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# Supplemental Data

# Bleach Activates a Redox-Regulated Chaperone by Oxidative Protein Unfolding

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## Supplemental Experimental Procedures

*Analysis of Hsp33's thiol status in vitro and in vivo* – To monitor Hsp33's redox status during the *in vitro* oxidation and reduction process, thiol trapping experiments with the thiol-specific probe AMS were performed (Hoffmann et al., 2004). To precisely quantify the oxidation status of Hsp33's two critical cysteines $Cys^{232}$ and $Cys^{234}$, we applied the OxICAT method exactly as previously described (Leichert et al., 2008). To analyze the *in vivo* oxidation status of Hsp33 upon HOCl treatment in phosphate buffer (Fig. 5), *E. coli* strain BB7222 was cultivated in LB medium at 37°C until an $OD_{600}$ of 0.5 was reached. Cells were harvested, washed with phosphate buffer and challenged with various concentrations of HOCl for 10 min as described. Then, 1 ml aliquots were removed and supplemented with 0.1 ml 5 x concentrated LB medium to quench residual oxidants and with 0.2 ml cell-permeable iodoacetamide (0.5 M in 1 M Tris-HCl, pH 8) to modify all accessible thiol groups *in vivo* (Zander et al., 1998). After 5 min of incubation at room temperature, ice-cold TCA was added to a final concentration of 10% (w/v) and the samples were kept on ice for 1 h. After TCA precipitation, sample pellets were dissolved in 50 µl denaturing buffer (6 M urea, 200 mM Tris-HCl (pH 8.5), 10 mM EDTA, 0.5 % [w/v] SDS) supplemented with 10 mM DTT and incubated at 25°C for 1 h to reduce all reversible thiol modifications. After TCA precipitation, the pellets were

resuspended in 50 µl denaturing buffer supplemented with 10 mM AMS to modify all newly accessible cysteines. After 1 h incubation at 25°C, the samples were loaded onto non-reducing SDS-PAGE and Hsp33 was detected using western blot analysis with antibodies against Hsp33. Based on this differential thiol trapping procedure, reduced cysteines are alkylated with IAM while oxidized cysteines are, upon DTT reduction, modified with the 490 Da probe AMS. To monitor the oxidation status of Hsp33 under cultivation conditions that allow cell recovery (Supplemental Fig. 3), BB7222 wild type cells were grown aerobically in MOPS minimal medium supplemented with 0.2% glucose and 10 µM thiamine at 37°C until $OD_{600}$ of 0.5 was reached. Then, the cultures were split and incubated with or without 2 mM HOCl. After 35 min of HOCl-stress treatment, cells were transferred to pre-warmed flasks containing 5 x concentrated LB +/- 200 µg/ml chloramphenicol. At defined time points during and after the stress treatment, 1 ml aliquots were removed and supplemented with 2 mM methionine and 0.2 ml of cell-permeable iodoacetamide (0.5 M in 1 M Tris-HCl, pH 8). Then, the differential thiol trapping was performed as described above.

*Electrospray (ESI) Mass Spectrometry* - For electrospray ionization MS analysis, $Hsp33_{red}$ was oxidized and activated for either 2 or 20 minutes incubation using a 10-fold molar excess of HOCl ($Hsp33_{HOCl}$). The oxidants were removed using NAP-5 (Amersham) columns. The buffer of the samples was exchanged to $ddH_2O$ just prior to the analysis to prevent any interference of the phosphate buffer with the measurements. ESI-MS was performed on a nanoAcuity/Qtof premier instrument at the Protein Structure Facility of the University of Michigan. The accuracy is +/- 0.01% of the molecular weight.

*Far-UV circular dichroism spectroscopy*- To determine the influence of HOCl-treatment on the conformation of GrpE or Hsp33, the purified proteins were incubated with either 250 µM (GrpE) or 500 µM HOCl (GrpE, Hsp33) for 20 min at 30°C in 40 mM potassium phosphate, pH 7.5. Then, the oxidants were removed using NAP-5 columns (Amersham Biosciences) and the far-UV CD spectra of untreated or HOCl-treated GrpE (10 µM) were recorded at 30°C using a Jasco circular dichroism spectrophotometer. To compare the HOCl-mediated conformational changes to the known temperature-induced conformational changes in GrpE (Grimshaw et al., 2003), the far-UV CD spectrum of untreated GrpE was recorded at 45°C. In the case of citrate synthase (CS), CD spectra of 3 µM CS were recorded directly upon addition of 450 µM of HOCl or $H_2O_2$ at 30°C until no further changes were observed (30 min). Then, 5 accumulations were recorded and corrected for the buffer signal. The temperature was controlled with a Peltier device.

*Construction of the hslO (Hsp33 gene) deletion in V. cholerae O395* - A DNA fragment spanning 658 nucleotides upstream and 547 nucleotides downstream of *V. cholerae hslO* (Hsp33 gene) was amplified by PCR and cloned into pUC19 using *Xba*I and *EcoR*I to generate the plasmid pJW21. pJW21 was then used in a PCR reaction, in which the start codon of *hslO* was fused to the stop codon of *hslO*, generating a pUC19 derived plasmid (pJW23) that contains an internal deletion of the *hslO* gene. This fragment of pJW23 was then cloned into the suicide vector pKAS32 generating pJW24 and transformed into *E. coli* SM10λpir (Skorupski and Taylor, 1996). Mating with *V. cholerae* strains O395 (Matson and DiRita, 2005) generated the Hsp33 deletion strain JW371 (O395 *hslO*$^-$). Recombination and loss of the wild type allele was confirmed by

PCR analysis using primers annealing 200 bp downstream and upstream of the *hslO* gene, respectively.

*Oxidative stress treatment of E. coli in LB medium* – Wild type *E. coli* BB7222 or the *hslO* deletion strain JW176 were grown at 30°C in LB medium until OD = 0.5-0.6 was reached. Then, cultures were split and supplemented with an equal volume of LB medium supplemented with HOCl (final concentration 6 mM). After the indicated time points, aliquots were removed, serially diluted in LB medium to quench the remaining HOCl, and spotted onto LB plates. LB plates were incubated at 30°C for 24 hours and the colony forming units were counted.

*Analysis of protein aggregation in vivo*-To analyze the substrate specificity of Hsp33, the absolute intensity for each of the detectable spots on the 2D gels of lysates prepared from wild type strain BB7222 and *hsp33* deletion strain JW176 was determined. Spot intensities were normalized to the four proteins YaeT, Imp, TolC, and DeoC, whose spot intensities did not significantly vary upon the individual stress treatments. For a protein to be considered to be a substrate protein of Hsp33, the average intensity of a protein spot in lysates of the Hsp33 deletion strain JW176 had to be at least 1.5-fold higher than of the respective protein spots under control conditions. This indicated that the protein aggregates significantly more when Hsp33 is absent. To identify HOCl-sensitive proteins, the Δ*rpoH* deletion strain BB7224 was challenged with 6 mM HOCl for 30 min at 30°C. Aggregated proteins were prepared and 2D gel analysis was conducted as described above. Then, data analysis was performed as before by comparing the extent of aggregation in HOCl-treated and non-treated BB7224 cells. Protein spots of interest were excised from 2D gels, digested with trypsin, and identified by peptide mass

fingerprinting at the Michigan Proteome Consortium as previously described (Winter et al., 2005).

*Protein aggregation in stress-treated cell lysates* - To analyze protein aggregation in cell lysates, the wild type *E. coli* strain BB7222 was cultivated until $OD_{600}$ of 0.5. Then, cells were harvested, washed twice in phosphate buffer (40 mM $KH_2PO_4$, 20 mM KCl pH 7.5) and lysed using two passages through a French Press Cell (1100 psi). After centrifugation (13,200 rpm, 45 min, 4°C), the soluble supernatant (final concentration 20 mg/ml) was split and either not supplemented with any additional protein or supplemented with 100 µM $Hsp33_{HOCl}$ or 100 µM lysozyme. Then, the cell lysates were either left untreated or treated with 10 mM HOCl or 10 mM $H_2O_2$ for 20 min at 30°C. Aggregated proteins were separated from the soluble supernatant by centrifugation (13,200 rpm, 30 min, 4°C). Then, the protein pellets were washed with phosphate buffer, resuspended in 50 µl reducing Laemmli buffer and analyzed using reducing 14% SDS-PAGE.

**Supplemental Figure Legends**

**Figure S1A.** Low molar ratios of HOCl rapidly activate Hsp33 *in vitro*. 50 µM of inactive $Hsp33_{red}$ was incubated for 2 minutes at 30°C with the indicated concentrations of HOCl. Then, Hsp33 was diluted 1:160 into the assay buffer and the influence of 0.3 µM Hsp33 on the aggregation of 75 nM chemically denatured citrate synthase at 30°C was determined as described in Fig. legend 1. Very similar results were obtained when Hsp33 was incubated with HOCl for 20 min at 30°C or when 10-fold lower concentrations of $Hsp33_{red}$ and HOCl were used instead.

**Figure S1B.** $H_2O_2$-mediated activation of Hsp33 is dependent on oxidant concentration. 20 µM inactive $Hsp33_{red}$ was incubated with either 200 µM $H_2O_2$ (circles), 2 mM $H_2O_2$ (triangles) or 10 mM $H_2O_2$ (squares) at 43°C. At various time points, aliquots were removed and the influence of Hsp33 on the aggregation of CS was determined as described above.

**Figure S2.** HOCl-mediated oxidation converts Hsp33 into natively unfolded protein. (**A**) Changes in secondary structure were determined by recording the far-UV circular dichroism (CD) spectra of $Hsp33_{red}$, $Hsp33_{HOCl}$, $Hsp33_{oxH2O2\text{-}43°C}$ at 30°C as previously described (Graf et al., 2004). (**B**) Changes in the surface hydrophobicity were monitored by recording the fluorescence spectra of bis-ANS-labelled $Hsp33_{red}$, $Hsp33_{oxHOCl}$ or $Hsp33_{oxH2O2\text{-}43°C}$ at 30°C ($\lambda_{ex}$ = 370 nm). Bis-ANS labeling of the proteins was conducted as previously described (Graf et al., 2004).

**Figure S3A**. Hsp33 increases HOCl-resistance of *E. coli*. Wild type *E. coli* BB7222 (closed circles) and the *hslO* deletion strain JW176 (open circles) were cultivated in LB medium until an $OD_{600}$ of 0.5 was reached. Then, cultures were diluted 1:2 into fresh LB

medium supplemented with HOCl (final concentration was 6 mM). Viability of cells was analyzed after the indicated time points by preparing serial dilutions of the cultures and spotting them onto LB plates.

**Figure S3B**. HOCl-mediated thiol modifications of Hsp33 are reversible *in vivo*. Wild type *E. coli* strain BB7222 was cultivated in MOPS minimal medium at 37°C until $OD_{600}$ of 0.5 was reached. Then, 2 mM HOCl was added and cells were incubated at 37°C. After 35 min, 5-fold concentrated LB medium supplemented with 200 µg/ml chloramphenicol was added to quench any residual oxidants and to stop protein synthesis, respectively. Samples were removed before the stress (time point 0) as well as at defined time points during and after the HOCl-stress treatment. All *in vivo* reduced cysteines were alkylated with iodoacetamide (IAM) while *in vivo* oxidized cysteines were reduced with DTT, and subsequently modified with the 490 Da thiol-specific AMS. Hsp33 was visualized by western blot analysis.

**Figure S4.** Hsp33 prevents HOCl-mediated protein aggregation in *E. coli* cell lysates. Aliquots of *E. coli* cell lysates (20 mg/ml) were either not supplemented with any additional proteins or supplemented with 100 µM activated $Hsp33_{HOCl}$ (Hsp33) or 100 µM lysozyme (Lys). Then, the lysates were incubated either in the absence of any oxidants (lane 1-3), or in the presence of 10 mM $H_2O_2$ (lane 4) or 10 mM HOCl (lane 5-7) for 20 min at 30°C. Aggregated proteins were separated from the soluble proteins by centrifugation and analyzed on reducing 14% SDS-PAGE.

**Table S1:** HOCl-aggregation sensitive substrate proteins of Hsp33

Increase in protein aggregation in the Δ*hslO* strain JW176 as compared to wild type BB7222. [a] denotes proteins, whose extent of aggregation is increased more than 1.5-

fold in the Hsp33 deletion strain as compared to WT. These proteins are considered substrate proteins of Hsp33. The extent of aggregation is given as the average of two independent experiments. [b] indicates genes that have been found to be essential for *E. coli* growth in rich media while [c] indicates genes preserved in over 80% diverse bacterial genomes (Gerdes et al., 2003). Asterisk (*) indicates the overlap with substrate proteins of Hsp33 under oxidative heat stress and substrate proteins of the DnaK-system under heat stress treatment (Mogk et al., 1999).

**Table S2:** Majority of HOCl-sensitive proteins are thermolabile *in vivo*. List of identified aggregation sensitive proteins in HOCl-treated Δ*rpoH* strain BB7224. [a] denotes all identified *E. coli* proteins, whose extent of aggregation is increased more than 1.5-fold upon treatment of Δ*rpoH* strain BB7224 with 6 mM HOCl for 30 min at 30°C as compared to non-treated BB7224. [b] denotes the corresponding *E. coli* proteins, whose extent of aggregation is either unchanged (-) or increases more than 1.5-fold (+) upon treatment of Δ*rpoH* strain BB7224 at 45°C for 30 min. Thermolabile proteins, which are HOCl-resistant are not shown.

**Table S1: HOCl-aggregation sensitive substrate proteins of Hsp33**

| Protein | Function | Δ*hslO*/MC4100 |
|---|---|---|
| AceE-IF1* | Pyruvate dehydrogenase E1-isoform 1 | 3.3 ± 0.3 |
| AceE-IF2* | Pyruvate dehydrogenase E1-isoform 2 | 4.5 ± 1.3 |
| AceK* | Isocitrate dehydrogenase kinase | 1.8 ± 0.2 |
| AdhE* | Aldehyde-alcohol dehydrogenase | 2.7 ± 0.1 |
| ClpX* | Clp protease ATP-binding subunit | 2.7 ± 0.8 |
| DacA | D-alanyl-D-alanine carboxypeptidase | 1.7 ± 0.0 |
| DeoB | Phosphopentomutase | 2.2 ± 0.2 |
| Efp*,b,c | Elongation factor P | 2.1 ± 0.6 |
| FabB*,b | 3-oxoacyl-[acyl-carrier-protein] synthase I | 2.8 ± 0.7 |
| Glf* | UDP-galactopyranose mutase | 1.8 ± 0.4 |
| Gnd | 6-phosphogluconate dehydrogenase | 1.9 ± 0.3 |
| MrsA | Phosphoglucosamine mutase | 1.9 ± 0.0 |
| NfnB | Oxygen-insensitive NAD(P)H nitroreductase | 2.2 ± 0.7 |
| NuoC-IF1 | NADH-quinone oxidoreductase chain-isoform 1 | 4.2 ± 0.1 |
| NuoC-IF2 | NADH-quinone oxidoreductase chain-isoform 2 | 3.5 ± 0.1 |
| OmpC* | Outer membrane protein C | 1.6 ± 0.2 |
| Pgk*,b,c | Phosphoglycerate kinase | 5.5 ± 3.2 |
| Pnp[b] | Polyribonucleotide nucleotidyltransferase | 1.9 ± 0.5 |
| PurB | Adenylosuccinate lyase | 1.8 ± 0.0 |
| RecA | Recombinase A | 2.2 ± 0.6 |
| RpsA | 30S ribosomal protein S1 | 1.7 ± 0.4 |
| SucA-IF1*,b | 2-oxoglutarate dehydrogenase E1-isoform 1 | 9.9 ± 1.9 |
| SucA-IF2*,b | 2-oxoglutarate dehydrogenase E1-isoform 2 | 16.6 ± 2.3 |
| Tig | Trigger factor | 1.6 ± 0.1 |
| TktA[b,c] | Transketolase 1 | 1.8 ± 0.4 |
| TnaA | Tryptophanase | 1.7 ± 0.4 |
| TrpD | Anthranilate synthase component II | 2.1 ± 0.8 |
| Tsf[b,c] | Elongation factor Ts | 1.7 ± 0.6 |
| TufB-IF1*,b,c | Elongation factor Tu-isoform 1 | 2.7 ± 1.3 |
| TufB-IF2*,b,c | Elongation factor Tu-isoform 2 | 2.3 ± 0.5 |
| TufB-IF3*,b,c | Elongation factor Tu-isoform 3 | 1.8 ± 0.3 |
| TufB-IF4v[b,c] | Elongation factor Tu-isoform 4 | 1.7 ± 0.1 |
| TypA-IF1* | GTP-binding protein TypA/BipA-isoform 1 | 1.5 ± 0.0 |
| TypA-IF2*, | GTP-binding protein TypA/BipA-isoform 2 | 1.6 ± 0.4 |
| Upp | Uracil phosphoribosyltransferase | 3.4 ± 1.0 |
| YleA*, | Function unknown | 1.7 ± 0.5 |

**Table S2: Majority of HOCl-sensitive proteins is thermolabile *in vivo*.**

| Proteins | Function | HOCl[a] | 45°C[b] |
|---|---|---|---|
| Pnp | Polyribonucleotide nucleotidyltransferase | + | - |
| RpsA | 30S ribosomal protein S1 | + | - |
| GroEL | Chaperone | + | - |
| TnaA | Tryptophanase | + | - |
| FliG | flagellar motor switch protein | + | - |
| Upp | Uracil phosphoribosyltransferase | + | - |
| HepA | RNA polymerase associated protein | + | + |
| AceE | Pyruvate dehydrogenase E1 component | + | + |
| AcnB | Aconitate hydratase 2 | + | + |
| FusA | Elongation factor G | + | + |
| TktA | Transketolase 1 | + | + |
| NuoC | NADH-quinone oxidoreductase chain C/D | + | + |
| AceK | Isocitrate dehydrogenase | + | + |
| PtsI | Phosphoenolpyruvate-phosphotransferase | + | + |
| YleA | Function unknown | + | + |
| LeuC | 3-isopropylmalate dehydratase large subunit | + | + |
| Tig | Trigger factor | + | + |
| ClpX | Clp protease ATP-binding subunit | + | + |
| AckA | Acetate kinase | + | + |
| Pgk | Phosphoglycerate kinase | + | + |
| FbaA | Fructose-bisphosphate aldolase class II | + | + |
| RecA | Recombinase A | + | + |
| Epd | D-erythrose-4-phosphate dehydrogenase | + | + |
| MreB | Rod shape-determining protein | + | + |
| AhpC | Alkyl hydroperoxide reductase subunit C | + | + |
| Efp | Elongation factor P | + | + |
| GapA | Glyceraldehyde-3-phosphate dehydrogenase | + | + |

**Supplemental References**

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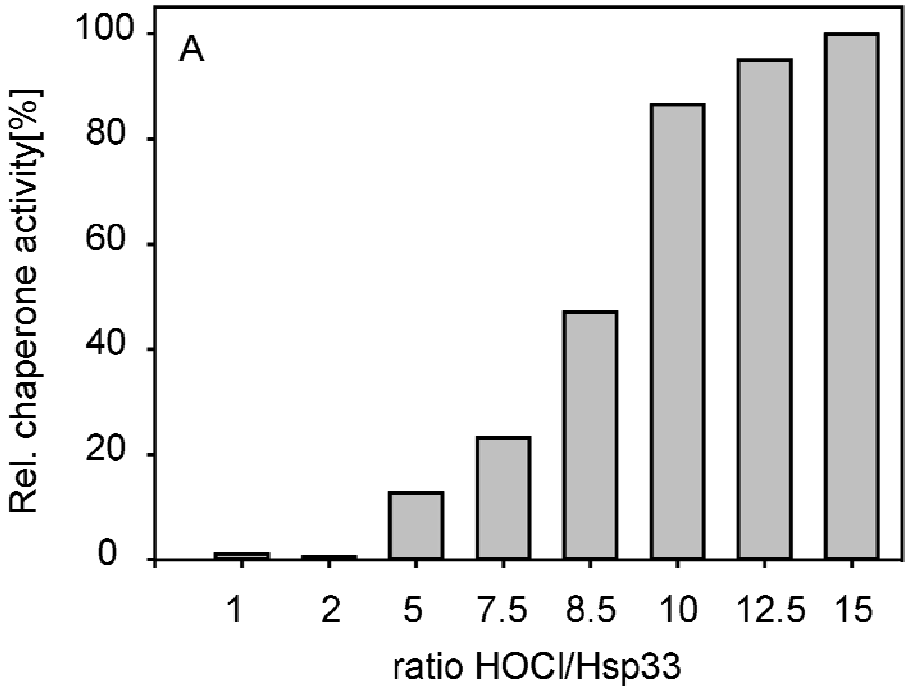
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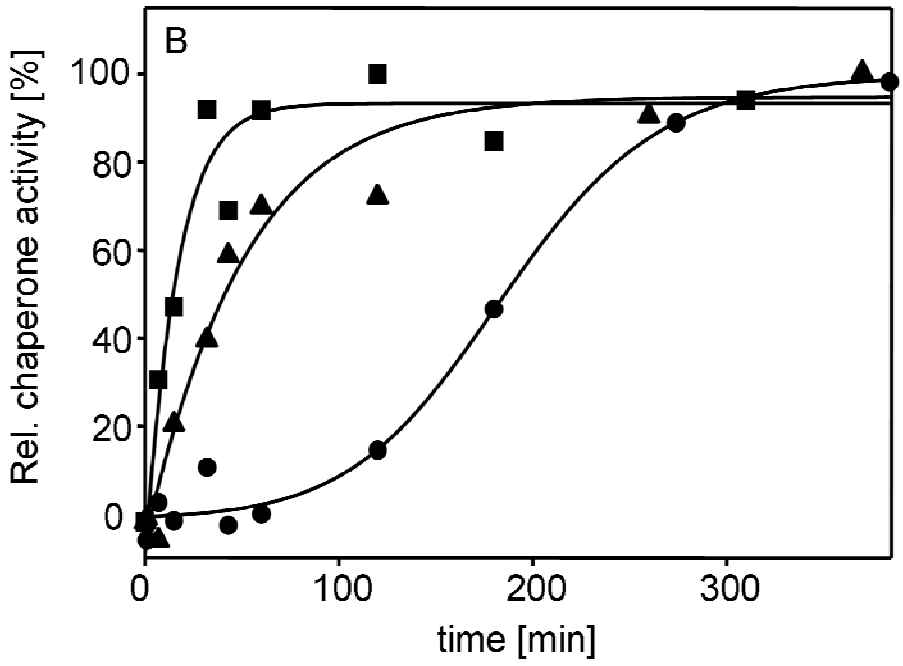
ratio HOCl/Hsp33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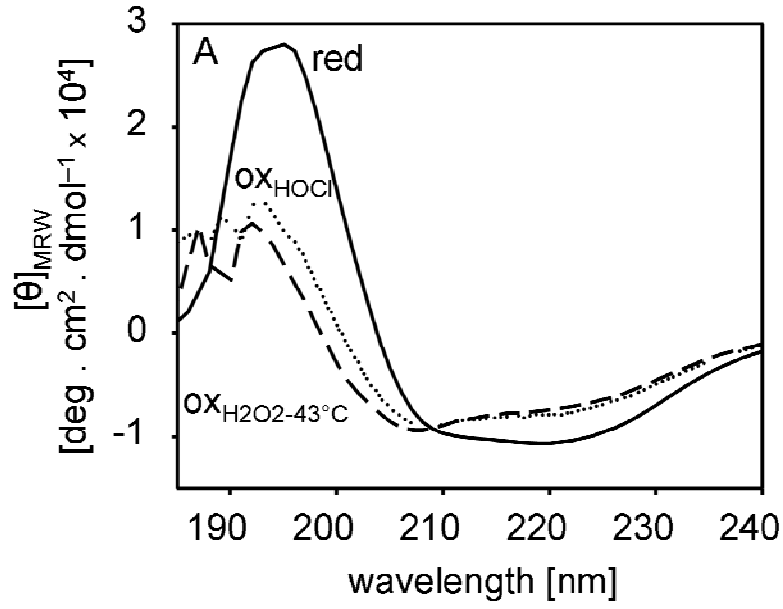
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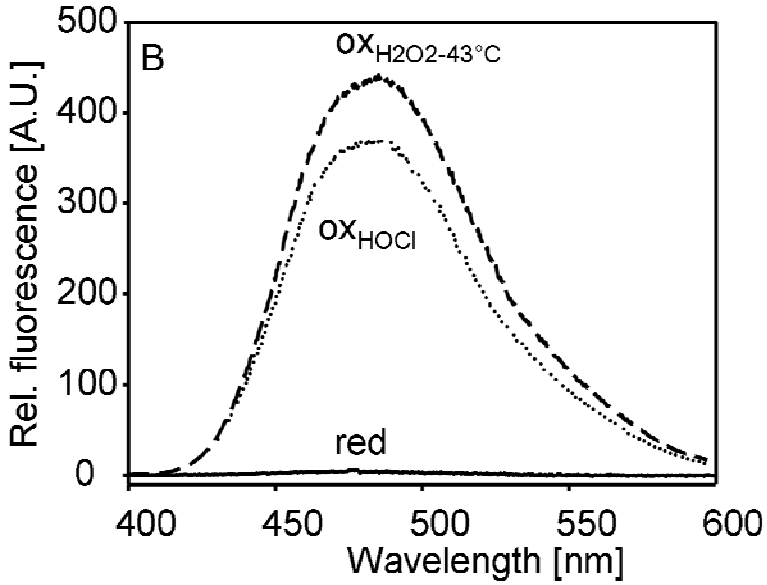
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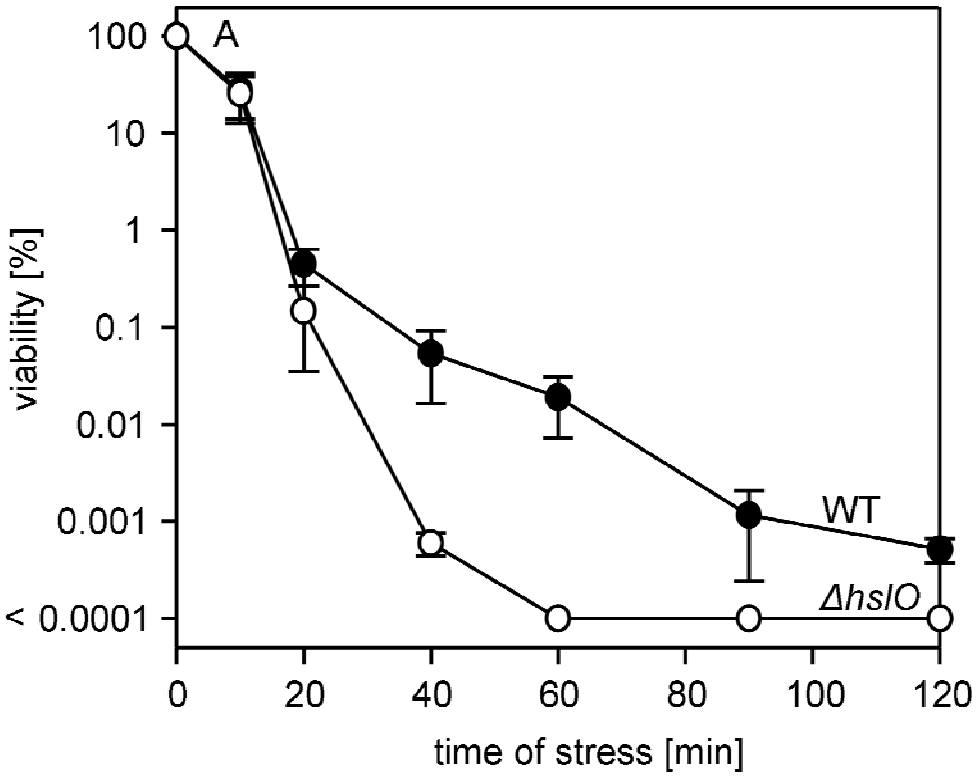
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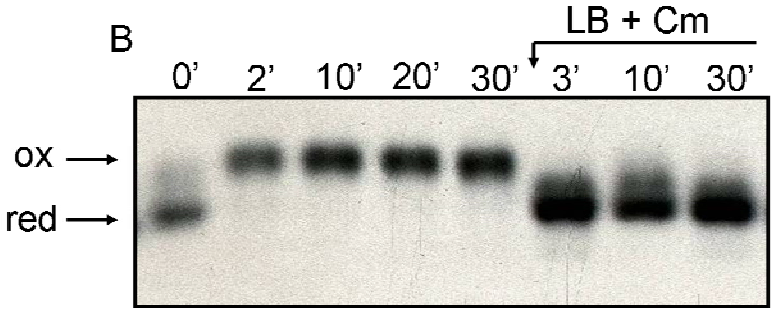
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*Winter et al., Supplemental Figure 4*