A universally conserved GTPase regulates the oxidative stress response in *Escherichia coli*

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Running title: YchF/hOla1 inhibit oxidative stress response

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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:** First functional description of the YchF protein family

**SUMMARY**

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 tumours. 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 employed *Escherichia coli* as model organism and found that YchF over-expression 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 a H2O2-dependent de-phosphorylation. 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 cells’ ability to scavenge damaging reactive oxygen species.

P-loop GTases execute and regulate essential biological processes and show high sequence conservation, resulting in a set of 8 GTase families that are universally conserved in eukaryotes, bacteria and archaea (1). These GTases include YihA and HlfX, which are involved in ribosome biogenesis, IF-2, EF-Tu and EF-G, which are required for protein synthesis, and the signal recognition particle (SRP) and its receptor (SR, FtsY), which are essential for co-translational protein targeting. It also includes YchF, a member of the Obg-family of universally conserved GTases. YchF differs from the other GTases because it preferentially hydrolyses 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 *E. 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. Due to 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-hydrolyzing 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 homolog of YchF, Ybr025c was suggested to interact with the elongation factor eEF1 (6). These data support a role of 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 human hOla1. Reducing the concentration of hOla1 in HeLa cells increased the cellular resistance to peroxide oxidants and thiol-depleting chemicals, while the over-expression of hOla1 increased the sensitivity of HeLa cells towards oxidative stress (7). This could indicate that hOla1 functions as a negative regulator of the oxidative stress response. Whether hOla1 inhibits directly one or more antioxidant enzymes or whether it acts by another mechanism, e.g. by influencing protein degradation, is not known. The latter possibility is supported by data showing an interaction of the yeast homologue Ybr025c with subunits of the proteasome (8).

The possible involvement of hOla1 in regulating stress response is consistent with data showing its down-regulation in cells treated with DNA-damaging agents or UV-light (9). hOla1 over-expression has been observed in many tumour cells (9, 10, 11), which could indicate that at high hOla1 concentrations, the cellular response to potentially mutagenic substances is impaired. A link between hOla1 and cancer is also deduced from the observation that down-regulation of hOla1 inhibits the motility and invasion of breast cancer cells (12) and the proliferation of neuronal and pancreatic cells (13). Thus, hOla1 appears to play an important role in cellular stress response, cell proliferation and tumour development, but details on its mode of action are entirely unknown.

Equally little is known about the bacterial YchF homologues. They are predicted to be located in the cytosol of bacterial cells and show high sequence similarity to hOla1, e.g. the E. coli YchF amino acid sequence is 45% identical and 62% similar to the amino acid sequence of hOla1. YchF is not essential in either E. coli or in B. subtilis and the only phenotype reported so far for an E. coli ΔychF mutant is weak cold-sensitivity (14, 15). A ΔychF mutant of the halophilic Gram-negative pathogen Vibrio vulnificus was shown to exhibit reduced cytotoxic effects on macrophages (16), suggesting an involvement of YchF in inactivating host defence. Reduced virulence was also observed in a ΔychF mutant of Streptococcus pneumonia (17). In contrast, a ΔychF mutant of the facultative intracellular pathogen Brucella melitensis did not show reduced cytotoxicity, but was, like the Vibrio vulnificus ΔychF mutant, impaired in iron metabolism (16, 18). Although these phenotypic reports link bacterial YchF homologues to important biological processes, details on its function are missing.

In the current study, we have analyzed the effect of ychF over-expression and ychF deletion on stress tolerance in E. coli. 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 (ΔychF) and JW3933 (ΔoxyR) were provided by NBRP (NIG 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 with 35 µg/ml chloramphenicol.

**Plasmid construction**

ychF was isolated from chromosomal E. coli DNA and cloned via XhoI and NdeI into the plasmid pET-22b. This construct was a gift from S. Angelini, Freiburg, and used for subcloning ychF into plasmid pTrc99a. The primers YchFfw and YchFrev (Table 1) were used to insert XbaI and HindIII restriction sites. pth-ychF was amplified from chromosomal DNA and also cloned into pTrc99a. The PCR was performed with Phusion High Fidelity PCR Master Mix (Finnzymes, Vantaa, Finland). PCR-fragment purification from agarose gel was done with 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 (NEB), Ipswich, USA. Vector and insert were ligated with the Quick Ligation Kit (NEB). 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 Tab. 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 primer OxyRFw and OxyRrev. After purification of the PCR product and Bsai digestion, the PCR product was cloned into Bsai digested pASK-IBA3C vector (IBA Tech., 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 Tab. 1. These constructs were transformed into *E. coli* Bl21 containing pSUP-BpaRS-6TRN (19), encoding the orthogonal aminoacyl-tRNA synthetase/tRNA pair for incorporation of pBpa into the position of the amber codon.

**Growth analyses**

Cells were grown over night and diluted 1:100 in liquid LB medium and further incubated until they reached an OD600 of 0.5-0.8. These cells were then used for measuring H₂O₂ sensitivity by the following methods: (A) Spot assay: The culture was adjusted to an OD600 of 0.5 and serially diluted. 10 µl of each dilution were spotted onto LB plates, supplemented with or without H₂O₂. After over night incubation at 37 °C plates were analyzed. (B) Cell viability assay: Cells were adjusted to OD600 of 0.5 and diluted 1:10 in phosphate-buffered saline (PBS) before treatment with 10 mM H₂O₂ in PBS for 50 min at 25 °C; control cells were treated with PBS. Subsequently cells were harvested by centrifugation and washed with PBS and resuspended in 1 ml 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 H₂O₂-treated wild type cells was set to 100%. (C) Inhibition assay: Cultures were adjusted to OD600 of 0.5, mixed with LB-Top agar and poured on LB plates. Subsequently, sterile filter discs were soaked in 2 mM H₂O₂ solution and placed on top of these plates. After approx. 5 hours 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 Mini Kit from GE Healthcare (Munich, Germany). RNA was eluted with RNase free water and tested for quality and purity by agarose gel electrophoresis and by absorption measurement. Possible DNA contaminations were detected by running a standard PCR reaction without prior reverse transcriptase treatment. If necessary, an additional DNase treatment was performed. RNA was stored at -80 °C. RT-PCR was performed with the RT-PCR Kit from Qiagen (Hilden, Germany), according to the enclosed manual in 25 µl reactions. The following primers were used: OxyR_RTforward/OxyR_RT reversed; rplB RTforward/ rplB_RT reversed; 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 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 purified OxyR and incubated for 20 min at 23 °C in the provided EMSA buffer. Subsequently, 5 µl of the provided loading dye were 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 OD600 of ~0.8 and then for additional 30' incubated with or without H₂O₂. Protein expression was not induced by IPTG 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 M NH₄Ac, 10 mM MgAc₂), using 1 ml buffer per 1 g wet cell pellet. Before...
cells were lysed by French pressing (two passes at 8000 psi), 0.5 mM PMSF (Roche, Mannheim, Germany) and 1x Complete protease inhibitor cocktail (Roche) were added to prevent protein degradation. Cell debris was removed by 30’ centrifugation at 15,500 rpm (Sorvall SS-34 rotor) and the supernatant was incubated with Talon metal affinity beads (Clonetech, Mountain View, USA) for one hour at 4 °C. After centrifugation at 1000 rpm for 10’ 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 KAc, 20 mM MgAc2) via a PD-10 column (GE healthcare). The quality of the purified protein was controlled via SDS-PAGE and PAGE-blue staining and was stored at -20 °C.

OxyR was purified from E. coli Bl21 cells carrying pASK-IBA3C-OxyR which were induced at OD600 0.3 with 200 µg anhydrotetracyclinchloride and cell extracts were prepared as described above with the exception that all steps were performed in buffer W (100 mM Tris/HC1, pH 8.0; 150 mM NaCl, 1 mM EDTA). Cell extracts were applied to buffer W equilibrated streptavidin columns and washed five times with one column-volume 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 OxyR, 20 mM Tris/HC1, pH 8.0, 100 mM NaCl, 2 mM CaC12, 1 µl Protease Xa (2 U/µl, Quiagen, Hilden Germany). The cleaved Strep tag was removed via streptavidin columns and complete cleavage was controlled via western blot and anti-strep antibodies (IBA Göttingen, Germany).

In vivo formaldehyde cross-linking

E. coli BW25113 pYchF was grown to an OD600 of 1.0 in LB medium and treated for 30 min with 20 mM H2O2. Subsequently paraformaldehyde in PFA-buffer (136 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.8) and centrifuged a second time. Subsequently, the cells were resuspended in 8 ml 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 M NH4Ac, 20 mM MgAc, 1% Triton X-100). Prior to french pressing 10 µl each of 0.5 mM PMSF (Roche) and 1x Complete protease inhibitor cocktail (Roche) 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 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 Na2HPO4, 1.76 mM KH2PO4, pH 7.8) and centrifuged a second time. Subsequently, the cells were resuspended in 8 ml 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 M NH4Ac, 20 mM MgAc, 1% Triton X-100). Prior to french pressing 10 µl each of 0.5 mM PMSF (Roche) and 1x Complete protease inhibitor cocktail (Roche) was added to each tube. The cells were then subjected three times to a French Pressure step and centrifuged at 15,800 rpm (Sorvall SS-34 rotor) for 20 min. YchF was purified by its C-terminal His-tag as described before. Protease inhibitors were again added to prevent protein degradation. 100µl of the eluted material was run on an SDS-PAGE and analysed by western blot.

Western blot analysis

After SDS-PAGE, proteins were electro-transferred by tank blotting to a nitrocellulose membrane (GE healthcare). Peptide antibodies were raised in rabbits by Genscript (New Jersey, USA). The following peptides were used: YchF: VNEDGFENNPYLDQC, OxyR: CRPGSPLRSRYIQLA. The Dps anti serum used in this
work was kindly supplied by Regine Hengge, Freie Universität Berlin, Germany. The RplB antibody was a gift from Richard Brimacombe, Max Planck Institute for Molecular Genetics, Berlin, Germany. The YidC antibody was raised in rabbits against the complete protein. KatG antibodies were obtained from Agrisera, Vännas, Sweden and the LexA antibody from Active Motif, La Hulpe, Belgium. All the antibodies were gained from rabbit serum, except for RplB, which was raised in goat. Horseradish peroxidase-coupled goat anti-rabbit antibodies or sheep anti-goat from Caltech Laboratories (Burlingame, USA) were used as secondary antibodies and enhanced chemiluminescence (ECL) reagent (GE Healthcare) was used as detection substrate.

Catalase activity assay
Over night grown cells were diluted 1:100 in LB medium and grown at 37 °C to an OD600 of 0.8 before H2O2 was added and the cells incubated for additional 30 min. The cell pellets were resuspended in lysis buffer (100 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA) and lysozyme was added to a final concentration of 100 µg/ml. After incubation at 37 °C for 30 min, samples were subjected to at least four cycles of freezing in liquid nitrogen and thawing at 37 °C. After 15 min incubation with DNase (final concentration 50 µg/ml) at 30 °C, samples were centrifuged for 10 min at 10,000x g and the supernatant was collected in a fresh tube. The samples were tested for catalase activity using the Catalase Assay kit (Cayman Europe, Tallinn, Estonia).

ATP hydrolysis assay
Purified YchF was incubated with γ-35P labelled ATP to analyze the ATP-hydrolysis rate. The radioactive nucleotide was diluted with non-labelled ATP 1:500 and 1 to 100 µM 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 MgCl2, 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 35P was added to 3 ml scintillation cocktail. The samples were measured in a scintillation counter (Perkin Elmer, USA) 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 Na-orthovanadate; 9.5 mM NaF, 10 mM β-glycerophosphate, 10 mM Na-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/ESI-MS using an UltiMate 3000 RSLCnano/LTQ-Orbitrap XL system (Thermo Fisher Scientific, Bremen, Germany) essentially as described previously (20). Mass spectrometric datasets were processed using the software suite MaxQuant (version 1.2.7.4) (21, 22). Proteins were identified by searching peaklists against the EcoProt database (v.31.01.2012, 4275 entries, tagged YchF sequence added) applying a false discovery rate of < 1% at both the peptide and protein level. 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 two minutes. Only razor and unique peptides were considered.

RESULTS
YchF over expression causes H2O2 hypersensitivity in E. coli
For analyzing the possible contribution of YchF to the oxidative stress response in E. coli, the H2O2 resistance of wild type (wt) E. coli cells, a YchF deletion strain (∆ychF) 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 H2O2, cell growth of all strains was comparable (Fig. 1A), but on plates containing 2 mM H2O2, wild type cells over-expressing YchF showed a significant growth inhibition. The same H2O2 concentration did not significantly impair the growth of wt E. coli cells lacking an additional plasmid copy of ychF. Growth of the ∆ychF strain in the presence of H2O2 was slightly better than that of the wt, but was also impaired by over-expression of ychF from a...
plasmid, although to a lower degree than wt *E. coli*.

The H$_2$O$_2$ hypersensitivity of *ychF* over-expressing cells was confirmed by two additional quantitative assays. In cell viability assays, the metabolic activity of cells after 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 cells was set to 100% and taken as reference. Cells over-expressing *ychF* showed a significantly reduced luminescence signal, indicating that viability of these cells was drastically compromised after H$_2$O$_2$ treatment (Fig. 1B), while the signal of the Δ*ychF* 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 LB-plates overlaid with *E. coli* cells in top agar. The diameter of the inhibition zone of wt+pYchF approximately doubled in comparison to wild type cells (Fig. 1C). The Δ*ychF* strain showed a smaller inhibition zone, indicating that in the absence of YchF cells are more resistant towards H$_2$O$_2$, which is also visible in the spot assay (Fig. 1A). The H$_2$O$_2$ sensitivity in the Δ*ychF* 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 Δ*ychF*+pYchF was lower than in wt+pYchF (Fig. 1D), which probably explains why H$_2$O$_2$ sensitivity was less pronounced in Δ*ychF*+pYchF (Figs 1A & 1C). Antibodies against Ffh, the protein component of the bacterial SRP, were used as control.

In *E. coli* and many enterobacteria, *ychF* is co-transcribed with *pth*, encoding an essential peptidyl-tRNA hydrolase (23, 24, 25). We therefore tested whether over-expression of *ychF*-pth caused the same H$_2$O$_2$ sensitivity as over-expression of *ychF* alone. The H$_2$O$_2$ hypersensitivity of wt and Δ*ychF* 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 over-expressing Ffh (pFfh), which is like YchF one of the 8 universally conserved NTPases (1). The ΔoxyR strain displayed an enhanced sensitivity towards H$_2$O$_2$, which was more pronounced than the H$_2$O$_2$ sensitivity of *ychF* over-expressing strains, suggesting that over-expressing *ychF* does not completely block the oxidative stress response. Over-expression 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 over-expression. In summary, our data show that high levels of YchF cause hypersensitivity towards oxidative stress in *E. coli* and thus results 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* over-expression influenced the expression of oxidative stress response proteins like OxyR or 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 Δ*ychF* cells or in cells over-expressing *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 for 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* over-expressing 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 over-expressing ycf (Fig. 2B). The membrane protein YidC served as 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 or OxyR and also 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, Δycf cells and ycf over-expressing cells. Catalase activity was low in wild type cells without H$_2$O$_2$ treatment (Fig. 3A), but was stimulated approx. 5-fold in extracts from cells treated with 0.5 mM 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 approx. 50% in cell extracts from ycf over-expressing 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 ycf over-expressing cells are H$_2$O$_2$-hypersensitive. In Δycf 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 mM 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 a FAD-dependent hydroperoxide reductase that can reduce H$_2$O$_2$ in a 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). While AhpCF and KatG expression are regulated by OxyR in a H$_2$O$_2$-dependent manner, the third catalase in *E. coli*, KatE, is also under the control of σ$^S$ 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 employed in our study also mainly determines KatG activity, because it is based on the peroxidatic activity of catalases and uses methanol as 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 which 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. The five most abundant proteins were GlmS, SlyD, and CRP, which are usual contaminants during His-tag purification in *E. coli* (32), LacI, which controls the expression of the lac promoter and is probably found because ycf was cloned under the control of the lac promoter, and IscS, which is involved in Fe-S cluster biosynthesis. The co-purification of GlmS, SlyD, CRP and LacI was not significantly influenced by H$_2$O$_2$ treatment, while IscS
slightly increased upon H₂O₂ treatment (Fig. 3C). IscS is a cysteine desulfurase and involved in the biosynthesis of Fe-S clusters, which are a main target of reactive oxygen. A possible direct interaction between YchF and IscS requires further analyses.

In *E. coli*, approx. 34 proteins are OxyR controlled and 22 of them are up-regulated upon oxidative stress (26). In our MS analysis, we found only 5 OxyR-regulated proteins (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 also a usual contamination during His-tag purification (32, 33). In particular KatG showed a strong H₂O₂-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 homo-dodecamers 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 homo-bifunctional cross-linker paraformaldehyde (PFA) before YchF purification, we noticed an additional band at approx. 120 kDa that was recognized by α-KatG antibodies and 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 employed an in vivo site-directed cross-linking approach using the phenylalanine derivative para-benzoyl-L-phenylalanine (pBpa). pBpa can be incorporated specifically at amber-stop codon positions in the presence of a specific plasmid-borne orthogonal amino acyl tRNA synthetase/ tRNA_CUA pair (19). We incorporated pBpa into position 20 of YchF, which is surface exposed and close to the phosphorylation site S16 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 UV-exposed 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 in which the C-terminal His-tag was cleaved off.

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 H₂O₂**

The YchF-dependent inhibition of KatG in the presence of H₂O₂ would be detrimental for the cell and we therefore addressed how cells prevented this inhibition under physiological conditions, i.e. when *ychF* was not over-expressed. First, we determined whether the cellular *ychF* mRNA concentration was influenced by H₂O₂. Total mRNA was isolated from either untreated wild type cells or from wild type cells treated with 0.5 or 20 mM H₂O₂. RT-PCR using a *ychF*-specific primer demonstrated that the *ychF* mRNA was slightly reduced in cells treated with 0.5 mM H₂O₂ and undetectable in cells treated with 20 mM H₂O₂ (Fig. 5A). As a control, we also analyzed the mRNA for the ribosomal protein L2 (*rplB*) and also noticed a slight decrease in cells treated with 20 mM H₂O₂, 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 to wild type cells, the addition of 20 mM H₂O₂ caused only a slight decrease in *ychF* mRNA levels in ΔoxyR cells, comparable to 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). Importantly, its
viability was tested after H\textsubscript{2}O\textsubscript{2} treatment. Cells phosphorylated state in the absence of H\textsubscript{2}O\textsubscript{2}. For significant portion of YchF exists in the performed on LB-grown cells and thus a residue 16 (34). This latter analysis was (3) and to contain a phosphate group at serine 16, or YchF(S16E) mutant. All three YchF derivatives (data not shown). This excludes that the phosphorylation state of YchF or its ATPase activity influence 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 H\textsubscript{2}O\textsubscript{2}. 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 to wild type YchF (Fig. 6B). Importantly, the phosphorylation-deficient YchF(S16A) mutant showed only very low activity (Fig. 6B). This suggested that either the phosphorylation of serine 16 was required for full ATP hydrolysis or that replacing it with alanine interfered with ATP binding because S16 is located within the ATP binding motif. For further analyzing this, we determined whether ATPase activity responded to H\textsubscript{2}O\textsubscript{2} by purifying YchF and the YchF(S16A)/YchF(S16E) mutants from H\textsubscript{2}O\textsubscript{2} treated and untreated cells. We observed a significant increase in ATPase activity when YchF was purified from H\textsubscript{2}O\textsubscript{2}-treated cells and a significant increase in ATPase activity was also observed for the YchF(S16A) mutant (Fig. 6C). This demonstrates that H\textsubscript{2}O\textsubscript{2} 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 H\textsubscript{2}O\textsubscript{2}. The YchF(S16E) mutant was also further stimulated by the presence of H\textsubscript{2}O\textsubscript{2}, 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 H\textsubscript{2}O\textsubscript{2} treatment could indicate that the ATPase activity is regulated by an additional H\textsubscript{2}O\textsubscript{2}-dependent modification. YchF contains, like OxyR, several cysteine residues and their oxidation by H\textsubscript{2}O\textsubscript{2} could influence the ATPase activity. However, we did not detect any influence of reducing agents like

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β-mercaptoethanol on the H$_2$O$_2$-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 was found co-purifying with wild type YchF from H$_2$O$_2$-treated cells (Fig. 6D). For YchF(S16A), we observed significant co-purification of KatG in the absence of H$_2$O$_2$, while in the presence of H$_2$O$_2$, 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 H$_2$O$_2$ probably explains why the over-expression of YchF(S16A) did not cause H$_2$O$_2$ hypersensitivity (Fig. 6A). These data further support the 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 in the 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 over-expression causes H$_2$O$_2$ hypersensitivity in *E. coli* and thus displays the same phenotype as over-expression of its homologue Ola1 in humans. Furthermore, we provide 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 a 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**

H$_2$O$_2$ response in most bacteria is coordinated by the LysR-type transcriptional regulator (LTTR) 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). Independent of its oxidation state, OxyR also functions as a repressor. It inhibits its own synthesis (37), and the synthesis of Antigen43 (Ag43), 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 0.1-1 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 micro-array study, which found ychF down-regulated 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 LTTRs. 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 over-expressed. 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 H₂O₂-dependent signalling (46, 47). Whether small amounts of H₂O₂ are beneficial also for unicellular organism is currently unknown, but it has been shown that bacterial cells that are treated with very low H₂O₂ concentrations survive a subsequent treatment with high H₂O₂ concentrations much better than non-adapted cells (48).

We also found Dps co-purifying with YchF by western blotting but, most likely due its small size, 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 is, like KatG, 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 on the ferro-oxidase activity of Dps for which H₂O₂ serves as oxidant (52). Thus, the protective role of Dps during oxidative stress is two-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 *Vibrio vulnificus* (16) and *Brucella 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) has 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 has also been observed, probably via the serine/threonine kinases PTK1 (48) and Yck2 (54). *E. coli* lacks orthologues of both kinases and 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) over-expression of the phosphorylation deficient YchF(S16A) derivative does not cause H₂O₂ hypersensitivity (2) KatG co-purification with YchF(S16A) is independent of H₂O₂ and (3) YchF(S16A) shows no basal ATPase activity. The first observation suggests that phosphorylation of YchF is required for KatG inhibition, but the second observation implies that the YchF-KatG interaction can occur independently of the S16 phosphorylation. This probably indicates that the inactivation of KatG by YchF is not only regulated by phosphorylation but additionally by a second modification. The presence of a second, so far unknown modification is also deduced from the following observations: (1) YchF(S16A) mimics the de-phosphorylated state but still requires H₂O₂ for ATPase activation and (2) although YchF(S16E) imitates a permanently phosphorylated state, it still shows H₂O₂-dependent ATPase activity. We currently favour a model in which the inhibitory effect is executed by phosphorylated YchF and de-phosphorylation attenuates this inhibition. This is also in line with the observation that phosphorylation of S16 was found in non-stressed, exponentially grown *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 SRP 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 over-expressing 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 (de-phosphorylation of S16 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 over-expression of YchF in *E. coli* and the over-expression of hOla1 in humans cause in both organisms hypersensitivity towards oxidative stress, 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 tumour cells and mutations that lead to enhanced hOla1 expression could be a primary cause for these tumours. High levels of hOla1 would suppress the ability of cells to cope 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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Footnotes: The abbreviation used are: DPS, DNA-protection during starvation; ECL, enhanced chemiluminescence; Ffh, fifty-four-homologue; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, lysogeny broth; MS, mass spectrometry; pBpa, para-benzoyl-L-phenylalanine; PBS, phosphate-buffered saline; PFA, para-formaldehyde; PMSF, phenylmethylsulfonyl fluoride, Pth, peptidyl-tRNA hydrolase; TBE, Tris-borate EDTA; TCA, trichloracetic acid

Figure Legends

**Figure 1:** Over-expression of YchF in *E. coli* leads to H$_2$O$_2$-hypersensitivity. (A) The indicated strains were inoculated in LB medium and grown to OD$_{600}$ of ~0.5 before step-wise dilution in LB medium. Of each dilution, 10 µl cell suspensions were spotted onto LB plates +/- 2 mM H$_2$O$_2$. Cell growth was analyzed after over night incubation at 37 °C. (B) Cells were adjusted to OD$_{600}$ of 0.5 and treated after a 1:10 dilution with 10 mM H$_2$O$_2$ in PBS for 50 min at 25 °C. After a wash step, 100 µl of the cell culture was transferred to a 96-well plate and 100 µl of the BacTiter-Glo Microbial cell viability assay solution was added and luminescence was recorded. The luminescence signal of wild type *E. coli* was set to 100%. Shown are the mean values of at least three independent experiments. (C) For the agar diffusion assay, *E. coli* cells were mixed with top agar and poured onto LB agar plates. Sterile discs of filter paper were soaked with 2 mM H$_2$O$_2$ and placed on top of the cell agar mixture. After four hours 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 (approx. 0.5*10^8 cells) precipitated on ice for 30’ 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 control, antibodies against Ffh were used. (E) The agar diffusion assay using the indicated strains was performed as described in (C).

Figure 2: YchF does not influence the steady-state stability of OxyR or KatG. (A) RNA was prepared from the indicated strains that were grown at 37 °C up to an OD_{600} of 0.5 and which were then further incubated for 15 min +/- 20 mM H_2O_2. RT-PCR was performed and products were analyzed on a 1% agarose gel. 100 ng of RNA was used per 25 µl reverse transcriptase reaction. Primers specific for the ribosomal protein L2 (rplB) served as a control. An aliquot of the same cells (approx. 0.5*10^6 cells) used for RNA preparation was directly TCA precipitated as described in the legend to Fig. 1 and separated on SDS-PAGE, transferred to a nitrocellulose membrane and subsequently decorated with antibodies against OxyR and L2 (rplB). (B) Cells were grown to OD_{600} of 0.5 and incubated for one additional hour in the presence or absence of 0.5 mM H_2O_2. 0.5*10^8 cells were processed as described in the legend to Fig. 1. Samples were taken immediately before H_2O_2 addition (0/-), 60 min after incubation without H_2O_2 (60/-) and 60 min after incubation with H_2O_2 (60/+). As loading control, the western blot was also decorated with antibodies against YidC.

Figure 3: YchF inhibits catalase activity in Escherichia coli. (A) The indicated strains were grown in the presence or absence of H_2O_2 and were subsequently lysed by lysozyme and freezing/thawing cycles. Catalase activity was measured as peroxidatic activity using methanol as substrate. The values represent the mean values of two independent experiments. (B) YchF was purified via metal-affinity chromatography from cells treated for 30 min with or without 20 mM H_2O_2. The purified protein (approx. 10 µg) was separated on SDS-PAGE and transferred to nitrocellulose for immune-detection using antibodies against YchF, KatG, Dps and LexA. (C) YchF was purified as described in (B) and co-purifying proteins were identified by mass spectrometry. Data were normalized to the intensity ratio of YchF from H_2O_2-treated versus non-treated cells. Shown are the ratios (+H_2O_2/-H_2O_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.

Figure 4: YchF cross-links to KatG in vivo. (A) Cells expressing Ychf were grown as described in material and methods and incubated after H_2O_2 treatment with the membrane permeable homobifunctional cross-linker para-formaldehyde (PFA). As 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 with α-YchF antibodies. (B) Crystal structure of Haemophilus influenzae YchF (4; 1JAL). The position S16 (green) and the position N20 (magenta), where para-benzoyl-L-phenylalanine (pBpa) was incorporated for in vivo site directed cross-linking, are indicated. (C) Cells expressing either YchF or YchF(N20pBpa) were UV-exposed 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.

Figure 5: YchF expression is negatively regulated by the transcription factor OxyR in response to H_2O_2. (A) RNA was prepared from the indicated strains that were grown at 37 °C to an OD_{600} of 0.5 and were then further incubated for 15 min with or without 0.5 or 20 mM H_2O_2. RT-PCR was performed and products were analyzed on a 1% agarose gel. 100 ng of RNA were used per 25 µl reverse transcriptase reaction. Primers specific for the ribosomal protein L2 served as control. (B) Upper panel; sequence logo based on previously identified OxyR binding sites (27, 32, 54). The katG OxyR binding motif and the putative ychF OxyR binding motif are shown below the sequence logo. Lower panel; localization of the OxyR binding sites (underlined) and the transcription start sites (σ^70) of katG and ychF. The transcription start sites were taken from the PortEco databank (http://porteco.org/). (C) Electrophoresis mobility shift assay using a biotinylated DNA fragment.
corresponding either to the promoter region of ychF or to the promoter region of yidC. Approx. 20 fmol of DNA were incubated with the indicated amount of purified OxyR and after incubation separated on TBE-PAGE and transferred to a nylon membrane. Binding of OxyR to biotinylated DNA was detected via streptavidin-conjugated horseradish peroxidase. (D) Immune-detection of YchF in cells grown as in (A). Approx. 0.5*10^8 cells were processed as described in the legend to Fig. 1. Antibodies against YidC served as control.

Figure 6: ATP hydrolysis and the phosphorylation state of YchF influence H_2O_2 hypersensitivity. (A) H_2O_2 hypersensitivity was tested by cell viability assays as described in Fig. 1. In pYchF(S16A), the phosphorylation site S16 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, encoding the protein component of the bacterial SRP. (B) Purified protein was incubated with γ-33P labelled 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-33P was determined by a scintillation counter. (C) ATPase activity was measured as in C in the presence of 50 ng purified protein. The values represent the mean values of three independent experiments. (D) Wild type YchF and YchF(S16A) or YchF(S16E) were purified as described in Fig. 3 and probed with antibodies against YidC and KatG.

Figure 7: Tentative model for YchF function in Escherichia coli. In the absence of H_2O_2, the transcription factor OxyR is kept in its reduced state by the glutaredoxin GlrX, which allows the basal expression of katG and ychF. Under these conditions, YchF inhibits KatG activity. In the presence of H_2O_2, OxyR is oxidized and induces the expression of oxidative stress response genes, like catalases. Simultaneously, it represses the expression of the catalase inhibitor YchF. In addition to this transcriptional regulation, the activity of YchF is also regulated by H_2O_2 at a post-translational level. YchF is phosphorylated at serine 16 in the absence of H_2O_2 (34) and a phosphorylation-deficient YchF mutant does not cause H_2O_2 hypersensitivity. Thus, KatG inhibition in the absence of H_2O_2 is probably caused by phosphorylated YchF. In the presence of H_2O_2, YchF is de-phosphorylated and undergoes a second H_2O_2-dependent modification, which needs to be indentified. These modifications stimulate the ATPase activity of YchF, resulting in the dissociation of KatG. KatG then detoxifies H_2O_2. At low H_2O_2 concentrations, repression of YchF by OxyR is relieved and phosphorylation of YchF increases its affinity for KatG, resulting in its inhibition.
Table 1: Nucleotide primer used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>YchFfw</td>
<td>5’-ACAA TTCCCCTCTAGAAATAATTTTG-3’</td>
</tr>
<tr>
<td>YchFrev</td>
<td>5’-CAACTCAGCAAGCTTTCCGGGCT-3’</td>
</tr>
<tr>
<td>Pthfw</td>
<td>5’-CGCCAGTTATCTAGACACTCAGG-3’</td>
</tr>
<tr>
<td>Pth+YchF_rev</td>
<td>5’-GATTAGCGAAAGCTTTATGAGAC-3’</td>
</tr>
<tr>
<td>YchF(S16A)fw</td>
<td>5’-TCGGGAAAGCAACCCCTG-3’</td>
</tr>
<tr>
<td>YchF(S16A)rev</td>
<td>5’-CGTTGGGCAAACCGAC-3’</td>
</tr>
<tr>
<td>YchF(S16E)fw</td>
<td>5’-TCGGGAAAGAGACCCCTG-3’</td>
</tr>
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<td>YchF(S16E)rev</td>
<td>5’-CGTTGGGCAAACCGAC-3’</td>
</tr>
<tr>
<td>YchF(P11AN12A)fw</td>
<td>5’-TTGGCCGCCGTCGGGAAATC-3’</td>
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<tr>
<td>YchF(P11AN12A)rev</td>
<td>5’-ACCGACGATACCGCATTGGAAATCC-3’</td>
</tr>
<tr>
<td>OxyRFw</td>
<td>5’ATGGTAGGCTCATAATGACTTTGATCTTGAGTACCTTG-3’</td>
</tr>
<tr>
<td>OxyRrev</td>
<td>5’ATGGTAGGCTCAGCGCTAAAACCGTGTTGAATCC-3’</td>
</tr>
<tr>
<td>OxyR_RTforward</td>
<td>5’-TGCAAGTCGCTTCCCGGCTTATGGATCCC-3’</td>
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<tr>
<td>OxyR_RTreversed</td>
<td>5’-TGCAAGTCGCTTCCCGGCTTATGGATCCC-3’</td>
</tr>
<tr>
<td>rplB_RTforward</td>
<td>5’-AGTTTGTAATGATTAACCGACAT-3’</td>
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<tr>
<td>rplB_RTreversed</td>
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</tr>
<tr>
<td>YchF_RTforward</td>
<td>5’-GCGGTATCGTCGGTTTGCCC-3’</td>
</tr>
<tr>
<td>YchF_RTreversed</td>
<td>5’-AAAAGGAAAGTTTACATCACATGCGCC-3’</td>
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<tr>
<td>YchF-300fw+bio</td>
<td>5’-CATCGGAATCGGGTCGCGGTCGCGG-3’</td>
</tr>
<tr>
<td>YchF-300rev</td>
<td>5’-AAGTTGGCTGCCTGATCAACCCGG-3’</td>
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<tr>
<td>YidC-300fw+bio</td>
<td>5’-ACCTCGATAACCGTGTTCTCGG-3’</td>
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<tr>
<td>YidC-300rev</td>
<td>5’-TGCGGCTGAGGTTGCCGCGG-3’</td>
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<tr>
<td>YchF(N20pBa)fw</td>
<td>5’-TCTACCCTGTCTACTGCGGTGACCATAAA-3’</td>
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### A

<table>
<thead>
<tr>
<th>H$_2$O$_2$ -</th>
<th>H$_2$O$_2$ +</th>
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<tbody>
<tr>
<td>10$^{-4}$</td>
<td>10$^{-5}$</td>
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<tr>
<td>wild type (wt)</td>
<td></td>
</tr>
<tr>
<td>ΔychF</td>
<td></td>
</tr>
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</table>

### B

- **wt**
- **wt + pYchF**

% luminescence signal

<table>
<thead>
<tr>
<th>wt</th>
<th>wt + pYchF</th>
<th>ΔychF</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>50</td>
<td>100</td>
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</tbody>
</table>

### C

- **wt**
- **wt + pYchF**
- **ΔychF**

Inhibition zone in cm

<table>
<thead>
<tr>
<th>wt</th>
<th>wt + pYchF</th>
<th>ΔychF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
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### D

<table>
<thead>
<tr>
<th>YchF</th>
<th>Ffh</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>wt + pYchF</td>
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<tr>
<td>45 kDa</td>
<td>45 kDa</td>
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</table>

### E

- **wt**
- **wt + pYchF**
- **ΔoxyR**

Inhibition zone in cm

<table>
<thead>
<tr>
<th>ΔoxyR</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt + pFfh</td>
</tr>
</tbody>
</table>

(Wenk et al., Fig. 1)
A

<table>
<thead>
<tr>
<th></th>
<th>- $\text{H}_2\text{O}_2$</th>
<th>+ $\text{H}_2\text{O}_2$</th>
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<tbody>
<tr>
<td></td>
<td>wild type</td>
<td>$\Delta\text{ychF}$</td>
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<tr>
<td>oxyR mRNA</td>
<td><img src="oxyR.png" alt="Image" /></td>
<td><img src="oxyR.png" alt="Image" /></td>
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<tr>
<td>rplB mRNA</td>
<td><img src="rplB.png" alt="Image" /></td>
<td><img src="rplB.png" alt="Image" /></td>
</tr>
<tr>
<td>OxyR</td>
<td><img src="OxyR.png" alt="Image" /></td>
<td><img src="OxyR.png" alt="Image" /></td>
</tr>
<tr>
<td>L2 (RplB)</td>
<td><img src="L2.png" alt="Image" /></td>
<td><img src="L2.png" alt="Image" /></td>
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B

<table>
<thead>
<tr>
<th>t in min/0.5 mM $\text{H}_2\text{O}_2$</th>
<th>wild type</th>
<th>wild type + pYchF</th>
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</thead>
<tbody>
<tr>
<td>0/-</td>
<td>60/-</td>
<td>60/+</td>
</tr>
<tr>
<td>0/-</td>
<td>60/-</td>
<td>60/+</td>
</tr>
<tr>
<td>KatG</td>
<td><img src="KatG.png" alt="Image" /></td>
<td><img src="KatG.png" alt="Image" /></td>
</tr>
<tr>
<td>YidC</td>
<td><img src="YidC.png" alt="Image" /></td>
<td><img src="YidC.png" alt="Image" /></td>
</tr>
</tbody>
</table>

(Wenk et al., Fig. 2)
**A**

![Bar graph](image)

**B**

![Western blot](image)

**C**

![Histogram](image)

(Wenk et al., Fig. 3)
(Wenk et al., Fig. 4)
A

<table>
<thead>
<tr>
<th></th>
<th>wild type</th>
<th>ΔoxyR</th>
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</thead>
<tbody>
<tr>
<td>H₂O₂ in mM</td>
<td>0 0.5 20</td>
<td>0 0.5 20</td>
</tr>
</tbody>
</table>

yChF

rplB

B

katG TAAGATCTCAACTATCGCATCCGTGGATTAATTCAAT

yChF GTTATTTCCATTTCTGCAATCTGTTAGCAATAATAAC

katG TATG TAAGATCTCAACTATCGCATCCGTGGATTAATTCAAT

(Δ)

yChF TTTTTGCCAGTGCCTGATTATAACAGTTTTGCAATCTGTTAGCAATAACAGTGTGATTATAAGATATTAA

katG TATGTAAGATCTCAACTATCGCATCCGTGGATTAATTCAAT

C

<table>
<thead>
<tr>
<th>DNA</th>
<th>yChF</th>
<th>yidC</th>
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</thead>
<tbody>
<tr>
<td>OxyR in ng</td>
<td>0 6 40</td>
<td>0 6 40</td>
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</table>

OxyR+DNA

DNA

D

<table>
<thead>
<tr>
<th></th>
<th>wild type</th>
<th>ΔoxyR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂ in mM</td>
<td>0 0.5 20</td>
<td>0 0.5 20</td>
</tr>
</tbody>
</table>

YchF

YidC

(Wenk et al., Fig. 5)
(Wenk et al., Fig. 6)
Glutaredoxin (GlrX)

**transcriptional regulation**
- basal expression *katG*
- basal expression *ychF*
- induction *katG*
- repression *ychF*

**post-translational regulation**
- phosphorylation
- $2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$
- $\text{ADP} + P_i \rightarrow \text{ATP}$
- $\text{H}_2\text{O}_2$-dependent dephosphorylation
- $\text{YchF} \rightarrow \text{KatG}$

(Wenk et al., Fig. 7)
A universally conserved GTPase regulates the oxidative stress response in 
Escherichia coli
Meike Wenk, Qiaorui Ba, Veronika Erichsen, Katherine MacInnes, Heike Wiese, 
Bettina Warscheid and Hans-Georg Koch

J. Biol. Chem. published online November 8, 2012

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