A Universally Conserved ATPase Regulates the Oxidative Stress Response in Escherichia coli

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Background: YchF is a universally conserved ATPase of unknown function.

Results: YchF inhibits catalase activity, and high YchF levels cause H₂O₂ hypersensitivity. YchF is negatively regulated by OxyR in response to H₂O₂.

Conclusion: YchF is a universally conserved negative regulator of the oxidative stress response that acts by a post-translational mechanism.

Significance: This is the first functional description of the YchF protein family.

YchF is an evolutionarily conserved ATPase of unknown function. In humans, the YchF homologue hOla1 appears to influence cell proliferation and was found to be up-regulated in many tumors. A possible involvement in regulating the oxidative stress response was also suggested, but details on the underlying mechanism are lacking. For gaining insight into YchF function, we used Escherichia coli as a model organism and found that YchF overexpression resulted in H₂O₂ hypersensitivity. This was not caused by transcriptional or translational down-regulation of H₂O₂-scavenging enzymes. Instead, we observed YchF-dependent inhibition of catalase activity and a direct interaction with the major E. coli catalase KatG. KatG inhibition was dependent on the ATPase activity of YchF and was regulated by post-translational modifications, most likely including an H₂O₂-dependent dephosphorylation. We furthermore showed that YchF expression is repressed by the transcription factor OxyR and further post-translationally modified in response to H₂O₂. In summary, our data show that YchF functions as a novel negative regulator of the oxidative stress response in E. coli. Considering the available data on hOla1, YchF/Ola1 most likely execute similar functions in bacteria and humans, and their up-regulation inhibits the ability of the cells to scavenge damaging reactive oxygen species.

P-loop GTPases execute and regulate essential biological processes and show high sequence conservation, resulting in a set of eight GTPase families that are universally conserved in eukaryotes, bacteria, and archaea (1). These GTPases include YihA and HflX, which are involved in ribosome biogenesis; initiation factor 2, elongation factor Tu, and elongation factor G, which are required for protein synthesis; and the signal recognition particle and its receptor (FtsY), which are essential for co-translational protein targeting. It also includes YchF, a member of the Obg family of universally conserved GTPases. YchF differs from the other GTPases because it preferentially hydrolyzes ATP over GTP (2), thereby functioning as an ATPase. This altered nucleotide specificity is due to the replacement of a conserved lysine residue within the nucleotide binding motif by valine in the Escherichia coli YchF or by leucine in the human YchF homologue hOla1 (2, 3).

Although the x-ray structures of YchF from Haemophilus influenzae and human Ola1 have been determined (2, 4), the exact function of these ATPases is unknown. Because of its structural characteristics, YchF was proposed to bind to ribosomes as well as to nucleic acids (4) and was classified as a member of the translation factor family of nucleotide-hydrolysing proteins (TRAFAC family). Consistent with this idea, YchF was shown to bind to ribosomes in the protozoan parasite Trypanosoma cruzi and in the proteobacterium E. coli (3, 5). Additionally, the yeast homologue of YchF, Ybr025c, was suggested to interact with the elongation factor eEF1 (6). These data support a role for YchF during translation, but the physiological significance of YchF binding to ribosomes and possible consequences for translation are currently unknown.

A translation-independent function was suggested for hOla1. Reducing the concentration of hOla1 in HeLa cells increased the cellular resistance to peroxide oxidants and thiol-depleting chemicals, whereas the overexpression of hOla1 increased the sensitivity of HeLa cells toward oxidative stress (7). This could indicate that hOla1 functions as a negative regulator of the oxidative stress response. Whether hOla1 inhibits

References:

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2. This article contains supplemental Table S1.

3. The abbreviations used are: hOla1, human Ola1; Dps, DNA protection during starvation; Ffh, fifty-four homologue; LB, lysogeny broth; pBpa, para-benzoyl-L-phenylalanine; PFA, para-formaldehyde; Pth, peptidyl-tRNA hydrolase.
YchF/hOla1 Inhibit Oxidative Stress Response

Table 1

| Name                  | Sequence                                                                 |
|-----------------------|--------------------------------------------------------------------------|
| YchF fw               | 5′-AACAATTCCCTTCTAGAAATAATTTTG-3′                                       |
| YchF rev              | 5′-CAATCTACGAACGTTTGGGCT-3′                                              |
| Pth fw                | 5′-GGCCATTTATCTAGACACTTGCGG-3′                                           |
| Pth+YchF rev          | 5′-GATTTCGAAAGCTTATAGGAC-3′                                              |
| YchF(S16A) fw         | 5′-TGGGAAAACCAACCTTG-3′                                                 |
| YchF(S16A) rev        | 5′-CTGGGAAACAAACCGAC-3′                                                 |
| YchF(S16E) fw         | 5′-TCCGGGAAACAGCGCT-3′                                                  |
| YchF(S16E) rev        | 5′-GTTGGGCAAAACGGAC-3′                                                  |
| YchF(P11/N12A) fw     | 5′-TTGGCAGCCGACGGCG-3′                                                  |
| YchF(P11/N12A) rev    | 5′-ACGGAGATACGGAATTTGAATCC-3′                                           |
| OxyRFw                | 5′-ATGTTAGTGTCGACTGGAATATTTGTAATATTTGATAGTCCTTGAGTAACCTTG-3′           |
| OxyRev                | 5′-ATCGTAACGGCAAGCTGATAGTGACAT-3′                                       |
| Oxy_R Forward         | 5′-CACAGCATCCGCGTGCCAG-3′                                                |
| Oxy_R Reverse         | 5′-GGGATGCGCCTGGGCGCC-3′                                                 |
| rPl_R Forward         | 5′-AAGTGTGTATATATAGTAAACCCGAT-3′                                        |
| rPl_R Reverse         | 5′-GATTTTGACGCGCGCGCCCG-3′                                               |
| Ychf_R Forward        | 5′-ATTTGCAGCGCATGCTGATAGTGACAT-3′                                       |
| Ychf_R Reverse        | 5′-GCGGTATGCGCTGGTTCGCC-3′                                               |
| Ychf-300fw+bio        | 5′-AAAAGAATGTTCTACATACGCCC-3′                                            |
| Ychf-300rev           | 5′-CATCGGAATCCGCGCTA-TACCAGG-3′                                         |
| Ychf-300rev           | 5′-AAGTGTGTATATATAGTAAACCCGAT-3′                                        |
| YidC-300fw+bio        | 5′-ACCTCAGTAACCCGCCGCTC-3′                                               |
| YidC-300rev           | 5′-CTGCCCTGAGGTGTCGCGG-3′                                                |
| YidC-300fW+bio        | 5′-TGGGCCCTGAGGTGTCGCGG-3′                                               |

Our data identify YchF as an important regulator of the oxidative stress response that functions by a post-translational mechanism.

**Experimental Procedures**

**Bacterial Strains and Growth Conditions**

*E. coli* BW25113 and BL21 were used as wild type *E. coli* strains and were routinely grown on LB medium at 37 °C. The *E. coli* strains JW1194 (Δ*ychF*) and JW3933 (Δ*oxyR*) were provided by the National BioResource Project (National Institute of Genetics, Japan). Both strains were grown on LB medium supplemented with 25 μg/ml kanamycin. Strains carrying pTrc99a-YchF (pYchF) plasmids were supplemented with 50 μg/ml ampicillin, and strains carrying pASK-IBA3C were supplemented with 35 μg/ml chloramphenicol.

**Plasmid Construction**

*ychF* was isolated from chromosomal *E. coli* DNA and cloned via Xhol and NdeI into the plasmid pET-22b. This construct was a gift from S. Angelini, Freiburg, Germany and used for subcloning *ychF* into plasmid pTrc99a. The primers YchF fw and YchF rev (Table 1) were used to insert XbaI and HindIII restriction sites. *pth-ychF* was amplified from chromosomal DNA and cloned into pTrc99a as well. The PCR was performed with Phusion High Fidelity PCR Master Mix (Finnzymes, Vantaa, Finland). PCR fragment purification from the agarose gel was done with a QiAquick gel extraction kit (Qiagen, Hilden, Germany), and the purified fragment as well as the plasmid pTrc99a was used for the restriction digest with HindIII and XbaI from New England Biolabs, Ipswich, MA. Vector and insert were ligated with the Quick Ligation kit (New England Biolabs). After ligation, pTrc99a-YchF was used to construct the mutants YchF(S16A), YchF(S16E), and YchF(P11/N12A) via inverse PCR with the primer pairs listed in Table 1. These constructs were transformed into E. coli BW25113 and JW1194. The plasmid pASK-IBA3C-OxyR was constructed by amplifying *oxyR* from chromosomal *E. coli* DNA using the primers OxyRFw and OxyRev. After purification of the PCR...
product and BsaI digestion, the PCR product was cloned into BsaI-digested pASK-IBA3C vector (IBA, Göttingen, Germany), resulting in OxyR with a C-terminal Strep tag under Tet promoter control. YchF amber stop codon mutants were constructed for in vivo cross-linking by inverse PCR on pTrc99a-YchF using the primers given in Table 1. These constructs were transformed into E. coli BL21 containing pSUP-BpaS-6TRN (19) encoding the orthogonal aminoacyl-tRNA synthetase/tRNA pair for incorporation of para-benzoyl-L-phenylalanine (pBpa) into the position of the amber codon.

**Growth Analyses**

Cells were grown overnight, diluted 1:100 in liquid LB medium, and further incubated until they reached an A_{600} of 0.5–0.8. These cells were then used for measuring H_{2}O_{2} sensitivity by the following methods.

**Spot Assay**—The culture was adjusted to an A_{600} of 0.5 and serially diluted. 10 µl of each dilution was spotted onto LB plates supplemented with or without H_{2}O_{2}. After overnight incubation at 37 °C, plates were analyzed.

**Cell Viability Assay**—Cells were adjusted to an A_{600} of 0.5 and diluted 1:10 in phosphate-buffered saline (PBS) before treatment with 10 mM H_{2}O_{2} in PBS for 50 min at 25 °C; control cells were treated with PBS. Subsequently, cells were harvested by centrifugation, washed with PBS, and resuspended in 1 ml of PBS. 100 µl of this cell culture was transferred to a 96-well plate, and 100 µl of the BacTiter-Glo microbial cell viability assay solution (Promega Corp., Mannheim, Germany) was added. The luminescence of H_{2}O_{2}-treated wild type cells was set to 100%.

**Inhibition Assay**— Cultures were adjusted to an A_{600} of 0.5, mixed with LB-Top agar, and poured on LB plates. Subsequently, sterile filter discs were soaked in 2 mM H_{2}O_{2} solution and placed on top of these plates. After approximately 5 h of incubation at 37 °C, the inhibition zones around the discs were quantified.

**Isolation of RNA and RT-PCR**

RNA isolation was performed by using the Illustra RNAspin minikit from GE Healthcare. RNA was eluted with RNase-free water and tested for quality and purity by agarose gel electrophoresis and absorption measurement. Possible DNA contaminations were detected by running a standard PCR without DNA amplification. RNA was stored at −80 °C. RT-PCR was performed with the RT-PCR kit from Qiagen according to the enclosed manual in 25-µl reactions. The following primers were used: OxyR_RTforward/OxyR_RT reversed, rplB_RTforward/rplB_RT reversed, and YchF_RTforward/YchF_RT reversed.

**Electrophoresis Mobility Shift Assays**

DNA binding of OxyR was detected by the Gelshift™ EMSA kit (Active Motif, La Hulpe, Belgium). In brief, the 300-nucleotide upstream region of *ychF* was amplified using the biotinylated primer YchF-300fw+bio and the primer YchF-300rev. The PCR product was purified using the QIAquick gel extraction kit (Qiagen). As a control, the 300-nucleotide upstream region of *yidC* was amplified using the biotinylated primer YidC-300fw+bio and the primer YidC-300rev. The DNA (20-fmol final concentration) was incubated with 20 fmol or 1,500 fmol of purified OxyR for 20 min at 23 °C in the provided EMSA buffer. Subsequently, 5 µl of the provided loading dye was added, and the sample was separated on a 5% Tris borate-EDTA acrylamide gel at 100 V and 4 °C. The gel was blotted onto a nylon membrane (Whatman Nytran SPC membrane), and the DNA was UV-cross-linked to the membrane for 1.5 min. Binding of OxyR to biotinylated DNA was detected by chemiluminescence using horseradish peroxidase-coupled streptavidin.

**Protein Purification**

YchF was purified via its C-terminal His tag. Cells were grown to an A_{600} of ~0.8 and then for additional 30 min incubated with or without H_{2}O_{2}. Protein expression was not induced by isopropyl β-D-1-thiogalactopyranoside because the basal expression level from the lac promoter was sufficient. Cells were collected by centrifugation and resuspended in extraction buffer (50 mM HEPES, pH 7.6, 1 mM ammonium acetate, 10 mM magnesium acetate) using 1 ml of buffer/1 g of wet cell pellet. Before cells were lysed by French pressing (two passes at 8,000 p.s.i.), 0.5 mM PMSF (Roche Applied Science) and 1 × Complete protease inhibitor mixture (Roche Applied Science) were added to prevent protein degradation. Cell debris was removed by 30-min centrifugation at 15,500 rpm (Sorvall SS-34 rotor), and the supernatant was incubated with Talon metal affinity beads (Clontech) for 1 h at 4 °C. After centrifugation at 1,000 rpm for 10 min, the supernatant was removed, and the Talon-bound material was washed four times with 5 mM imidazole in extraction buffer. After washing, the material was placed on a column and eluted with 200 mM imidazole in extraction buffer. This buffer was exchanged with HT buffer (100 mM HEPES, pH 7.6, 200 mM potassium acetate, 20 mM magnesium acetate) via a PD-10 column (GE Healthcare). The quality of the purified protein was controlled via SDS-PAGE and PAGE blue staining, and the purified protein was stored at −20 °C.

OxyR was purified from *E. coli* BL21 cells carrying pASK-IBA3C-OxyR that were induced at an A_{600} of 0.3 with 200 µg of anhydrotriacetylcyclone H_{2}O_{2} and cell extracts were prepared as described above with the exception that all steps were performed in buffer W (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). Cell extracts were added to buffer W-equilibrated streptavidin columns and washed five times with 1 column volume of buffer W. Proteins were eluted with 2.5 mM desthiobiotin in buffer W, and purification was controlled via SDS-PAGE. Fractions containing pure OxyR were pooled and subjected to protease Xa cleavage to remove the Strep tag. The protease digestion was performed in a 50-µl reaction at 23 °C overnight under the following conditions: 50 µg of OxyR, 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl_2, 1 µl of Protease Xa (2 units/µl; Qiagen). The cleaved Strep tag was removed via streptavidin columns, and complete cleavage was controlled via Western blot and anti-Strep antibodies (IBA).
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In Vivo Formaldehyde Cross-linking

*E. coli* BW25113 pYchF was grown to an A_{600} of 1.0 in LB medium and treated for 30 min with 20 mM H_{2}O_{2}. Subsequently para-formaldehyde (PFA) in PFA buffer (136 mM NaCl, 2.7 mM KCl, 1.8 mM KH_{2}PO_{4}, 10 mM Na_{2}HPO_{4}, pH 6.5) was added to a final concentration of 0.1%, and cells were incubated for 30 min at 37 °C before the cells were harvested. For cell fractionation, cells were resuspended in extraction buffer (see above) supplemented with 50 μM PMSF and 100 μg/μl lysozyme. Cells were incubated for 30 min at 37 °C and subjected to repeated freezing and thawing cycles before French pressing. Further processing and YchF purification followed the protocol described above.

In Vivo Site-directed Cross-linking

*E. coli* BL21 cells carrying plasmids pSUP-BpaRS-6TRN and pYchF or its derivatives were grown overnight and diluted 1:200 in 400 ml of minimal medium (19). 440 ml of 0.8 M pBpa in 1 M NaOH was added to each culture, and the cells were grown at 30 °C for 22 h. The expression of YchF and the YchF amber stop codon mutants was then induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside, and growth was continued for another 4 h. Subsequently, the cells were harvested by centrifugation at 15,800 rpm (Sorvall SS-34 rotor) and resuspended in 20 ml of 50 mM triethanolamine acetate, pH 7.5, 30% glycerol for storage at −80 °C. Cells were thawed and centrifuged for 12 min at 5,000 rpm before the pellet was washed with 1× PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_{2}HPO_{4}, 1.76 mM KH_{2}PO_{4}, pH 7.8) and centrifuged a second time. Subsequently, the cells were resuspended in 8 ml of PBS buffer and transferred into two 6-well microtiter plates for photocross-linking. One plate was exposed to UV light for 20 min, and the other remained unexposed. The cells were then centrifuged again for 7 min at 5,000 rpm and resuspended in EPTx buffer (50 mM HEPES, 1 mM ammonium acetate, 20 mM magnesium acetate, 1% Triton X-100). Prior to French pressing, 10 μl each of 0.5 mM PMSF (Roche Applied Science) and 1× Complete protease inhibitor mixture (Roche Applied Science) was added to each tube. The cells were then subjected three times to a French pressure step and centrifuged at 15,800 rpm (Sorvall SLA-3000 rotor) and resuspended in 2.7 mM KCl, 1.8 mM KH_{2}PO_{4}, 10 mM Na_{2}HPO_{4}, pH 6.5) for 10 min on ice. After 10-min centrifugation at 13,000 rpm in a table top centrifuge, 200 μl of the supernatant, which contained the hydrolyzed 33P, was added to 3 ml of scintillation mixture. The samples were measured in a scintillation counter (PerkinElmer Life Sciences), and after preparing a calibration curve, the hydrolyzed ATP was calculated.

ATP Hydrolysis Assay

Purified YchF was incubated with γ-33P-labeled ATP to analyze the ATP hydrolysis rate. The radioactive nucleotide was diluted 1:500 with non-labeled ATP, and a 1–100 μM concentration of this solution was incubated with YchF (50 ng if not indicated otherwise) in reaction buffer (50 mM Tris-Cl, pH 7.5, 5 mM MgCl_{2}, 200 mM NaCl) at 37 °C for 10 min. Subsequently, 800 μl of 5% charcoal in 20 mM phosphoric acid was added for absorbing ATP and ADP, and the samples were incubated for 10 min on ice. After a 10-min centrifugation step at 13,000 rpm in a table top centrifuge, 200 μl of the supernatant, which contained the hydrolyzed 33P, was added to 3 ml of scintillation mixture. The samples were measured in a scintillation counter (PerkinElmer Life Sciences), and after preparing a calibration curve, the hydrolyzed ATP was calculated.

Mass Spectrometric Analysis

For the identification of putative interaction partners, YchF was affinity-purified as described above in the presence of phosphatase inhibitors (2 mM sodium orthovanadate, 9.5 mM NaF, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate), and eluates were subjected to tryptic in-solution digestion (trypsin to protein ratio, 1:50) for 4 h at 42 °C and analyzed by LC/electrospray ionization-MS using an UltiMate 3000 RSLCnano/LTQ-Orbitrap XL system (Thermo Fisher Scientific, Bremen, Germany) essentially as described previously (20). Mass spectrometric data sets were processed using the software suite MaxQuant (version 1.2.7.4) (21, 22). Proteins were identified by searching peak lists against the EcoProt database (v.31.01.2012; 4,275 entries; tagged YchF sequence added) applying a false discovery rate of <1% at both the peptide and protein levels. Criteria for protein identification were as follows: MS and MS/MS mass tolerances of 6 ppm and 0.5 Da, respectively; at least one unique peptide (>7 amino acids); a maximum of two missed cleavages; methionine oxidation and
phosphorylation at serine, threonine, and tyrosine were considered as variable modifications. Quantitative information about proteins was obtained using the label-free protein quantification option in MaxQuant with default settings and the “match between runs” option with a retention time window of 2 min. Only razor and unique peptides were considered.

RESULTS

**YchF Overexpression Causes H$_2$O$_2$ Hypersensitivity in E. coli**—For analyzing the possible contribution of YchF to the oxidative stress response in E. coli, the H$_2$O$_2$ resistance of wild type (WT) E. coli cells, a YchF deletion strain (ΔychF), and E. coli cells expressing YchF under the control of the lac promoter from the plasmid pTrc99A (pYchF) was determined. On LB agar plates without H$_2$O$_2$, cell growth of all strains was comparable (Fig. 1A), but on plates containing 2 mM H$_2$O$_2$, wild type cells overexpressing YchF showed a significant growth inhibition. The same H$_2$O$_2$ concentration did not significantly impair the growth of WT E. coli cells lacking an additional plasmid copy of ychF. Growth of the ΔychF strain in the presence of H$_2$O$_2$ was slightly better than that of the WT but was also impaired by overexpression of ychF from a plasmid although to a lower degree than WT E. coli.

The H$_2$O$_2$ hypersensitivity of ychF-overexpressing cells was confirmed by two additional quantitative assays. In cell viability assays, the metabolic activity of cells after a 50-min exposure to 10 mM H$_2$O$_2$ was analyzed by measuring their ATP content via luciferase luminescence. The luminescence signal of H$_2$O$_2$-treated wild type E. coli was set to 100% and taken as reference. Cells overexpressing YchF showed a significantly reduced luminescence signal, indicating that viability of these cells was drastically compromised after H$_2$O$_2$ treatment (Fig. 1B), whereas the signal of the ΔychF strain was slightly stronger than in the wild type (Fig. 1B). These data confirm the results from the spot test (Fig. 1A). An agar disk diffusion test further validated that increased YchF concentrations caused increased H$_2$O$_2$ sensitivity in E. coli. Paper disks were soaked in H$_2$O$_2$ and placed on top of the cell agar mixture. After 4 h of incubation, inhibition zones were measured. The data were obtained in at least three independent experiments, and the mean values are shown. D, Western blot using whole cells (approximately 0.5 × 10$^8$ cells) precipitated on ice for 30 min with trichloroacetic acid (TCA). After centrifugation, the pellet was resuspended in SDS loading buffer and separated by SDS-PAGE. After Western transfer, the membrane was probed with α-YchF antibodies. As a control, antibodies against Ffh were used. E, the agar diffusion assay using the indicated strains was performed as described in C.
visible in the spot assay (Fig. 1A). The H$_2$O$_2$ sensitivity in the ΔychF strain containing pYchF was weaker than in the pYchF-carrying wild type strain, which is probably related to the lower amount of ychF in this strain (Fig. 1D).

For correlating the observed growth defects with the cellular YchF concentrations, we performed Western blotting using peptide antibodies against YchF. The YchF concentration was significantly increased in cells carrying a plasmid-borne copy of ychF (pYchF), but the concentration in ΔychF+pYchF was lower than in WT+pYchF (Fig. 1D), which probably explains why H$_2$O$_2$ sensitivity was less pronounced in ΔychF+pYchF (Fig. 1, A and C). Antibodies against Ffh, the protein component of the bacterial signal recognition particle, were used as a control.

In E. coli and many enterobacteria, ychF is co-transcribed with pth, which encodes an essential peptidyl-tRNA hydrolase (23–25). We therefore tested whether overexpression of ychF-pth caused the same H$_2$O$_2$ sensitivity as overexpression of ychF alone. The H$_2$O$_2$ hypersensitivity of WT and ΔychF strains expressing either ychF alone or ychF-pth was comparable (Fig. 1E), indicating that H$_2$O$_2$ hypersensitivity is correlated with the YchF concentration and that Pth had no significant impact. As additional controls, we analyzed the H$_2$O$_2$ hypersensitivity of a strain lacking OxyR (ΔoxyR), the major transcriptional regulator of the oxidative stress response (26), and a wild type strain overexpressing Ffh (pFfh), which is, like YchF, one of the eight universally conserved NTPases (1). The ΔoxyR strain displayed an enhanced sensitivity toward H$_2$O$_2$, which was more pronounced than the H$_2$O$_2$ sensitivity of ychF-overexpressing strains, suggesting that overexpressing ychF does not completely block the oxidative stress response. Overexpression of Ffh did not significantly influence the H$_2$O$_2$ sensitivity of E. coli, further substantiating that H$_2$O$_2$ hypersensitivity is specifically the result of YchF overexpression. In summary, our data show that high levels of YchF cause hypersensitivity toward oxidative stress in E. coli and thus result in the same phenotype as the overexpression of hOla1 in humans (7).

YchF Does Not Influence the Cellular Concentrations of KatG and OxyR—Based on the presence of a putative nucleic acid binding motif within YchF (2, 4) and its ability to bind to E. coli ribosomes (3), it appeared likely that ychF overexpression influenced the expression of oxidative stress response proteins like OxyR and the catalase KatG (26). The expression of these proteins was therefore analyzed by RT-PCR and Western blot analyses. Treating E. coli cells with H$_2$O$_2$ did not significantly influence the amount of the oxyR mRNA (Fig. 2A, upper panels). This is in agreement with previous data showing that H$_2$O$_2$ activates OxyR by specific cysteine oxidation (27) but does not significantly change the cellular OxyR concentration (28). H$_2$O$_2$ stress also did not change the amount of oxyR mRNA in ΔychF cells or in cells overexpressing ychF (Fig. 2A, upper panels). As a control, the mRNA level of the ribosomal protein L2 was analyzed, and this revealed that comparable amounts of total mRNA were used in these assays. Like the mRNA level, we did not notice any difference in the OxyR protein levels (Fig. 2A, lower panels). This demonstrated that the amount of YchF does not significantly influence the transcription or translation of OxyR. Thus, YchF probably functions downstream of OxyR,

which would also explain why H$_2$O$_2$ hypersensitivity in the ΔoxyR strain is more pronounced than in the ychF-overexpressing strain (Fig. 1E).

KatG is the major catalase during exponential growth of E. coli, and its expression is induced by OxyR in response to H$_2$O$_2$ (26, 27). When cells were treated with H$_2$O$_2$, we observed an increase in the cellular amounts of KatG by Western blotting using α-KatG antibodies (Fig. 2B). However, a similar increase in the cellular KatG concentration was also observed in cells overexpressing ychF (Fig. 2B). The membrane protein YidC served as a loading control. In summary, these data demonstrate that the H$_2$O$_2$ hypersensitivity at high YchF concentrations is not the result of a YchF-dependent down-regulation of major oxidative stress response proteins like KatG and OxyR and not the result of increased KatG degradation.

YchF Suppresses the Oxidative Stress Response by Direct Interaction with KatG—We next analyzed whether H$_2$O$_2$ hypersensitivity was the result of a direct YchF-dependent inhibition of H$_2$O$_2$-detoxifying enzymes like catalases. Catalase activity was measured in cell extracts from H$_2$O$_2$-treated wild type cells, ΔychF cells, and ychF-overexpressing cells. Catalase activity was low in wild type cells without H$_2$O$_2$ treatment (Fig. 3A) but was stimulated approximately 5-fold in extracts from cells treated with 0.5 or 20 mM H$_2$O$_2$ (Fig. 3A). This increased activity is in agreement with the higher KatG concentration in
H$_2$O$_2$-treated cells as observed by Western blotting (Fig. 2B). In comparison, the basal catalase activity was reduced by approximately 50% in cell extracts from ychF-overexpressing cells (Fig. 3A). Importantly, we did not observe a strong H$_2$O$_2$-dependent stimulation of catalase activity in these cells, which explains why ychF-overexpressing cells are H$_2$O$_2$-hypersensitive. In ΔychF cell extracts, the basal catalase activity was about 4-fold higher than in wild type extracts and only slightly increased further when cells were treated with 0.5 or 20 mM H$_2$O$_2$. In summary, these data demonstrate that high concentrations of YchF inhibit catalase activity in *E. coli*.

H$_2$O$_2$ is detoxified in *E. coli* by three different enzymes. AhpCF functions as an FAD-dependent hydroperoxide reductase that can reduce H$_2$O$_2$ in an NADH/H$^+$-dependent reaction. However, AhpCF is easily saturated when intracellular H$_2$O$_2$ concentrations exceed 20 μM, and subsequently, KatG becomes the primary scavenging enzyme (26). Although AhpCF and KatG expression is regulated by OxyR in an H$_2$O$_2$-dependent manner, the third catalase in *E. coli*, KatE, is also under the control of σ$^B$ and is induced during stationary phase (29). Thus, under our experimental conditions, KatG is probably mainly responsible for H$_2$O$_2$ elimination. The catalase assay system used in our study also mainly determines KatG activity because it is based on the peroxidatic activity of catalases and uses methanol as a substrate. This activity is very low in KatE and AhpCF but high in catalase-peroxidases like KatG (30).

For elucidating whether YchF interacts directly with KatG, *E. coli* cells expressing YchF were treated with H$_2$O$_2$, and YchF was purified via its C-terminal His tag. As a control, YchF was also purified from cells that were not H$_2$O$_2$-treated, and both YchF preparations were analyzed for co-purifying proteins. Western blot analysis revealed that comparable amounts of YchF were purified from both cultures (Fig. 3B). Purified YchF from the control cells (–H$_2$O$_2$) contained only a small amount of KatG (Fig. 3B), but the amount of KatG co-purifying with YchF increased significantly when YchF was purified from H$_2$O$_2$-treated cells (Fig. 3B). Like KatG, the iron-scavenging protein Dps also co-purified with YchF in H$_2$O$_2$-treated cells (Fig. 3B). As a control, we used antibodies against LexA, another stress response protein that is not directly linked to oxidative stress but involved in the SOS DNA damage control pathway. LexA did not co-purify with YchF, supporting a specific interaction of YchF with proteins of the oxidative stress response.

It is important to emphasize that the intracellular levels of KatG and Dps increase upon H$_2$O$_2$ treatment (26), and therefore, their co-purification with YchF could just reflect this increase in concentration. On the other hand, LexA expression is also increased upon H$_2$O$_2$ treatment (31), but it did not co-purify with YchF. Nevertheless, we also followed an unbiased mass spectrometry-based approach for identifying proteins that co-purified with YchF in the presence or absence of H$_2$O$_2$ (Fig. 3C). Data were normalized to the intensity ratio of YchF from H$_2$O$_2$-treated versus non-treated cells. Shown are the ratios (+H$_2$O$_2$/-H$_2$O$_2$) of the five most abundant proteins detected in the YchF sample as well as the ratios of all identified OxyR-regulated proteins. Note that Dps could not be identified by mass spectrometry.
which are a main target of reactive oxygen. A possible direct interaction between YchF and IscS requires further analyses.

In *E. coli*, approximately 34 proteins are OxyR-controlled, and 22 of them are up-regulated upon oxidative stress (26). In our MS analysis, we found only five OxyR-regulated proteins (supplemental Table S1): all three catalases of *E. coli* (AhpF, KatG, and KatE); OxyR itself, which is down-regulated by oxidative stress; and Fur, which is up-regulated by OxyR but is also a usual contaminant during His tag purification (32, 33). In particular, KatG showed a strong H$_2$O$_2$-dependent co-purification with YchF (Fig. 3C). Thus, the co-purification of KatG with YchF does not simply reflect OxyR-induced up-regulation of stress response proteins but instead is the result of a selective interaction between both proteins. Surprisingly, we did not detect Dps by MS, although Dps is the protein that is most strongly induced upon oxidative stress (26). Whether this is related to its small size or to its tendency to form homodimers upon oxidative stress is currently unknown.

The proposed interaction between YchF and KatG was further verified by two *in vivo* cross-linking approaches. When *E. coli* cells expressing YchF were treated with the membrane-permeable homobifunctional cross-linker PFA before YchF purification, we noticed an additional band at approximately 120 kDa that was recognized by α-KatG antibodies and was PFA-dependent (Fig. 4A). The size of the cross-linking product fits with a cross-link between the 40-kDa YchF and the 80-kDa KatG. We also used an *in vivo* site-directed cross-linking approach using the phenylalanine derivative pBpa. pBpa can be incorporated specifically at amber stop codon positions in the presence of a specific plasmid-borne orthogonal aminoacyl-tRNA synthetase/tRNA$_{	ext{CUA}}$ pair (19). We incorporated pBpa into position 20 of YchF, which is surface-exposed and close to the phosphorylation site Ser-16 of YchF that has been identified in a recent phosphoproteome study of *E. coli* (34) (Fig. 4B). Whole cells expressing YchF(N20pBpa) or wild type YchF were exposed to UV light to activate pBpa, and YchF and possible cross-linked partner proteins were then purified. For YchF(N20pBpa), we observed several UV-specific cross-linking products that were not present in cells expressing wild type YchF (Fig. 4C). This demonstrates that the N-terminal helix of YchF is a major protein-protein interaction site. When the same material was probed with KatG antibodies, we observed a UV-dependent double band at 120 kDa, demonstrating that KatG is in close proximity to the N-terminal helix of YchF. The reason for the double band is currently unknown, but it is likely that the second band corresponds to an YchF-KatG cross-linking product from which the C-terminal His tag was cleaved. In summary, these data strongly support that YchF interacts directly with KatG and that this interaction is probably responsible for KatG inhibition.

**YchF Expression Is Controlled by OxyR in Response to H$_2$O$_2$—** The YchF-dependent inhibition of KatG in the presence of H$_2$O$_2$ would be detrimental for the cell, and therefore, we addressed how cells prevented this inhibition under physiological conditions, *i.e.* when *ychF* was not overexpressed. First, we determined whether the cellular *ychF* mRNA concentration was influenced by H$_2$O$_2$. Total mRNA was isolated from either untreated wild type cells or from wild type cells treated with 0.5 or 20 mM H$_2$O$_2$. RT-PCR using a *ychF*-specific primer demonstrated that the *ychF* mRNA was slightly reduced in cells treated with 0.5 mM H$_2$O$_2$, and undetectable in cells treated with 20 mM H$_2$O$_2$ (Fig. 5A). As a control, we also analyzed the mRNA for the ribosomal protein L2 (*rplB*) and noticed a slight decrease in cells treated with 20 mM H$_2$O$_2$, but this reduction was significantly less pronounced than the reduction of *ychF* mRNA. Thus, the transcription of *ychF* is down-regulated upon oxidative stress, which would be in line with the possible role of YchF as an inhibitor of the oxidative stress response. Whether down-regulation of *ychF* was OxyR-dependent was analyzed by determining the *ychF* mRNA concentration in a ΔoxyR strain. In comparison with wild type cells, the addition of 20 mM H$_2$O$_2$ caused only a slight decrease in *ychF* mRNA levels in ΔoxyR cells comparable with the decrease of the L2 control. This suggests that *ychF* expression is regulated by OxyR. The binding sites of OxyR-dependent genes are not completely conserved, but based on known target sites in *E. coli*, a consensus sequence has been identified (28) (Fig. 5B). A sequence very similar to this consensus sequence is present immediately downstream from the transcription start site of *ychF* (Fig. 5B).

**FIGURE 4. YchF cross-links to KatG in vivo.** A, cells expressing YchF were grown as described under “Experimental Procedures” and incubated after H$_2$O$_2$ treatment with the membrane-permeable homobifunctional cross-linker PFA. As a control, cells were treated with PFA buffer. Cells were subsequently fractionated, and YchF and possible cross-linking products were purified and processed as described in Fig. 3. The upper panel was decorated with α-KatG antibodies, and the lower panel was decorated with α-YchF antibodies. B, crystal structure of *H. influenzae* YchF (Ref. 4; Protein Data Bank code 1JAL). The position Ser-16 (green) and the position Asn-20 (magenta) where pBpa was incorporated for *in vivo* site-directed cross-linking are indicated. C, cells expressing either YchF or YchF(N20pBpa) were exposed to UV light and fractionated before YchF was purified as described in the legend to Fig. 3. The left panel shows the immune detection using α-YchF antibodies, and putative UV-dependent cross-linking products are indicated by arrows. The right panel shows the immune detection of the same material using α-KatG antibodies.
Binding of OxyR to the YchF promoter was verified by electrophoresis mobility shift assays using purified OxyR and a biotinylated DNA fragment comprising the promoter region of ychF. Incubation of the DNA fragment with purified OxyR resulted in a reduced electrophoretic mobility that was detected by horseradish peroxidase coupled to streptavidin after transfer to a nylon membrane (Fig. 5C). As a control, we used a biotinylated DNA fragment comprising the promoter region of the OxyR-independent yidC. Here, no shift was observed. In summary, these data demonstrate that ychF is negatively regulated by OxyR in response to oxidative stress.

The H$_2$O$_2$-dependent decrease of YchF was also detectable by Western blotting, whereas the protein concentration of YidC, an integral membrane protein not involved in stress response, was unchanged (Fig. 5D). However, the effects at the protein level were less pronounced than at the mRNA level, which could indicate that the YchF turnover is rather slow under these conditions.

**The Function of YchF Is Regulated by Phosphorylation and by Its ATPase Activity**—YchF belongs to the Obg family of NTPases and has been shown to hydrolyze ATP (3) and to contain a phosphate group at serine residue 16 (34). This latter analysis was performed on LB-grown cells, and thus a significant portion of YchF exists in the phosphorylated state in the absence of H$_2$O$_2$. For determining the influence of ATP hydrolysis and phosphorylation on H$_2$O$_2$ hypersensitivity, we constructed three plasmid-encoded YchF mutants. In YchF(S16A), serine residue 16 was replaced by alanine, and in YchF(P11AN12A), two residues within the conserved G1 ATP binding motif were mutated to alanine. In addition, we constructed a phosphomimetic YchF(S16E) mutant. All three YchF derivatives were expressed in wild type E. coli cells, and cell viability was tested after H$_2$O$_2$ treatment. Cells expressing only the endogenous chromosomal ychF copy or cells overexpressing either wild type ychF or ffh from a plasmid served as controls. Cells overexpressing the YchF(S16A) or YchF(P11AN12A)
derivatives did not show H2O2 hypersensitivity (Fig. 6A). In contrast, cells overexpressing YchF(S16E) were hypersensitive like cells overexpressing YchF (Fig. 6A). This indicates that the phosphorylation of Ser-16 and ATP hydrolysis are important for the inhibitory effect of YchF on the oxidative stress response. Expressing wild type YchF did not impede the H2O2-induced up-regulation of KatG (Fig. 2B), and this was also confirmed for cells expressing the YchF derivatives (data not shown). This excludes that the phosphorylation state of YchF or its ATPase activity influences the expression of KatG and further supports the hypothesis that YchF functions at the post-translational level.

The ATPase activity of YchF was measured directly for the wild type protein and the YchF mutants purified from cells that were not treated with H2O2. The YchF(P11AN12A) mutant displayed only very low activity even when analyzed at high protein concentration (Fig. 6B). On the other hand, the ATPase activity of the phosphomimetic YchF(S16E) derivative was comparable with wild type YchF (Fig. 6B). This suggested either that the phosphorylation of serine 16 was required for full ATP hydrolysis or that replacing it with alanine interfered with ATP binding because Ser-16 is located within the ATP binding motif. For further analysis, we determined whether ATPase activity responded to H2O2 by purifying YchF and the YchF(S16A)/YchF(S16E) mutants from H2O2-treated and untreated cells. We observed a significant increase in ATPase activity when YchF was purified from H2O2-treated cells, and a significant increase in ATPase activity was also observed for the YchF(S16A) mutant (Fig. 6C). This demonstrates that H2O2 treatment results in increased ATPase activity of YchF. It also shows that YchF(S16A) is able to bind and hydrolyze ATP but has a very low basal activity in the absence of H2O2. The YchF(S16E) mutant was also further stimulated by the presence of H2O2, but the stimulation appeared to be weaker than for wild type YchF (Fig. 6C).

YchF(S16A) probably mimics the dephosphorylated state of YchF, and the increase of ATPase activity upon H2O2 treatment could indicate that the ATPase activity is regulated by an additional H2O2-dependent modification. Like OxyR, YchF contains several cysteine residues, and their oxidation by H2O2 could influence the ATPase activity. However, we did not detect any influence of reducing agents like β-mercaptoethanol on the H2O2-dependent stimulation of ATP hydrolysis (data not shown).

Finally, we tested whether co-purification of KatG and YchF was also observed for YchF(S16A) or YchF(S16E). KatG co-purified with wild type YchF from H2O2-treated cells (Fig. 6D). For YchF(S16A), we observed significant co-purification of KatG in the absence of H2O2, whereas in the presence of H2O2, the amount of KatG co-purifying with YchF(S16A) was significantly reduced. The reduced KatG co-purification with the YchF(S16A) mutant in the presence of H2O2 probably explains why the overexpression of YchF(S16A) did not cause H2O2 hypersensitivity (Fig. 6A). These data further support the

**FIGURE 6. ATP hydrolysis and the phosphorylation state of YchF influence H2O2 hypersensitivity.** A, H2O2 hypersensitivity was tested by cell viability assays as described in Fig. 1. In pYchF(S16A), the phosphorylation site Ser-16 in YchF was replaced by alanine, and YchF(S16E) corresponds to a phosphomimetic mutant. In pYchF(P11N12), two residues within the G1 nucleotide binding motif were replaced. pFfh corresponds to plasmid-borne copy of ffh, which encodes the protein component of the bacterial signal recognition particle. Shown are the mean values of two independent experiments and the error bars indicate the standard deviation. B, purified protein was incubated with γ-<sup>32</sup>P-labeled ATP in reaction buffer for 10 min at 37 °C. ATP and ADP were removed by the addition of 5% charcoal in 20 mM phosphoric acid and a 10-min centrifugation step. The released γ-<sup>32</sup>P was determined by a scintillation counter. C, ATPase activity was measured as in B in the presence of 50 ng of purified protein. The values represent the mean values of three independent experiments. The error bars indicate the standard deviation. D, wild type YchF, YchF(S16A), and YchF(S16E) were purified as described in Fig. 3 and probed with antibodies against YchF and KatG.
hypothesis that YchF function is not only controlled by phosphorylation/dephosphorylation but probably also by additional, so far unknown modifications. KatG co-purified with YchF(S16E) both in the presence and absence of H$_2$O$_2$, but the amount was higher after H$_2$O$_2$ treatment. This explains why cells expressing this phosphomimetic YchF mutant are H$_2$O$_2$-hypersensitive.

**DISCUSSION**

YchF is a member of the universally conserved Obg-related NTPase family (2), but despite its conservation, the cellular role of YchF and its homologues in different species remained unclear. Our current study has revealed that YchF overexpression causes H$_2$O$_2$ hypersensitivity in *E. coli* and thus displays the same phenotype as overexpression of its homologue Ola1 in humans. Furthermore, we provide the first mechanistic details on YchF function because we show that YchF does not influence the expression of major oxidative stress response proteins but instead inhibits stress response proteins like KatG by direct interaction. This interaction is regulated by the phosphorylation state of YchF and by ATP hydrolysis. Finally, we show that YchF expression is repressed by the transcription factor OxyR in an H$_2$O$_2$-dependent manner. Based on these observations, we propose that YchF/Ola1 is a universally conserved negative regulator of the oxidative stress response.

**YchF Is Part of the OxyR Regulon**—The H$_2$O$_2$ response in most bacteria is coordinated by the LysR-type transcriptional regulator OxyR (32, 35). OxyR interacts in its oxidized state with RNA polymerase and stimulates the expression of at least 30 proteins (35). Additional genes are indirectly controlled by OxyR as it induces the synthesis of OxyS, a small non-coding RNA that controls the expression of additional genes (36). Independently of its oxidation state, OxyR also functions as a repressor. It inhibits its own synthesis (37) and the synthesis of Antigen 43, a self-recognizing surface adhesin found in most *E. coli* strains (38, 39). We now show that OxyR also inhibits *ychF* synthesis, but this inhibition is only observed after exposing cells to H$_2$O$_2$. H$_2$O$_2$ concentrations between 0.1 and 1 mM have been shown to oxidize cysteine residues within OxyR (40, 41), and it is therefore likely that only oxidized OxyR is able to repress YchF synthesis. This observation is also in agreement with a global microarray study, which found that *ychF* is downregulated after H$_2$O$_2$ treatment (31). The putative OxyR binding site upstream of YchF overlaps with the RNA polymerase binding site, which is typical for genes that are negatively regulated by LysR-type transcriptional regulators. In contrast, activating OxyR binding sites as in KatG are located 40–60 nucleotides upstream of the RNA polymerase binding site (32). Thus, upon H$_2$O$_2$ exposure, OxyR activates the transcription of H$_2$O$_2$-scavenging enzymes like KatG and at the same time represses the transcription of the KatG inhibitor YchF (Fig. 7).

**YchF Acts as Negative Regulator of H$_2$O$_2$-scavenging Enzymes**—YchF inhibits catalase activity and interacts directly with KatG, leading to H$_2$O$_2$ hypersensitivity when YchF is overexpressed. Although the requirement for an oxidative stress response is apparent, it is less obvious why cells need to inhibit KatG and possibly other proteins of the oxidative stress response in the absence of H$_2$O$_2$. YchF-inhibited KatG could serve as a reservoir that can be rapidly activated when cells encounter H$_2$O$_2$. KatG of *E. coli* belongs to the catalase-peroxidase family of catalases (30), which can also catalyze a peroxidatic reaction in which ethanol or short-chain aliphatic alcohols are oxidized to potentially toxic aldehydes (42). Catalase-peroxidases also efficiently bind and oxidize NADH, and this NADH oxidase activity is linked to the formation of the reactive superoxide radical (43). Thus, the inhibition of KatG in the absence of oxidative stress might be required for reducing the accumulation of potentially toxic KatG side products and for reducing futile NADH oxidation. Studies in mammalian cells have shown that high levels of catalase activity can have adverse physiological effects (44, 45), which probably also reflects the importance of intracellular H$_2$O$_2$-dependent signaling (46, 47). Whether small amounts of H$_2$O$_2$ are also beneficial for unicellular organisms is currently unknown, but it has been shown that bacterial cells that are treated with very low H$_2$O$_2$ concentrations survive a subsequent treatment with high H$_2$O$_2$ concentrations much better than non-adapted cells (48).

We also found that Dps co-purified with YchF by Western blotting (most likely due its small size) but not by MS. Thus, it is currently unknown whether YchF specifically interacts with Dps and if so whether this interferes with Dps activity. Dps is a ferritin-like protein and, like KatG, is under OxyR control (49, 50). Dps in *E. coli* forms a shell-like dodecamer that may contain up to 400–500 iron atoms (51). Iron sequestration depends...
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on the ferro-oxidase activity of Dps for which H₂O₂ serves as oxidant (52). Thus, the protective role of Dps during oxidative stress is 2-fold: it reduces the H₂O₂ concentration and the free iron concentration. As iron availability is a major determinant for bacterial growth, rapid inactivation of Dps by YchF might be important for allowing growth after H₂O₂ stress ceases. A possible link between iron availability and YchF has already been proposed for V. vulnificus (16) and B. melitensis (18), and it was suggested that YchF is involved in iron metabolism. In these organisms, the absence of YchF has been shown to interfere with growth under iron-limiting conditions. Although this would be in line with increased Dps-dependent iron sequestration in the absence of YchF, further experiments are needed to determine the impact of YchF on Dps function. The in vivo site-directed cross-linking approach (Fig. 4C) revealed that YchF interacts with many more partner proteins, and MS analyses are currently in progress for identifying these additional partner proteins.

Post-translational Regulation of YchF Activity—YchF is phosphorylated in E. coli (34), and phosphorylation of the yeast homologue, probably via the serine/threonine kinases PTK1 (48) and Yck2 (53, 54), has also been observed. E. coli lacks orthologues of both kinases; therefore, the kinase responsible for YchF phosphorylation and the cognate phosphatase need to be identified. The function of YchF is clearly influenced by its phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state. 1) Overexpression of the phosphorylation-deficient YchF(S16A) derivative did not cause H₂O₂ phosphorylation state.

The presence of a second, so far unknown modification is also deduced from the following observations. 1) YchF(S16A) mimicked the dephosphorylated state but still required H₂O₂ for ATPase activation. 2) Although YchF(S16E) imitated a permanently phosphorylated state, it still showed H₂O₂-dependent ATPase activity. We currently favor a model in which the inhibitory effect is executed by phosphorylated YchF and dephosphorylation attenuates this inhibition. This is also in line with the observation that phosphorylation of Ser-16 was found in non-stressed, exponentially growing E. coli cells (34). The stimulated ATPase activity in the presence of H₂O₂ could induce the dissociation of YchF from KatG, subsequently allowing the detoxification of H₂O₂ (Fig. 6). A similar mechanism is used for the dissociation of signal recognition particle from its receptor, which is induced by GTP hydrolysis (55). The observation that KatG binds close to the phosphorylation site and ATPase domain of YchF also supports this hypothesis.

In summary, the H₂O₂ hypersensitivity of YchF overexpressing cells is the result of (i) a continuously high YchF level that is disconnected from the OxyR regulation and (ii) most likely an inefficient post-translational modification (dephosphorylation of Ser-16 and a so far unknown modification). This results in persistent inhibition of KatG and possibly other proteins of the oxidative stress response. Our data do not exclude that YchF also regulates the translation of some target genes, which would explain the ribosome interaction that has been observed (3, 5).

E. coli YchF as a Model for Human Ola1 Function—The overexpression of YchF in E. coli and the overexpression of hOla1 in humans cause hypersensitivity toward oxidative stress in both organisms, indicating that YchF function has been conserved during evolution. This is further corroborated by our data that experimentally prove that YchF inhibits oxidative stress by a post-translational mechanism, which was also put forward for human Ola1 (7).

Whether hOla1 also functions by inhibiting catalases is so far unknown. Catalases in mammalian cells are found in the cytosol, in peroxisomes, and probably in mitochondria (30, 56, 57). hOla1 is located in the cytoplasm and does not contain a typical peroxisomal or mitochondrial localization signal. Thus, a direct inhibition of cytosolic catalases by hOla1 is possible and needs to be experimentally verified. However, in plants, stress exposure was shown to influence the localization of YchF homologues (58), and it is therefore possible that hOla1 does not exclusively function in the cytosol.

Increased hOla1 expression is observed in many tumor cells, and mutations that lead to enhanced hOla1 expression could be a primary cause for these tumors. High levels of hOla1 would suppress the ability of cells to cope with and inactivate damaging reactive oxygen species, resulting in the accumulation of DNA damage and oncogenic transformation. Thus, our study on E. coli YchF provides an important framework for further exploring the mechanism and the biological and medical significance of the YchF/Ola1 family of ATPases.

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