK59 strains expressed dramatically reduced β -galactosidase activity, due to a lack of RpoS. Interestingly, CK59 (*fur*⁻, Δ rpoS) harboring pBP195 revealed levels of β -galactosidase that were lower (50%) than was observed with CK32, indicating that Fur, in addition to RpoS, is required for full *dps* expression.

In order to provide direct evidence for Dps expression, we conducted Western blotting in order to detect the

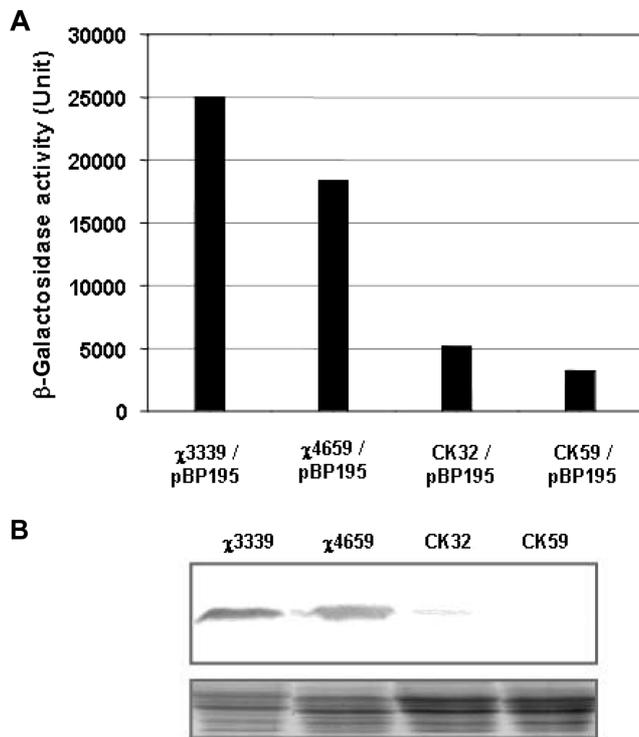


Fig. 3. Requirement of Fur for the full expression of *dps*. **A.** β -Galactosidase activities in the *Salmonella* defecting regulatory proteins. β -Galactosidase activities were measured in *S. typhimurium* cells harboring pBP195 cultured in LB broth until the stationary phase. Levels are expressed as the mean values of triplicate measurements. *S. typhimurium* strains; wild-type, χ 3339; *fur* mutant, χ 4659; *rpoS* deletion mutant, CK32; double mutant (*fur*⁻, Δ *rpoS*), CK59. **B.** Western blot analysis of Dps protein. *Salmonella* cells cultured in LB broth until stationary phase were subjected to Western blotting. The immunoreactive Dps protein was detected using the Dps-specific polyclonal antibody generated in another study. Equal amount of sample loading was confirmed by comparisons of the intensity of Coomassie-stained bands after SDS-PAGE. Strains (bottom panel). The same *Salmonella* strains designated in panel A were used.

protein using Dps-specific polyclonal antibody. The χ 4659 *fur* mutant expressed a reduced quantity of Dps protein as compared with the wild-type strain (Fig. 3B). Although dramatically reduced levels of Dps were detected in the CK32 strain, only a small amount of Dps was detected. No Dps protein was detected in the CK59 strain. An equal amount of sample loading for Western blotting was demonstrated by comparing the intensity of Coomassie-stained bands following SDS-PAGE. All of the indirect (by β -galactosidase assay) and direct (by Western blot) evidence suggested that Fur performs a function as a subsidiary regulator in RpoS-mediated *dps* expression.

Growth Phase-dependent Hypersensitivity of *Salmonella* Against Oxidative Stress

In order to characterize the function of the Dps protein in *Salmonella*, we attempted to determine the survival ability of cells after exposure to the oxidative stress conditions

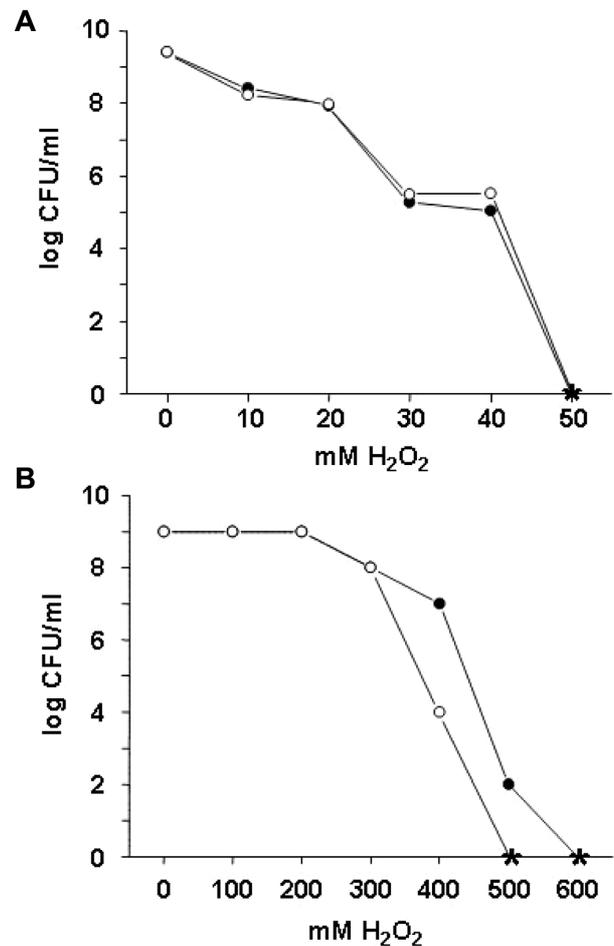


Fig. 4. Sensitivity of *dps* mutant to oxidative stress. *S. typhimurium* χ 3339 (solid symbols), or CK24 (open symbols) cells grown to exponential (A) or stationary (B) phase in LB broth were exposed to various H₂O₂ concentrations for 1 h. Viable-cell counting was conducted to determine the number of survived cells. Asterisks indicate that no cells survived under those conditions. All assays were conducted at least three times.

induced by hydrogen peroxide (H₂O₂). Both *S. typhimurium* χ 3339 and CK24 strains grown at the exponential phase proved to be extremely sensitive to oxidative stress (Fig. 4A). Approximately 10% of the cells survived after treatment with 10 mM H₂O₂. Treatment with 50 mM H₂O₂ killed all of the cells. By way of contrast with the cells in exponential phase, the cells grown at the stationary phase maintained viability even in the presence of 200 mM H₂O₂ (Fig. 4B). However, the χ 3339 and CK24 strains showed apparently different viability following treatment with more than 400 mM H₂O₂. The viable counts of the CK24 strain were 3 log CFU lower than that of χ 3339 after treatment with 400 mM H₂O₂. These results demonstrate that the Dps protein performs a pivotal function in the survival of stationary-phase *S. typhimurium* against oxidative stress.

Requirement of Dps for Survival Against Oxidative Stress Under Iron-restricted Condition

In general, bacterial cells exploit a variety of protection systems to protect cells against attack by reactive oxygen species, which are generated principally after reaction with H_2O_2 [10]. Therefore, cells exposed to iron-depleted or iron-replete conditions may develop different levels of protection ability against oxidative stress. Little or no *in vivo* evidence has been discovered regarding the responses of *Salmonella* grown under a variety of iron conditions to oxidative stress, with regard to the Dps protein. In order to examine the survival ability of iron-stressed *Salmonella* against oxidative stress, *S. typhimurium* χ 3339 or CK24 (Δ *dps*) grown to the stationary phase under iron-replete or -depleted conditions was treated with various H_2O_2 concentrations. The survived cells were counted by viable counting procedures. The wild-type strain evidenced resistance against H_2O_2 exposure, regardless of preexposure to iron (Fig. 5). Interestingly, the CK24 (Δ *dps*) cells grown under iron-replete condition survived at a similar level as was observed in the wild-type strain, suggesting the induction of some protection mechanism(s) other than Dps in this condition. The CK24 grown under iron-depleted condition evidenced profound sensitivity to H_2O_2 . The CK24 cells grown under iron-depleted condition survived at a rate of 3 log CFU lower than the cells grown in iron-replete condition coupled with treatment with 150 mM H_2O_2 . These results indicated that *Salmonella* cells exposed

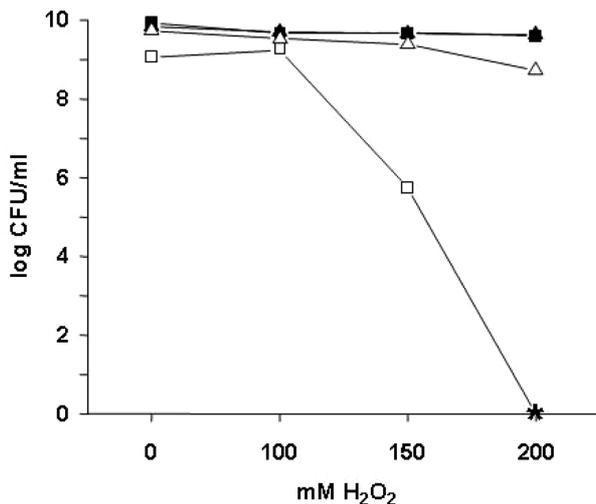


Fig. 5. Survival of iron preexposed *Salmonella* against oxidative stress.

S. typhimurium χ 3339 (solid symbols) and CK24 (Δ *dps*) (open symbols) cells grown until the stationary phase under iron-replete (triangles) or depleted (squares) conditions were exposed to various H_2O_2 concentrations for 1 h. Viable-cell counting was conducted to determine the numbers of survived cells. The asterisk indicates no cells survived under that condition. All assays were conducted at least three times.

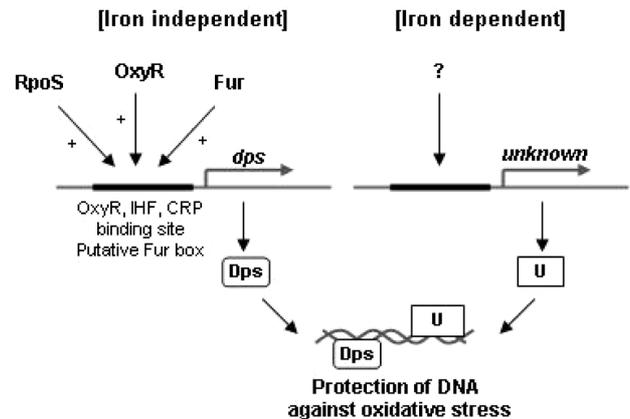


Fig. 6. Model for the *Salmonella* protection mechanism against oxidative stress combined with iron stress.

OxyR, RpoS, and Fur positively regulate *dps* gene expression in response to oxidative stress. Dps-mediated protection occurs regardless of iron. In the presence of iron, an unknown component (designated as U) in addition to Dps may be involved in the protection. The question mark indicates a putative regulator responding to iron restriction.

to high-iron condition operate multiple mechanisms, including Dps, to protect cells against oxidative stress, and cells grown under iron-restricted condition required Dps for full protection against oxidative stress condition.

DISCUSSION

The association of iron metabolism with oxidative stress [29] and the existence of two putative Fur binding boxes (Fig. 1) were two key facts that compelled us to conduct this research. Although Fur functions as either an activator or as a repressor, the repressor function has been studied to a significantly higher degree than have the activator functions. When Fur works as a repressor in the transcription of a gene, the Fur binding sites locate at the -35 and -10 sequences in the promoter of the gene, thereby allowing for the occlusion of the RNA polymerase access [3, 5, 14]. The putative Fur binding sites in the *dps* promoter are positioned at least 35 nucleotides upstream from the -35 sequences (Fig. 1), thereby suggesting that if Fur does function in *dps* transcription, it will not function as a repressor. The association of Fur with the transcription of *dps* was demonstrated by both β -galactosidase assays with *dps::ZYA* transcriptional fusion (Figs. 2 and 3A) and by Western blot analyses (Fig. 3B). In the β -galactosidase analysis, it was apparent that the promoter activity in a strain defecting Fur was far lower than that observed in the wild-type strain. In order to verify our results, we checked the phenotype of the *S. typhimurium* χ 4659 *fur* mutant strain. The χ 4659 strain secreted siderophores under iron-replete condition (data not shown), which is a typical phenotype of the *fur* mutant [14]. The existence of a small

amount of Dps protein in CK32 ($\Delta rpoS$) and the absence of the Dps protein in CK59 (fur^- , $\Delta rpoS$) clearly indicate the roles of Fur and RpoS in *dps* expression; RpoS is the major regulator and Fur functions as a subsidiary regulator in the transcription of *dps* in *Salmonella*. Fur has been reported to function as a positive regulator [6]. The requirement of Fur for the full induction of *dps* expression is described for the first time in this study. However, the question as to the possibility of direct interaction between the Fur protein and the putative Fur binding sites remains to be answered.

The hypersensitivity of cells in the exponential phase against oxidative stress as compared with cells in the stationary phase (Figs. 4A and 4B) was consistent with the results described in previous studies [26], thereby indicating the normal operation of our oxidative stress assay. The *in vivo* role of Dps to protect cells against oxidative stress was confirmed by the lower survival ability of the CK24 strain after exposure to H_2O_2 (Fig. 4B). Although the presence or absence of iron in the media did not influence *dps* expression (Fig. 1), the *dps* deletion mutant cells grown in iron-restricted condition were significantly more sensitive than were the cells grown in iron-replete condition against oxidative stress (Fig. 5), thereby indicating that unknown protection factor(s) other than Dps may be induced in iron-replete condition.

Based on the evidences presented in this study, we hypothesized that *Salmonella* exploits a series of complicated protection mechanisms to protect cells against oxidative stress: multiple protectors including Dps are involved, and these are differentially expressed in different environments (Fig. 6). These complicated systems may allow for the survival of *Salmonella* under a variety of environments occurring in the host during *Salmonella* infection.

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