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Hsp33 confers bleach resistance by protecting elongation factor Tu against oxidative degradation in *Vibrio cholerae*

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Summary

The redox-regulated chaperone Hsp33 protects bacteria specifically against stress conditions that cause oxidative protein unfolding, such as treatment with bleach or exposure to peroxide at elevated temperatures. To gain insight into the mechanism by which expression of Hsp33 confers resistance to oxidative protein unfolding conditions, we made use of *Vibrio cholerae* strain O395 lacking the Hsp33 gene *hslO*. We found that this strain, which is exquisitely bleach-sensitive, displays a temperature-sensitive (*ts*) phenotype during aerobic growth, implying that *V. cholerae* suffers from oxidative heat stress when cultivated at 43°C. We utilized this phenotype to select for *Escherichia coli* genes that rescue the *ts* phenotype of *V. cholerae ΔhslO* when overexpressed. We discovered that expression of a single protein, the elongation factor EF-Tu, was sufficient to rescue both the *ts* and bleach-sensitive phenotypes of *V. cholerae ΔhslO*. *In vivo* studies revealed that *V. cholerae* EF-Tu is highly sensitive to oxidative protein degradation in the absence of Hsp33, indicating that EF-Tu is a vital chaperone substrate of Hsp33 in *V. cholerae*. These results suggest an 'essential client protein' model for Hsp33's chaperone action in *Vibrio* in which stabilization of a single oxidative stress-sensitive protein is sufficient to enhance the oxidative stress resistance of the whole organism.

Introduction

The heat shock protein Hsp33 is a highly conserved, redox-regulated chaperone, which has been shown to specifically protect bacteria against a variety of different oxidative stress conditions that are accompanied by protein unfolding. These stress conditions include exposure to hypochlorous acid (HOCl), the active ingredient of household bleach and a known physiological antimicrobial, produced by cells of the innate immune response to kill invading microorganisms (Miller and Britigan, 1997). HOCl is a fast acting oxidant, which directly induces protein unfolding both *in vitro* and *in vivo* (Winter *et al.*, 2008). Other physiological oxidants, such as peroxide or nitric oxide, do not cause widespread protein unfolding in organisms and induce activation of Hsp33 only when combined with protein unfolding conditions, such as heat shock treatment (i.e. oxidative heat stress) (Winter *et al.*, 2005). The reason why bacteria require Hsp33 particularly under oxidative protein unfolding conditions is likely due to the fact that enzymes involved in ATP-generation fall victim to oxidative inactivation (Hyslop *et al.*, 1988) causing ATP-dependent chaperones, commonly used to protect against protein aggregation, to lose their *in vivo* function (Winter *et al.*, 2005). Hsp33, which functions as an ATP-independent chaperone and is activated by oxidative unfolding apparently compensates for this loss of ATP-dependent chaperone activity by protecting hundreds of different proteins against protein aggregation in *Escherichia coli* (Ilbert *et al.*, 2007; Winter *et al.*, 2008). At this point, it is still unresolved whether the protective role of chaperones such as Hsp33 results from the general decrease in the pool of aggregated proteins, from the protection of a single essential protein whose stress sensitivity dictates the stress sensitivity of the organism, or from something in-between.

To investigate how expression of the chaperone Hsp33 confers resistance to oxidative protein unfolding conditions in bacteria, we made use of *V. cholerae* strain O395 lacking the Hsp33 gene *hslO*. We found that this strain, which has been previously shown to be highly HOCl stress-sensitive, displays a temperature-sensitive phenotype under aerobic growth conditions, implying that cultivation of *V. cholerae* at 43°C causes oxidative protein unfolding. We utilized this phenotype to select for *E. coli*-specific system(s) that compensate for the deletion of *Vibrio hslO*. We discovered that expression of the *E. coli* elongation factor EF-Tu fully rescues the temperature-sensitive phenotype of *V. cholerae hslO* deletions and restores bleach resistance to wild-type levels. *In vivo*

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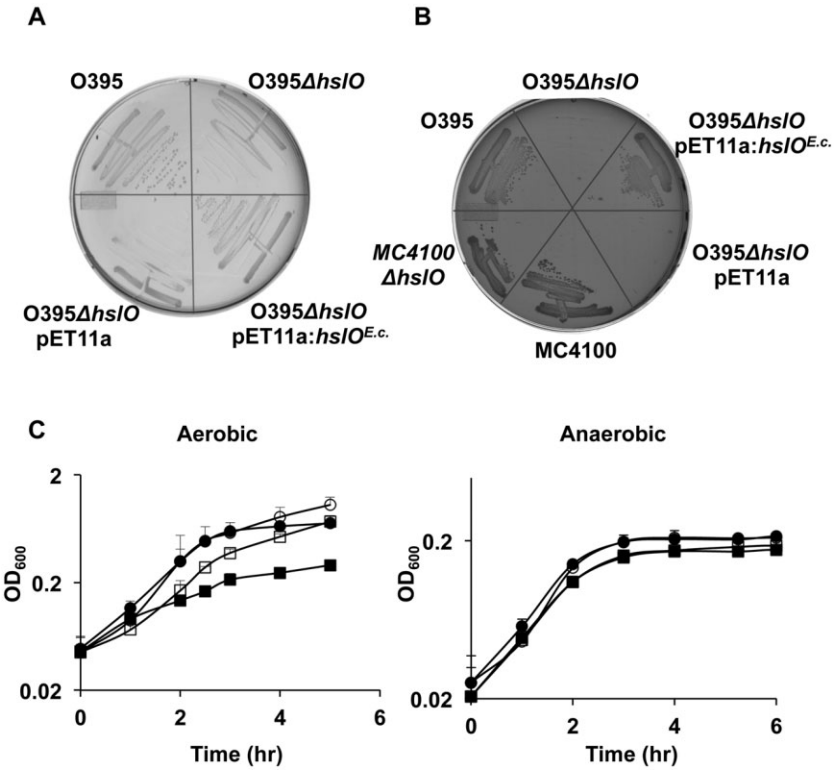

Fig. 1. Aerobically grown *V. cholerae* Δ*hslO* strain has *ts* phenotype.
A and B. Wild-type *V. cholerae* O395, O395 Δ*hslO* or O395 Δ*hslO* expressing either the empty pET11a plasmid or *E. coli* Hsp33 from a pET11a plasmid were grown on LB plates (A) or MacConkey plates (B) for 24 h at 43°C. Wild-type *E. coli* MC4100 and the corresponding MC4100 Δ*hslO* mutant strain are shown as controls.
C. *V. cholerae* O395 (circles) or O395 Δ*hslO* (squares) were cultivated in LB growth medium at either 37°C (open symbols) or 43°C (filled symbols) in the presence (left panel) or absence (right panel) of air oxygen. Bacterial growth was monitored by optical density measurements at 600 nm.

studies revealed that *V. cholerae* EF-Tu is rapidly degraded in the absence of Hsp33. Expression of *E. coli* EF-Tu compensates for the lack of Hsp33, suggesting that the cytoprotective effect of the general chaperone Hsp33 in *Vibrio* comes from guarding a single stress-sensitive protein, EF-Tu, whose presence is essential for the survival of the organism.

Results

V. cholerae *Hsp33 null mutants reveal a temperature-sensitive (*ts*) phenotype*

In vitro studies showed that the highly specialized bacterial chaperone Hsp33 contains a dual stress-sensing mechanism, which mediates activation of the chaperone function specifically under oxidative stress conditions that lead to protein unfolding (Ilbert *et al*., 2007; Winter *et al*., 2008). *In vivo* studies confirmed these results and showed that absence of the Hsp33 gene *hslO* significantly decreases *E. coli*'s resistance to HOCl stress or oxidative heat stress treatment but does not affect *E. coli*'s survival at high concentrations of peroxide or at elevated temperatures alone (Winter *et al*., 2005). It thus came as a surprise when we tested the *hslO* deletion phenotype in *V. cholerae* O395 and found this strain to be severely temperature-sensitive (*ts*) for growth. As shown in Fig. 1A and B, compared with wild-type cells, *V. cholerae* Δ*hslO* forms significantly smaller colonies on Luria–Bertani (LB) plates and fails to form any colonies on MacConkey plates after 24 h of incubation at 43°C. The temperature sensitivity of *hslO* null mutants in *Vibrio* and the finding that Hsp33 functions as an oxidative stress-regulated chaperone in *E. coli* suggested that heat treatment of *V. cholerae* either causes or exacerbates oxidative stress conditions that induce the activation of Hsp33, which in turn enhances the survival of *V. cholerae* at high temperatures. To investigate whether reactive oxygen species (ROS) indeed affect *Vibrio*'s survival at elevated temperatures, we compared the growth of *V. cholerae* wild type with that of the *hslO* deletion mutant in liquid media under both aerobic and anaerobic growth conditions (Fig. 1C). When cultivated under aerobic conditions, we observed a slight growth disadvantage in Δ*hslO* strains at 37°C and a significant reduction in growth rate at 43°C when compared with the growth of wild-type O395. This result was fully consistent with the *ts* phenotype of this mutant strain on plates. In contrast, however, when we cultivated the same strains under anaerobic conditions, the growth rates of wild type and Δ*hslO* mutants strains were not significantly different at either 37°C or 43°C (Fig. 1C). These results suggest that at elevated temperatures *V. cholerae* suffers from oxidative heat stress, which requires activation of Hsp33's chaperone function for survival. Expression of *E. coli* Hsp33 in the *V. cholerae* Δ*hslO* deletion strain was able to fully complement the *ts*

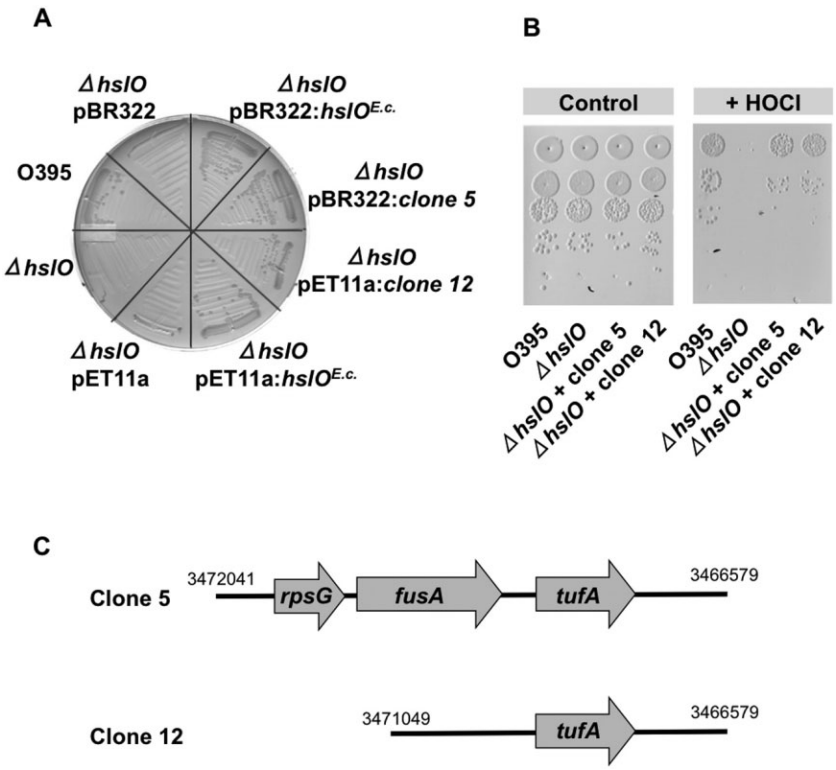

Fig. 2. *E. coli* expression library contains clones that rescue *ts* and HOCl-sensitive phenotypes of O395 *ΔhslO* mutant.
A. *E. coli* gene expression library from MG1655 *ΔhslO* mutant strain was constructed in either pET11A or pBR322 plasmids and transformed into the *V. cholerae* O395 *ΔhslO* mutant strain. Transformants were cultivated on MacConkey plates for 24 h at 43°C. Two independently identified transformants, clone 5 from the pBR322 library and clone 12 from the pET11a library, were selected for further analysis. Growth of these two strains was tested on MacConkey plates for 24 h at 43°C and compared with O395 wild type, O395 *ΔhslO* and O395 *ΔhslO* expressing the respective empty plasmids.
B. To test the bleach sensitivity of these strains, wild-type O395, O395 *ΔhslO* or O395 *ΔhslO* expressing either clone 5 or clone 12 were cultivated in LB medium until mid-log phase was reached. Cells were washed, resuspended in phosphate buffer, and treated with 10 μM HOCl for 20 min. Cell viability was analysed by preparing serial dilutions of the cultures and spotting them onto LB plates.
C. Schematic presentation of *E. coli* genomic sequences with indicated chromosomal positions that were inserted into clone 5 or clone 12.

phenotype of this strain (Fig. 1A and B), excluding significant differences in the activation requirements between the two Hsp33 homologues.

Identification of E. coli *genes that rescue the* ts *phenotype of O395* ΔhslO

We reasoned that the severe *ts* phenotype of the O395 *ΔhslO* mutant strain on MacConkey plates might serve as an *in vivo* selection system to identify *E. coli* proteins that, when overexpressed in *V. cholerae*, protect against oxidative heat stress and, by extension, against HOCl-mediated protein damage. The complementing *E. coli* gene could encode Hsp33 itself or proteins that function in a way analogous to Hsp33 in parallel pathways. Alternatively, rescuing genes could encode *E. coli* homologues for important *Vibrio* Hsp33 substrates, which are either Hsp33-independent in *E. coli* or become Hsp33-independent in *Vibrio* simply by increasing their steady-state concentrations. Because we were searching for proteins that are potent in rescuing *hslO* null mutants at relatively low levels of expression, we constructed genomic expression libraries from wild-type *E. coli* MG1655 using either pBR322 (15–20 copies per cell) or pET11a plasmids as expression vectors. We had previously observed that pET11a mediated expression of *E. coli* Hsp33 in *hslO* null strains lacking the T7 DNA polymerase provides sufficient Hsp33 levels to allow complementation (Fig. 1A and B). We transformed the genomic library into the O395*ΔhslO* mutant strain and selected for transformants showing robust growth on MacConkey plates after 24 h incubation at 43°C. All six investigated transformants encoded the *E. coli hslO* gene. To avoid repeated cloning and identification of the *hslO* gene, we next constructed a genomic library using chromosomal DNA of the MG1655 *ΔhslO* deletion mutant WC126, and performed the same selection procedure. We found two independent clones, clone 5 from the pBR322 library and clone 12 from the pET11a library, which conferred a high degree of complementation (Fig. 2A). Cultivation of *V. cholerae ΔhslO* strain mutants expressing either clone 5 or 12 on MacConkey plates at 43°C yielded colonies that were similar in size to colonies formed by *V. cholerae* wild type or by *V. cholerae ΔhslO* strains expressing *E. coli* Hsp33 from either pBR322 or pET11a plasmid (Fig. 2A). In contrast, a *V. cholerae ΔhslO* mutant strain expressing the empty vector failed to form colonies under these conditions. As mentioned previously, Hsp33 protects bacteria against oxidative protein unfolding conditions induced by either high concentrations of peroxide at elevated temperatures (Winter *et al.*, 2005) or by low concentrations of HOCl (Winter *et al.*, 2008). To test whether expression of either one of the two identified *E. coli* clones rescues the HOCl-sensitive phenotype of the O395 *ΔhslO* mutant as well, we exposed O395 wild type, O395 *ΔhslO* or the O395 *ΔhslO* mutant

strains expressing either clone 5 or clone 12 to a 10 μM HOCl treatment for 20 min and tested their survival (Fig. 2B). Consistent with earlier studies (Winter *et al*., 2008), O395 *ΔhslO* was significantly more sensitive to HOCl treatment than wild type. Importantly, O395 *ΔhslO* mutant strains expressing either clone 5 or 12 were resistant to HOCl treatment. These results indicate that the gene(s) encoded on these plasmids are capable of protecting O395 *ΔhslO* against a variety of different stress conditions that cause oxidative protein unfolding.

E. coli *EF-Tu expression rescues the* ts *phenotype of* V. cholerae *O395* ΔhslO

To investigate which *E. coli* genes are responsible for rescuing the temperature- and HOCl-sensitive phenotype of the *V. cholerae* O395 *ΔhslO* mutant, we sequenced the inserts in clones 5 and 12. While the pBR322:clone 5 contained a sequence spanning three separate genes (*rpsG*, *fusA* and *tufA*), the pET11a:clone 12 contained only one complete gene (*tufA*) that encodes elongation factor Tu (EF-Tu) (Fig. 2C). Phenotypical analysis of O395 *ΔhslO* expressing subclones of pBR322:clone 5 with either *rpsG* or *rpsG*/*fusA* deleted verified that the presence of the *E. coli tufA* gene, including its upstream regions, is sufficient to rescue the *ts* phenotype of the O395 *ΔhslO* mutant strain. Erase-A-Base (Promega) was used to identify the minimal sequence sufficient to complement the *ts* phenotype of O395 *ΔhslO*. The shortest sequence capable of rescuing the *ts* phenotype contained 405 bases upstream of the *tufA* gene as well as the complete *tufA* gene. This result is in excellent agreement with previous studies showing that the promoter region of *tufA* is about 400 bases upstream of the start codon (Zengel and Lindahl, 1990). We concluded from these studies that expression of a single protein, *E. coli* EF-Tu, is necessary and sufficient to rescue the *ts* phenotype and, by extension, the HOCl-sensitive phenotype of a *V. cholerae* mutant strain lacking Hsp33. We noted that *E. coli* EF-Tu was not massively overexpressed; rather, its expression levels were comparable with the endogenous level of EF-Tu in wild-type *V. cholerae* (see below).

Hsp33 is essential for maintaining high levels of soluble EF-Tu in V. cholerae

The elongation factor EF-Tu is one of the most abundant proteins in the bacterial cytosol and is essential for cell growth (Pedersen *et al*., 1978). To begin to understand how overexpression of *E. coli* EF-Tu can rescue the temperature-sensitive growth defect of the O395 *ΔhslO* mutant strain on plates and in cultures, we compared the levels of endogenous EF-Tu in *V. cholerae* and O395 *ΔhslO* mutant strains under both non-stress and heat shock conditions in the absence of additional *E. coli* EF-Tu. We found that lack of Hsp33 did not cause a noticeable change in the steady-state concentration of endogenous *Vibrio* EF-Tu at 30°C, but led to a reproducible decrease in EF-Tu levels at 37°C and a very substantial decrease at 43°C when compared with EF-Tu levels in either wild type or the O395*ΔhslO* mutant expressing *E. coli* Hsp33 from a plasmid (Fig. 3A). When we cultivated the strains anaerobically; however, no difference in the steady-state levels of EF-Tu was detected at any temperature (Fig. 3A). This result is entirely consistent with the lack in phenotype of *hslO* null mutants in *Vibrio* under anaerobic growth conditions (Fig. 1C) and serves to show that elevated temperatures alone do not affect EF-Tu levels in *V. cholerae*. These results suggest that under aerobic heat shock conditions, presence of the redox-regulated chaperone Hsp33 is necessary to maintain EF-Tu at cellular levels that are sufficient for cell growth. Note that we saw no differences in the steady-state levels of EF-Tu in *E. coli* wild-type vs. *E. coli ΔhslO* strains at 43°C, which is consistent with the fact that the *E. coli ΔhslO* deletion strains are not temperature-sensitive for growth (Winter *et al*., 2005).

Absence of Hsp33 leads to accelerated degradation of EF-Tu

Changes in the steady-state levels of a protein are either the result of decreased rates of transcription and translation and/or are caused by increased rates of proteolysis. To assess the mRNA levels of *tufA* in *V. cholerae* and *V. cholerae ΔhslO* strains, we performed RT-PCR under aerobic conditions at three different temperatures: 30°C, 37°C and 43°C. No significant difference in *tufA* transcript levels was observed (Fig. S1), arguing against the possibility that Hsp33 either directly or indirectly affects the expression of *tufA*. Our results strongly suggested that Hsp33 acts at the post-transcriptional level, presumably by protecting EF-Tu against premature degradation.

To determine the proteolytic stability of endogenous EF-Tu in the presence and absence of Hsp33, we conducted pulse-chase experiments combined with 2D gel electrophoresis. We performed these experiments at non-stress temperatures to exclude that major differences in the growth rates of *V. cholerae* wild-type and *ΔhslO* deletion strains affect our data analysis. We reproducibly identified the same 300 protein spots on 2D gels and autoradiographs and used them as reference spots for our analysis (see *Experimental procedures* for details). Analysis of the Coommassie stained 2D gels confirmed our previous observations and showed a 26% reduction in EF-Tu steady-state levels in the O395 *ΔhslO* mutant strain as compared with wild-type O395 at non-stress temperatures (Fig. 3B, left panel). Western blot analysis failed to detect any fragments of EF-Tu. When we compared the

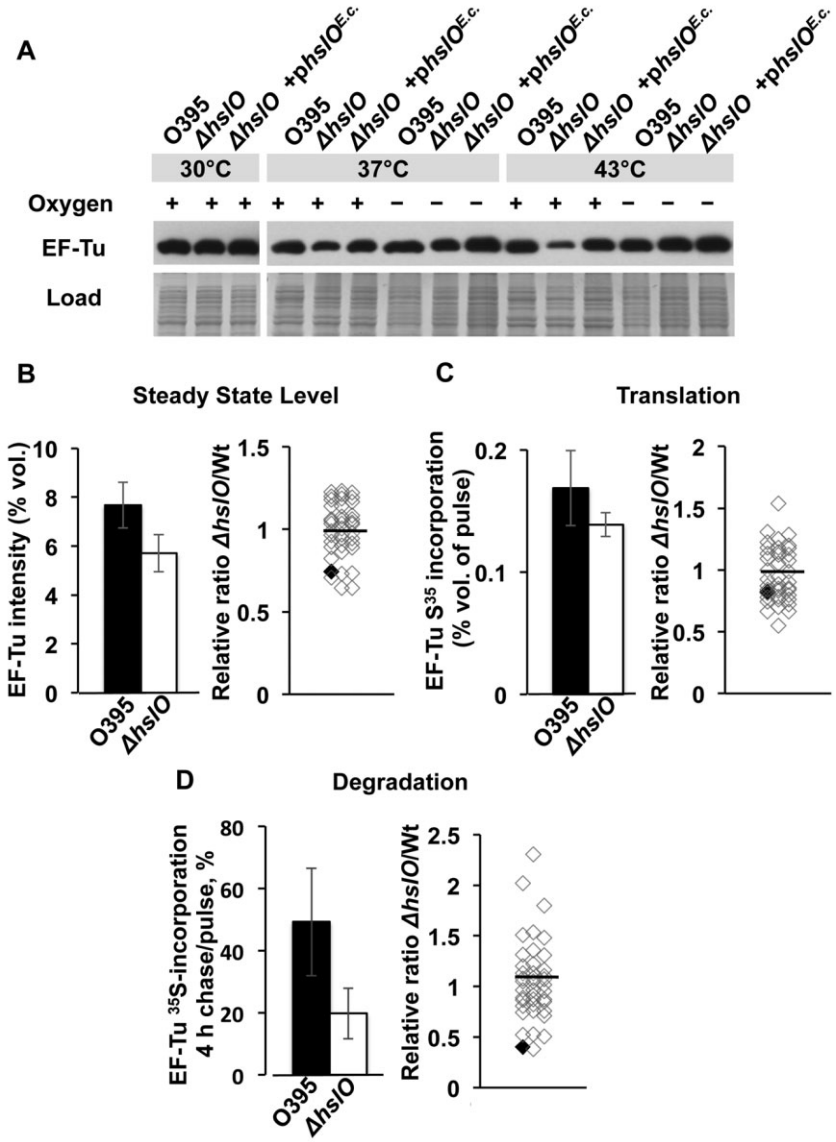

Fig. 3. Hsp33 protects *V. cholerae* EF-Tu against protein degradation.
A. Wild-type O395, O395 *ΔhslO* mutant or O395 *ΔhslO* mutant encoding *E. coli hslO* on a pBR322 plasmid (*ΔhslO* + p*hslO*$^{E.c.}$) were cultivated in LB medium at the indicated temperature in the presence and absence of air oxygen. Cells were harvested at mid-log growth and lysed. Steady-state levels of EF-Tu were visualized using Western blot analysis with polyclonal antibodies against *E. coli* EF-Tu.
B–D. *V. cholerae* wild-type and *V. cholerae ΔhslO* mutant strains were cultivated in minimum MOPS medium supplemented with all amino acids except methionine and cysteine. Cells were pulsed for 2 min with radioactive ^{35}S-methionine, flushed with cold unlabelled methionine, and chased for 4 h. The cell lysates were prepared and proteins were separated by 2D PAGE and scanned for ^{35}S incorporation. The error bars represent standard errors from four individual experiments.
B. Left panel: To compare the steady-state EF-Tu levels in *V. cholerae* wild type (black bar) and *ΔhslO* mutant (white bar), the relative spot intensity of EF-Tu on Coommassie-stained 2D gels was determined for both strain backgrounds. Right panel: The relative spot intensity of the 40 most abundant protein spots (see Fig. S2A) was determined in both *ΔhslO* and wild-type *V. cholerae* and compared. Individual proteins are represented by open diamonds. EF-Tu is indicated as black diamond.
C. Left panel: To compare the ^{35}S incorporation into EF-Tu from *V. cholerae* wild type and *ΔhslO* mutant, the relative spot intensity of EF-Tu on the respective autoradiographs was determined. Right panel: The relative ratio of ^{35}S-incorporation into the 40 most abundant protein spots in *ΔhslO* and wild-type *V. cholerae* was determined. Individual proteins are represented by open diamonds. EF-Tu is indicated with a black diamond.
D. Left panel: To determine the rates of protein degradation, ^{35}S incorporation after 4 h of chase relative to ^{35}S incorporation after 2 min pulse was calculated for EF-Tu in *V. cholerae* wild type and *ΔhslO* mutant. Right panel: Protein degradation rates were determined for the 40 most abundant protein spots in both *ΔhslO* and wild-type *V. cholerae* and compared. Individual proteins are represented by open diamonds. EF-Tu is indicated with a black diamond.

ratio of EF-Tu steady-state levels in O395 wild type and the corresponding *ΔhslO* deletion mutant with the 40 most abundant protein spots (Fig. S2A), we found the ratio of EF-Tu to be significantly below the mean (Fig. 3B, right panel and Fig. S3), suggesting that Hsp33 affects EF-Tu levels rather specifically.

Analysis of the autoradiographs revealed that EF-Tu translation was not significantly different in wild-type and mutant cells, confirming that Hsp33 has no effect on transcription or translation of EF-Tu (Figs 3C and S2B). In contrast, we found that the rate of EF-Tu degradation in wild-type and *ΔhslO* mutant strains was dramatically different. We detected almost 50% of the original ^{35}S-label after 4 h of pulse in EF-Tu isolated from wild-type cells, whereas only 20% of the original label was detected in EF-Tu isolated from cells lacking Hsp33. These results indicate that EF-Tu was degraded 2.5-fold faster in the *hslO* deletion strain than in wild-type cells (Figs 3D, S2C and D). Compared with the 40 most abundant protein spots on the 2D gel, EF-Tu showed again the largest difference in degradation rates between wild type and the *hslO* deletion mutant (Fig. 3D, right panel, and Fig. S3). These results strongly suggest that EF-Tu is a key Hsp33 client protein, a conclusion, which is consistent with global protein–protein interaction studies in *E. coli* that showed that Hsp33 is an interaction partner of EF-Tu (Butland *et al*., 2005).

V. cholerae *EF-Tu is exquisitely sensitive to oxidative stress treatment*

Our studies demonstrated that during aerobic growth of *V. cholerae*, presence of Hsp33 is required to maintain the stability of the EF-Tu protein. The fact that plasmid-driven expression of *E. coli* EF-Tu is sufficient to complement the *ts* phenotype of the O395 *ΔhslO* mutant furthermore suggested that *V. cholerae* EF-Tu might exhibit higher oxidative stress sensitivity than *E. coli* EF-Tu. It has been previously shown that *E. coli* EF-Tu, although apparently insensitive to peroxide-mediated thiol modifications, quickly responds to HOCl stress treatment with the reversible modification of at least one of its three cysteine residues (Leichert *et al*., 2008). To compare the *in vivo* redox status of *E. coli* and *V. cholerae* EF-Tu before and after HOCl treatment, we performed differential thiol-trapping experiments using MC4100 and O395 strains. Both strains were cultivated in LB medium at 37°C to mid-log phase. Cell aliquots were removed before and 20 min after HOCl treatment, and lysed in the presence of 10% TCA to prevent any further thiol oxidation (Zander *et al*., 1998). All reduced cysteine thiols were irreversibly alkylated with N-ethylmaleimide (NEM), whereas all reversibly oxidized cysteines were reduced with DTT and subsequently labelled with the 500 Da thiol-specific alkylation reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). The latter labelling step introduces a 500 Da molecular mass to every cysteine residue that was originally oxidized *in vivo*. This mass addition significantly slows the migration of AMS-labelled proteins and allows direct visualization of the *in vivo* redox status of proteins on one-dimensional SDS-PAGE. We observed a striking difference in the *in vivo* redox status of *V. cholerae* and *E. coli* EF-Tu, particularly in exponentially growing bacteria. While *E. coli* EF-Tu was almost completely reduced during logarithmic growth, the majority of *V. cholerae* EF-Tu was already partially oxidized (Fig. 4, compare lanes 5 and 7). Treatment of *V. cholerae* with sublethal concentrations of HOCl shifted almost all of the endogenous EF-Tu into the fully oxidized species. Comparative analysis of NEM-trapped samples on reducing and non-reducing gels suggested the formation of both inter- and intramolecular disulfide bonds in HOCl-treated *V. cholerae* EF-Tu (Fig. S4). In contrast, the same treatment of *E. coli* cells led only to a partial oxidation of *E. coli* EF-Tu and no visible intermolecular disulfide bond formation (Figs 4 and S4). These results suggest that *V. cholerae* EF-Tu is highly susceptible to oxidation and appears to be exposed to substantial levels of ROS during aerobic exponential growth even at non-stress temperatures.

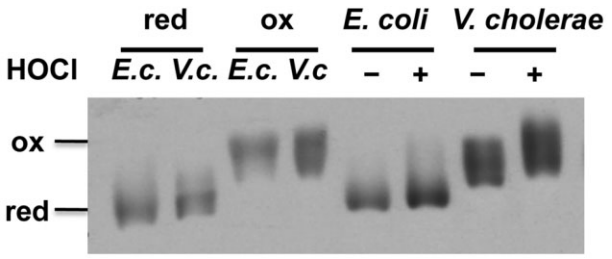

Fig. 4. *V. cholerae* EF-Tu is exquisitely sensitive to oxidative thiol modifications. To determine the redox status of EF-Tu *in vivo*, *E. coli* MC4100 and *V. cholerae* O395 were grown in LB medium at 37°C under aerobic conditions until mid-log phase was reached. Cells were either left untreated or were treated with 3 mM HOCl for 20 min. Samples were taken and cysteines were labelled with NEM, followed by the reduction of all oxidized thiols and the labelling of all newly reduced cysteines with the 500 Da thiol-alkylating molecule AMS. Addition of AMS molecules is visualized as migration difference on SDS-PAGE, whose extent directly reflects the number of *in vivo* oxidized cysteines. To define the migration behaviour of fully NEM-labelled EF-Tu (equivalent to reduced species) and fully AMS-labelled EF-Tu (equivalent to completely oxidized species), aliquots of non-stressed MC4100 or O395 cells (indicated by *E.c. and V.c.* respectively) were reduced with DTT and labelled exclusively with either NEM (lanes 1 and 2) or AMS (lanes 3 and 4). Proteins were separated on SDS-PAGE and EF-Tu was visualized with Western blot analysis using antibodies against *E. coli* EF-Tu.

Expression of E. coli *EF-Tu affects* V. cholerae *EF-Tu levels* in vivo

Our results suggested that *V. cholerae* EF-Tu is a highly oxidative stress-sensitive protein whose loss in steady-

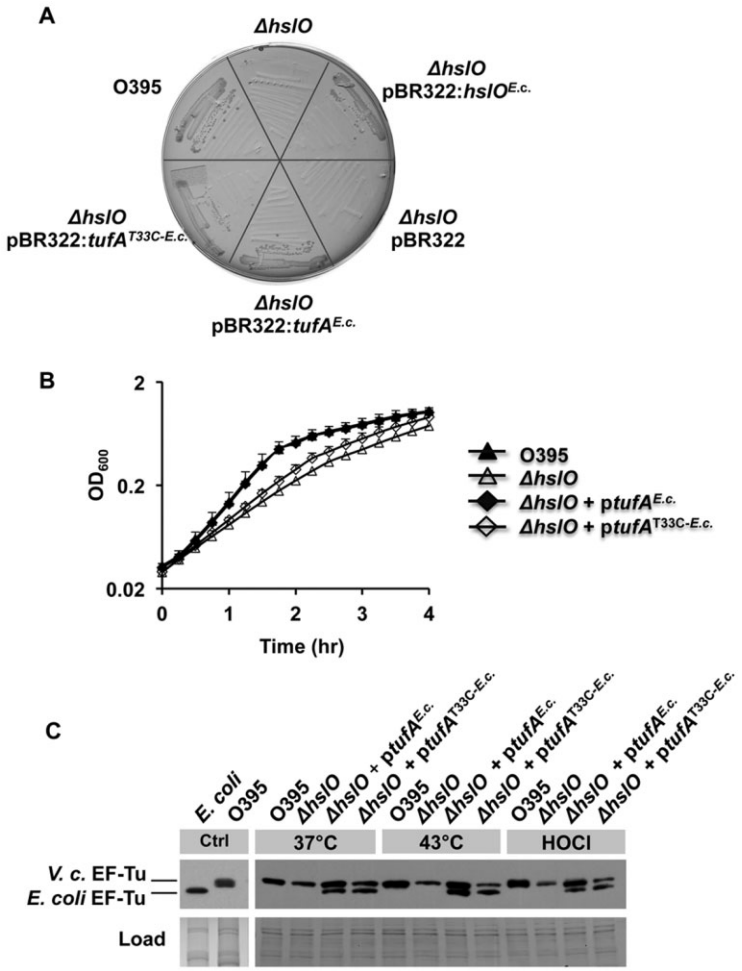

Fig. 5. *E. coli* EF-Tu compensates for lack of Hsp33 by protecting *V. cholerae* EF-Tu against oxidative protein degradation.
A. *V. cholerae* O395 wild type, O395 *ΔhslO* mutant or O395 *ΔhslO* expressing the empty pBR322 vector, *E. coli* Hsp33, *E. coli* EF-Tu or the *E. coli* EF-Tu^{T33C} variant were cultivated on MacConkey plates for 24 h at 43°C.
B. *V. cholerae* O395 wild type (black triangles), O395 *ΔhslO* mutant (white triangles), and O395 *ΔhslO* expressing either *E. coli* EF-Tu (black diamonds) or the *E. coli* EF-Tu^{T33C} variant (white diamonds) were cultivated in LB medium at 43°C aerobically. Bacterial growth was monitored by optical density measurements at 600 nm.
C. To analyse the steady-state levels of EF-Tu in these bacterial strains, the different strains were cultivated in LB medium at either 37°C or 43°C until mid-log phase was reached. To evaluate the effects of HOCl on cellular EF-Tu levels, the 37°C cultures were split and either left untreated or incubated with 3 mM HOCl for 20 min. Cell aliquots were taken and the proteins were separated by SDS-PAGE. EF-Tu was visualized by Western blot using antibodies against *E. coli* EF-Tu.

state levels in the absence of Hsp33 is compensated by expressing the potentially more oxidative stress-resistant *E. coli* EF-Tu homologue. Amino acid sequence comparison between *E. coli* and *V. cholerae* EF-Tu revealed that in addition to the three cysteine residues that are present in the two EF-Tu homologues, *V. cholerae* EF-Tu encodes one additional cysteine residue that is located at position 33 (Cys33) (Fig. S5). To elucidate whether this cysteine is responsible for the increased oxidative stress sensitivity of *V. cholerae* EF-Tu, we generated a variant of *E. coli* EF-Tu that carries an additional cysteine residue at this position. For these experiments we used *E. coli* EF-Tu as we were unable to express detectable levels of *V. cholerae* EF-Tu from plasmid constructs in either *V. cholerae* or *E. coli* despite the use of various expression vectors. We expressed the *E. coli* EF-Tu^{T33C} variant in the *Vibrio* O395 *ΔhslO* mutant strain to test its ability to rescue its *ts* phenotype. We expected that any increase in oxidative stress sensitivity or overall decrease in EF-Tu stability caused by introducing the T33C mutation into *E. coli* EF-Tu should lead to lower steady-state levels of EF-Tu and thus to a lower capacity of this mutant protein to rescue the *ts* or bleach-sensitive phenotype of the *V. cholerae* O395 *ΔhslO* mutant. As shown in Fig. 5A, the O395 *ΔhslO* mutant expressing *E. coli* EF-Tu^{T33C} formed slightly smaller colonies on MacConkey plates at 43°C than O395 wild-type or O395 *ΔhslO* mutant strains expressing wild-type *E. coli* EF-Tu from the same plasmid. Moreover, monitoring the aerobic growth of these strains in LB medium at 43°C revealed that expression of the *E. coli* EF-Tu^{T33C} variant was only partially able to rescue the growth defect of O395 *ΔhslO* (Fig. 5B). We then decided to analyse the levels of soluble *E. coli* EF-Tu and *E. coli* EF-Tu^{T33C} variant upon plasmid-mediated expression in O395 *ΔhslO* at 43°C, which was made possible by the fact that *V. cholerae* EF-Tu and *E. coli* EF-Tu differ significantly in their migration behaviour on SDS-PAGE and hence can be distinguished even when coexpressed (Fig. 5C). We did not find any significant difference in the cellular levels of the two *E. coli* EF-Tu variants, suggesting that the simple presence of additional EF-Tu might not be sufficient to rescue the *ts* phe-

notype of O395 *ΔhslO* (Fig. 5C). What we did notice, however, was a significant difference in the levels of endogenous *Vibrio* EF-Tu. Whereas expression of wild-type *E. coli* EF-Tu in O395 *ΔhslO* mutant strains raised the endogenous EF-Tu levels to those observed in wild-type O395 at both 37°C and 43°C, expression of the *E. coli* EF-Tu^{T33C} variant did not affect the endogenous levels of *V. cholerae* EF-Tu (Fig. 5C). These results suggested that wild-type *E. coli* EF-Tu, but not *E. coli* EF-Tu^{T33C}, confers stability to *Vibrio* EF-Tu, thus increasing its steady-state levels and potentially contributing to the enhanced stress survival observed in these bacteria. We obtained very similar results when we analysed the steady-state levels of *V. cholerae* EF-Tu in response to bleach treatment at 37°C. While expression of either Hsp33 or *E. coli* EF-Tu significantly stabilized the levels of endogenous EF-Tu in the presence of HOCl, absence of Hsp33 or presence of the *E. coli* EF-Tu^{T33C} mutant led to substantially increased bleach-mediated degradation of endogenous EF-Tu. These results thus suggest that expression of *E. coli* EF-Tu functionally replaces Hsp33 at least in part by protecting *V. cholerae* EF-Tu against oxidative protein degradation. Furthermore, our study demonstrates that the oxidative stress sensitivity of the single bacterial protein EF-Tu is sufficient to determine the cellular survival of *V. cholerae* at elevated temperatures and in the presence of the physiological antimicrobial bleach.

Discussion

Hsp33 is a highly specialized chaperone, which appears to be selectively activated by protein unfolding conditions in the presence of elevated ROS levels, such as experienced by organisms during hypochlorous acid stress or oxidative heat stress (Winter *et al*., 2008). Absence of Hsp33 in bacteria exposed to these specific stress conditions causes a substantial growth disadvantage, allowing us to use the *hslO* deletion phenotype as an indicator for oxidative protein unfolding *in vivo*. Here we report the surprising finding that deletion of the Hsp33 gene *hslO* in *V. cholerae*, a mutant strain with a high sensitivity to HOCl stress (Winter *et al*., 2008), displays a temperature-sensitive (*ts*) growth defect. Importantly, we found that the *ts* phenotype was fully abrogated when the mutant bacteria were cultivated under anaerobic conditions. One reasonable explanation is that *V. cholerae* is exposed to significant ROS production during aerobic growth and requires Hsp33 as an alternative chaperone once it encounters protein unfolding induced by stress conditions, such as elevated temperatures.

The severe *ts* phenotype of *V. cholerae hslO* deletion mutants provided us with the opportunity to use a genetic approach to shed light on the *in vivo* mechanism of Hsp33's chaperone action. We reasoned that by searching for genes in addition to *hslO* that are capable of rescuing the *ts* phenotype of *V. cholerae ΔhslO* mutants, we might discover alternative *E. coli* chaperones or antioxidant systems that are able to replace Hsp33 under oxidizing protein unfolding stress conditions. Alternatively, we might identify bacterial proteins whose high sensitivity to oxidative protein unfolding causes the observed phenotype and can be compensated by an increase in their steady-state levels. We independently selected two EF-Tu clones that rescued both the temperature-sensitive and bleach-sensitive phenotypes of *V. cholerae* lacking Hsp33. EF-Tu promotes binding of aminoacyl-tRNA to the ribosome and therefore allows peptide chain elongation during protein biosynthesis (Thompson *et al*., 1986). Previous *in vivo* studies provided evidence that *E. coli* EF-Tu is a redox-sensitive protein that shows elevated levels of thiol oxidation during aerobic growth and undergoes additional oxidative thiol modifications in response to HOCl treatment (Leichert *et al*., 2008). Moreover, exposure of *E. coli* cells to near-lethal HOCl stress conditions or oxidative heat shock caused EF-Tu's aggregation, suggesting that excessive thiol modifications might induce protein unfolding (Winter *et al*., 2008). Finally, it was found that decreasing the levels of EF-Tu by deleting the *tufA* gene increased *E. coli*'s bleach sensitivity (Leichert *et al*., 2008). Our studies in *V. cholerae* were fully consistent with the results in *E. coli*. Yet, our finding that EF-Tu's cysteines become oxidized simply by growing *V. cholerae* under aerobic conditions suggested an even higher oxidation sensitivity of *Vibrio* EF-Tu as compared with *E. coli* EF-Tu. Absence of the redox-regulated chaperone Hsp33 then leads to the premature degradation of EF-Tu, which causes a decrease in growth rates and, by a yet to be defined mechanism, a significant increase in bleach sensitivity.

At this point, it is unclear how expression of *E. coli* EF-Tu confers enhanced bleach resistance to *V. cholerae*. The simplest and most straightforward explanation of our results would be that *E. coli* wild-type EF-Tu has increased oxidative stress resistance, hence functionally replacing *V. cholerae* EF-Tu in protein translation and promoting *E. coli*'s recovery after bleach stress by rapidly resuming protein translation. Introduction of the additional Cys33 would increase EF-Tu's oxidative stress sensitivity and hence abrogate this protective function. However, our studies led to a very unexpected finding: we showed that presence of *E. coli* EF-Tu not only increased the steady-state levels of *V. cholerae* EF-Tu during aerobic growth but significantly stabilized *Vibrio* EF-Tu both during heat stress and upon short-term treatment with bleach. Hence the beneficial effects of *E. coli* EF-Tu expression were less likely simply due to an increase in the translation rate of *V. cholerae* EF-Tu but appeared to involve stabilization of *Vibrio* EF-Tu towards oxidative protein degradation.

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant genotype	Plasmid	Reference
JW370	*V. cholerae* O395 WT		Winter *et al*. (2008)
JW371	O395 Δ*hslO*		Winter *et al*. (2008)
WC022	JW371	pET11a	This study
WC025	JW371	pET11a:*hslO*$^{E.c.}$	This study
WC038	JW371	pET11a:clone 12	This study
WC028	JW371	pBR322	This study
WC159	JW371	pBR322:*hslO*$^{E.c.}$	This study
WC037	JW371	pBR322:clone 5	This study
WC157	JW371	pBR322:*tufA*$^{E.c.}$	This study
WC187	JW371	pBR322:*tufA*$^{T33C\text{-}E.c.}$	This study
WC126	MG1655		Lab collection
WC127	WC126 *hslO*::kan		Lab collection
BB7222	MC4100		Winter *et al*. (2008)
JW176	BB7222 *hslO*::kan		Winter *et al*. (2008)

This conclusion was also consistent with our analysis of protein translation in *V. cholerae* strains lacking Hsp33, which appeared to not be affected by the decreased EF-Tu levels. It has long been known that even during exponential growth EF-Tu molecules outnumber ribosomes by a factor of seven (Furano, 1975; Pedersen *et al*., 1978). This finding has fuelled the idea that EF-Tu plays more than one role in the cell. For instance, it has been demonstrated that EF-Tu polymerizes with other proteins to form filamentous, actin-like structure that function to maintain cell shape in *E. coli* and *Bacillus subtilis* (Beck, 1979; Defeu Soufo *et al*., 2010). Moreover, previous *in vitro* studies showed that purified elongation factor EF-Tu protects thermally unfolding citrate synthase, a commonly used *in vitro* chaperone substrate, against protein aggregation and supports the refolding of citrate synthase upon return to non-stress temperatures (Kudlicki *et al*., 1997; Caldas *et al*., 1998). This potential chaperone function of *E. coli* EF-Tu might compensate for the lack of Hsp33 and hence stabilizes *V. cholerae* EF-Tu. We were unable to find increased stabilization or decreased aggregation in response to heat or bleach stress for any other *Vibrio* protein(s) in *hslO* deletion mutants expressing *E. coli* EF-Tu, suggesting that EF-Tu might act specifically with the *Vibrio* EF-Tu pool, for instance by forming intermolecular dimers (Weijland and Parmeggiani, 1994). Another proposed function of EF-Tu includes an antioxidant scavenger role to buffer oxidants through the use of its 10 methionine residues (Luo and Levine, 2009). This scavenging function of EF-Tu has been proposed to decrease the levels of ROS *in vivo* and hence protect oxidation-sensitive proteins against oxidative damage. As the *E. coli* EF-Tu^{T33C} variant has the same number of methionines and is expressed to the same extent, this non-specific antioxidant function appears to play only a minor role in conferring bleach resistance in *V. cholerae*. Future studies are clearly needed to address these fundamental questions regarding EF-Tu's alternative *in vivo* functions.

In summary, our study revealed that the stress sensitivity of a whole organism is determined in large part by the stress sensitivity of a single essential protein. In the case of *Vibrio*, this single essential protein appears to be EF-Tu. The stabilization of ET-Tu by the chaperone Hsp33 is sufficient to significantly enhance the stress resistance of the whole organism. It remains now to be determined what essential protein(s) are the 'weakest links' in other organisms under the same or other stress conditions. Given the many known stress conditions, which vary in effects and biological targets, it is likely to be different proteins for different stress conditions, justifying the wide substrate specificity of molecular chaperones.

Experimental procedures

Strain and growth condition

The *V. cholerae* and *E. coli* strains used in this study can be found in Table 1. Strains were cultivated in LB medium at the indicated temperatures. Ampicillin (100 μg ml^{-1}) was added to those cultures that contained pET11a or pBR322 plasmids. Growth on MacConkey agar at 43°C was used to select for temperature-sensitive phenotypes.

Preparation of genomic overexpression library

Genomic DNA from the *E. coli* wild-type strain MG1655 or the Δ*hslO* deletion strain JW176 was prepared using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). The genomic DNA (2 μg) was partially digested with 0.05–0.2 units of BfuCI (New England Biolabs) in a total volume of 50 μl for 10 min at 37°C. Digested products were analysed on 1% agarose gels and showed fragment sizes ranging between 1 and 8 kb. After heat inactivation of enzyme, the DNA fragments were ligated into linearized pBR322/BamH1 or pET11a/BamH1 plasmid vectors using T4 ligase (New

England Biolabs). The overexpressing plasmids were transformed into ultracompetent XL10-Gold cells (Stratagene) following the manufacturer's protocol. Colony-forming units of transformants were counted the next day to calculate the size of the libraries (1×10^5 colonies for pBR322:Δ*hslO* library; 5×10^4 colonies for pET11a:Δ*hslO* library). The transformants of each library were combined and the plasmids were purified using Wizard Plus SV Minipreps kit (Promega). The plasmids were then transformed into *V. cholerae* Δ*hslO* mutant strain. The transformants were plated on MacConkey agar and incubated for 24 h at 43°C to select for clones that rescue the *ts* phenotype of the *V. cholerae* Δ*hslO* mutant strain. Transformants that formed healthy looking colonies on plates were re-streaked on MacConkey plates and grown at 43°C. Plasmids of 12 transformants per library were purified and re-transformed into *V. cholerae* Δ*hslO* mutant to eliminate the possibility of mutations in the strain background.

HOCl survival assay

To determine the HOCl stress resistance, bacterial stains were cultivated in LB media at 37°C until OD_{600} of 0.4–0.5 was reached. Due to the reactivity of HOCl, cells were harvested, washed twice with 83 mM sodium phosphate buffer, pH 7.0, and resuspended in the same buffer. The cell density in each sample was normalized to 2×10^8 cells per millilitre and treated with the indicated concentrations of sodium hypochlorite (Sigma-Aldrich). After 20 min incubation at room temperature, the treated cells were diluted 1:10 into fivefold concentrated LB medium to quench the remaining HOCl (Winter *et al.*, 2008). Serial tenfold dilutions of treated cells were prepared and spotted onto LB plates. The colony-forming units after overnight incubation at 37°C were counted and used for determination of cell survival.

Pulse-chase labelling and 2D gel electrophoresis

Vibrio cholerae O395 wild type and *V. cholerae* Δ*hslO* were cultivated in MOPS minimal medium supplemented with 0.2% glucose and all amino acids except methionine and cysteine for 24 h at 37°C. Cells were then diluted 1:80 into fresh media and grown at 35°C until $OD_{600} = 0.4–0.5$ was reached. Then, 15 μCi ml^{-1} radioactive ^{35}S-methionine (Easytag Expre^{35}S Protein Labeling Mix, PerkinElmer) was added to each culture for 2 min (i.e. pulse) followed by the addition of 2.7 mM unlabeled methionine (i.e. chase). Aliquots of 1.8 ml cells were collected immediately after the pulse as well as 1, 2, and 4 h during the chase. All samples were washed twice with ice-cold 60 mM KCl buffer and lysed in DAB buffer (6 M Urea, 200 mM Tris-HCl pH 8.5, 10 mM EDTA, and 0.5% w/v SDS). To determine the protein amount in the cell lysates, the D_C Protein Assay Kit (Bio-Rad) was applied using BSA as standard. 90 μg of protein from each sample were then pelleted using trichloroacetic acid precipitation and redissolved in 450 μl of loading buffer [7 M urea, 2 M thiourea, 1% (w/v) Serdolit MB-1, 1% (w/v) dithiothreitol, 4% (w/v) Chaps, and 0.5% (v/v) Pharmalyte 3–10]. The 2D gel electrophoresis, staining of the gels and autoradiography were performed as previously described (Leichert and Jakob, 2004).

Image and data analysis

The protein pattern on the stained 2D gels and autoradiographs were compared and analysed using the Delta2D 3.6 Software (Decodon). Spot detection, background correction and normalization were performed according to the software's instructions. Spot matching and alignments across the autoradiographs and stained gels of at least four independent pulse-chase experiments were performed. A master fusion gel, which retained all spots located on all individual images (Fig. S2) was generated for spot labelling, visualization and cross-reference between gels. With the built-in settings of Delta2D, the spot quality, pixel intensity (i.e. volume) and % volume (volume of individual spot over total volume of all spots) for each protein spot was determined and exported as a spreadsheet table. To compare the steady-state concentration and translation of EF-Tu within a 2 min time frame in *V. cholerae* and *V. cholerae* Δ*hslO*, we calculated the mean % volume of EF-Tu as well as of the 40 most abundant protein spots on the Coomassie-stained 2D gels or autoradiographs (Fig. S2). To analyse the protein degradation rates in *V. cholerae* and *V. cholerae* Δ*hslO*, we divided the % volume of each spot on the autoradiograph by the % volume of the respective spot on the Coommassie stained 2D gel and determined the fold decrease of ^{35}S incorporation from the 2 min pulse to 4 h of chase. The mean degradation rate for EF-Tu and the each of the 40 pre-selected spots was calculated from four independent pulse-chase experiments (Fig. S3). Standard errors were calculated and are shown in the figures.

Differential in vivo *thiol trapping with NEM and AMS*

Bacterial strains were cultivated in LB media at 37°C until OD_{600} of 0.4–0.5 was reached. Then, 3 mM HOCl was added to the medium directly and incubation was continued for 20 min. Before and after the stress treatment, aliquots of 1 ml were taken and acidified with trichloroacetic acid (TCA) to a final concentration of 10%. After 30 min of incubation on ice, precipitated proteins were pelleted by centrifugation (13 000 r.p.m., 20 min, 4°C). The protein pellet was resuspended in DAB buffer (6 M Urea, 200 mM Tris-HCl pH 8.5, 10 mM EDTA, and 0.5% w/v SDS) supplemented with 100 mM NEM to irreversibly alkylate all reduced cysteines. Samples were incubated for 30 min at 25°C. The proteins were again precipitated with TCA to remove any unbound NEM, and pelleted by centrifugation. For differential thiol trapping with AMS, protein pellets were resuspended in DAB buffer supplemented with 10 mM DTT to reduce all *in vivo* oxidized cysteines, and incubated for 1 h at 25°C. Excess DTT was removed by TCA precipitation and centrifugation. All newly accessible cysteines were then modified with 10 mM of the thiol-specific alkylation reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), which adds 500 Da mass to every modified cysteine. As control, we also prepared fully NEM-labelled proteins, which represent the fully reduced species and fully AMS-labelled proteins, which represent the fully oxidized protein species. Cell aliquots were taken, precipitated with TCA and resuspended in DAB buffer supplemented with 10 mM DTT to reduce all *in vivo* thiol modifications. After incubation of the samples for 1 h

at 25°C, proteins were precipitated with TCA, centrifuged and resuspended in DAB buffer supplemented with either 100 mM NEM or 10 mM AMS. Proteins were separated on SDS-PAGE and EF-Tu was visualized by Western blot analysis using polyclonal antibodies against EF-Tu (provided by Dr. Beckwith).

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