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Report

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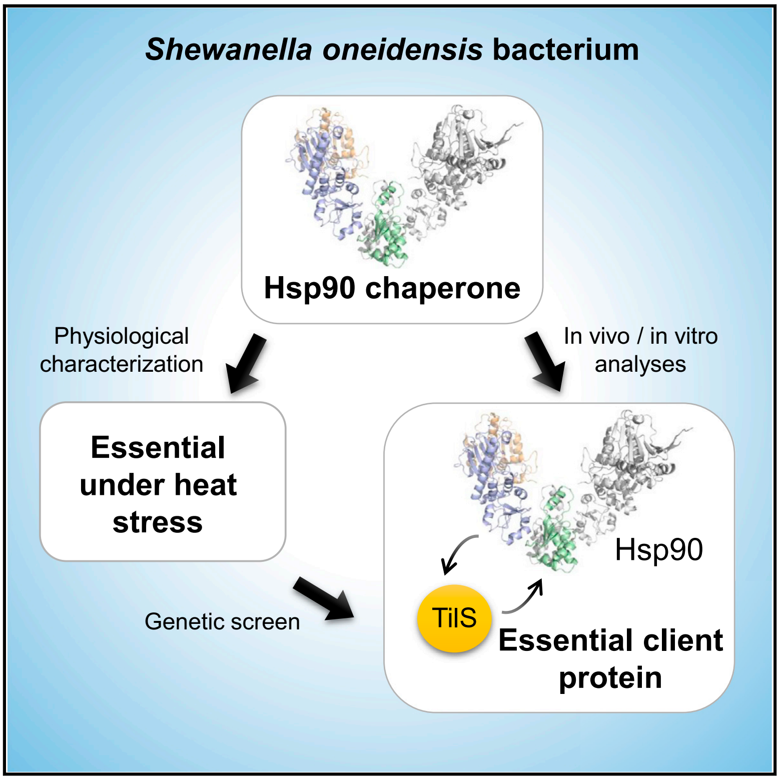
Graphical Abstract



Authors

Flora Ambre Honoré, Vincent Méjean, Olivier Genest

Correspondence

ogenest@imm.cnrs.fr

In Brief

Honoré et al. show that Hsp90 is essential in the model bacterium *Shewanella oneidensis* under heat stress, which is contrary to previously published reports. They also identify a tRNA maturation factor TilS as a client of Hsp90 that explains the essentiality of the chaperone.

Highlights

- Hsp90 is essential in the model bacterium *S. oneidensis* under heat stress
- A genetic screen uncovered an Hsp90 client (TilS) involved in tRNA maturation
- Hsp90 increases the amount and activity of the TilS client under heat stress
- Hsp90 interacts with TilS and protects it from aggregation in vitro

Honoré et al., 2017, Cell Reports *19*, 680–687
April 25, 2017
http://dx.doi.org/10.1016/j.celrep.2017.03.082

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Hsp90 Is Essential under Heat Stress in the Bacterium *Shewanella oneidensis*

Flora Ambre Honoré,[1] Vincent Méjean,[1] and Olivier Genest[1,2,*]
[1]Aix Marseille Univ, CNRS, BIP UMR 7281, 31 Chemin Joseph Aiguier, 13402 Marseille, France
[2]Lead Contact
*Correspondence: ogenest@imm.cnrs.fr
http://dx.doi.org/10.1016/j.celrep.2017.03.082

SUMMARY

The Hsp90 chaperone is essential in eukaryotes and activates a large array of client proteins. In contrast, its role is still elusive in bacteria, and only a few Hsp90 bacterial clients are known. Here, we found that Hsp90 is essential in the model bacterium *Shewanella oneidensis* under heat stress. A genetic screen for Hsp90 client proteins identified TilS, an essential protein involved in tRNA maturation. Overexpression of TilS rescued the growth defect of the *hsp90* deletion strain under heat stress. In vivo, the activity and the amount of TilS were significantly reduced in the absence of Hsp90 at high temperature. Furthermore, we showed that Hsp90 interacts with TilS, and Hsp90 prevents TilS aggregation in vitro at high temperature. Together, our results indicate that TilS is a client of Hsp90 in *S. oneidensis*. Therefore, our study links the essentiality of bacterial Hsp90 at high temperature with the identification of a client.

INTRODUCTION

All living organisms have developed several response mechanisms to survive in stress conditions. One of them involves a class of proteins, the chaperones, that are essential to maintain cellular proteostasis (Balchin et al., 2016). The Hsp90 chaperone is conserved in bacteria and in eukaryotes and participates, in collaboration with the Hsp70 chaperone system, in protein folding and activation (Johnson, 2012; Stankiewicz and Mayer, 2012; Taipale et al., 2010).

Hsp90 is a dimer and each protomer consists of three domains (Figure 1A) (Stankiewicz and Mayer, 2012; Taipale et al., 2010). The N-terminal domain binds and hydrolyses ATP. Residues from the M- and the C-terminal domains encompass a region involved in client binding, and the C-terminal domain allows the dimerization of the protein. Hsp90 is a highly dynamic protein (Flynn et al., 2015; Krukenberg et al., 2011; Li et al., 2012; Mayer and Le Breton, 2015; Shiau et al., 2006) with several conformations that are influenced by the presence of nucleotides (ATP or ADP) bound to the N-domain. In eukaryotes, Hsp90 is essential and more than 300 client proteins have been identified (Taipale et al., 2010). In addition, Hsp90 participates in tumorigenesis by allowing the folding and activation of oncoproteins (Neckers, 2007; Vartholomaiou et al., 2016). Therefore, Hsp90 is a target for cancer treatments.

In contrast to eukaryotes, the role of Hsp90 is still highly elusive in bacteria, and Hsp90 seems not to be essential in proteobacteria. In *E. coli*, the absence of Hsp90 (HtpG) leads to slight effects at very high temperature (Bardwell and Craig, 1988; Grudniak et al., 2013; Press et al., 2013; Thomas and Baneyx, 2000). In addition, Hsp90 participates in bacterial immunity via the CRISPR system (Yosef et al., 2011), in the virulence of some bacteria like extraintestinal pathogenic *E. coli* strains, *Edwardsiella tarda*, or *Salmonella typhimurium* and plays a role in the biosynthesis of antibiotic and toxins (Dang et al., 2011; Garcie et al., 2016; Verbrugghe et al., 2015; Vivien et al., 2005). However, the precise function of Hsp90 in virulence, i.e., the client proteins involved, is unknown. In contrast, in cyanobacteria that are phylogenetically distant from proteobacteria, Hsp90 is essential in heat stress and contributes to the resistance to oxidative stress (Hossain and Nakamoto, 2003; Tanaka and Nakamoto, 1999). In addition, only few Hsp90 clients have been identified so far in bacteria (Buchner, 2010). In *E. coli*, they include the L2 ribosomal protein, Cas3 from the CRISPR system, possibly CheA and FliN that participate in motility, and in cyanobacteria, a 30-kDa linker polypeptide of a large complex involved in photosynthesis, the phycobilisome (Motojima-Miyazaki et al., 2010; Press et al., 2013; Sato et al., 2010; Yosef et al., 2011). In vitro, Hsp90 from *E. coli* and from cyanobacteria has been shown to collaborate with the DnaK chaperone system to remodel denatured proteins (Genest et al., 2011; Nakamoto et al., 2014).

Shewanella are ubiquitous Gram-negative γ-proteobacteria found in oceans, lakes, and sediments (Hau and Gralnick, 2007). Given the wide changing conditions in which these bacteria evolve, they possess a great ability to adapt to stress. Therefore, we chose to study chaperone proteins in the model *Shewanella oneidensis*. We found that Hsp90 from *S. oneidensis* ($Hsp90_{So}$) is essential for growth at high temperature. We also identified an essential client of $Hsp90_{So}$ involved in tRNA maturation. When overproduced, this client restored the growth defect of the $Hsp90_{So}$ deletion strain, therefore explaining why Hsp90 is essential in *S. oneidensis*. This work will pave the way for a better understanding of the Hsp90 chaperone in bacteria.

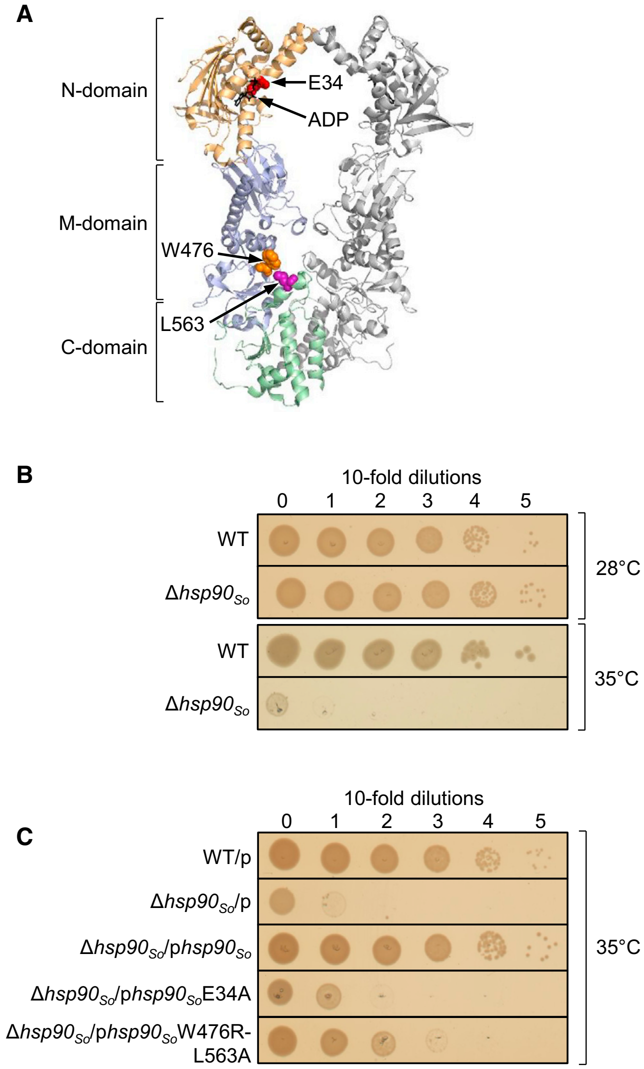

Figure 1. $Hsp90_{So}$ Is Essential for *S. oneidensis* Growth at High Temperature

(A) Model of the structure of Hsp90 from *E. coli* ($Hsp90_{Ec}$) crystalized in the presence of ADP (PDB: 2iop) (Shiau et al., 2006). $Hsp90_{Ec}$ is shown as a dimer. In one protomer, the N-domain, M-domain, and C-domain are colored yellow, blue, and green, respectively. The ADP molecule is represented in black. The conserved E34 residue important for ATP hydrolysis and the conserved W476 and L563 residues ($Hsp90_{So}$ numbering) important for client binding are colored and shown as CPK models.

(B) WT and Δ*hsp90*$_{So}$ strains grown at 28°C were diluted to OD_{600} = 1. 10-fold serial dilutions were spotted on LB plates that were incubated at 28°C or 35°C as indicated.

(C) WT strain containing the pBad33 vector (WT/p), and Δ*hsp90*$_{So}$ strain containing either pBad33 (Δ*hsp90*$_{So}$/p) or the p*hsp90*$_{So}$ WT or mutant plasmids grown at 28°C with chloramphenicol were diluted to OD_{600} = 1. 10-fold serial dilutions were spotted on LB plates containing 0.02% arabinose that were incubated for 24 hr at 35°C. In (B) and (C), plates are representative of at least three experiments.

See also Figure S1.

RESULTS AND DISCUSSION

$Hsp90_{So}$ Is Essential at High Temperature in *S. oneidensis*

To evaluate the role of Hsp90 in *S. oneidensis*, we deleted the gene coding for $Hsp90_{So}$. The wild-type (WT) and Δ*hsp90*$_{So}$ strains were grown aerobically at 28°C, and serial dilutions were spotted on plates. When the plates were incubated at 28°C or at lower temperatures, no growth difference was observed between the WT and the Δ*hsp90*$_{So}$ strains (Figures 1B and S1A). However, when the plates were incubated at a high temperature for *S. oneidensis* (35°C), the growth of the Δ*hsp90*$_{So}$ strain was dramatically affected, whereas the WT strain grew as well as at 28°C (Figure 1B). Similar results were obtained when the cells were grown in liquid media at 38°C (Figure S1B). Moreover, addition of the Hsp90 inhibitors geldanamycin or radicicol strongly reduced the growth of the WT strain at 38°C (Figure S1B).

To confirm that the growth defect was due to the absence of $Hsp90_{So}$, we performed a complementation assay. The Δ*hsp90*$_{So}$ strain containing an empty vector grew very poorly at 35°C on plate (Figure 1C). In contrast, in the presence of the p*hsp90*$_{So}$ plasmid producing $Hsp90_{So}$, the growth was restored to the same level as the WT strain containing an empty vector. We also checked that the three strains grew similarly at 28°C (Figure S1C). Altogether, these results indicate that $Hsp90_{So}$ is required for the growth of *S. oneidensis* at high temperature.

This growth defect is reminiscent of the phenotype observed with the *Synechococcus* cyanobacteria (Tanaka and Nakamoto, 1999). While *Synechococcus* and the γ-proteobacteria *S. oneidensis* are distant evolution-wise, their Hsp90 chaperone have a common essential role at high temperature. We thus propose that Hsp90 could be essential in many other bacteria in stressful conditions.

ATP Hydrolysis and Client Binding Are Important for the Chaperone Function of $Hsp90_{So}$ In Vivo

It has been shown that the ATPase activity of Hsp90 in eukaryotes is essential for its function (Obermann et al., 1998; Panaretou et al., 1998). Taking advantage of the essential role of $Hsp90_{So}$ at high temperature, we tested whether an $Hsp90_{So}$ mutant that is affected for ATPase activity could complement the Δ*hsp90*$_{So}$ strain. Based on previous works (Genest et al., 2011; Graf et al., 2009; Obermann et al., 1998; Panaretou et al., 1998), we constructed the $Hsp90_{So}$ mutant E34A (Figure 1A). The mutant protein was purified and its ATPase activity was dramatically affected compared to the WT protein (Figure S1D). Then, the plasmid containing the *hsp90*$_{So}$ mutant gene, p*hsp90*$_{So}$E34A, was introduced into the Δ*hsp90*$_{So}$ strain. We found that the mutant protein $Hsp90_{So}$E34A did not complement the growth defect of the Δ*hsp90*$_{So}$ strain at high temperature (Figure 1C). As controls, we observed that the mutation did not affect growth at 28°C (Figure S1C) and we checked by western blot with an anti-Hsp90 antibody that the mutant protein was produced at the same level as the WT protein (Figure S1E).

We also mutated residues located in the region of $Hsp90_{So}$ that is known to participate in client binding in $Hsp90_{Ec}$ and yeast Hsp82 (Genest et al., 2013; Street et al., 2012). The W476 and

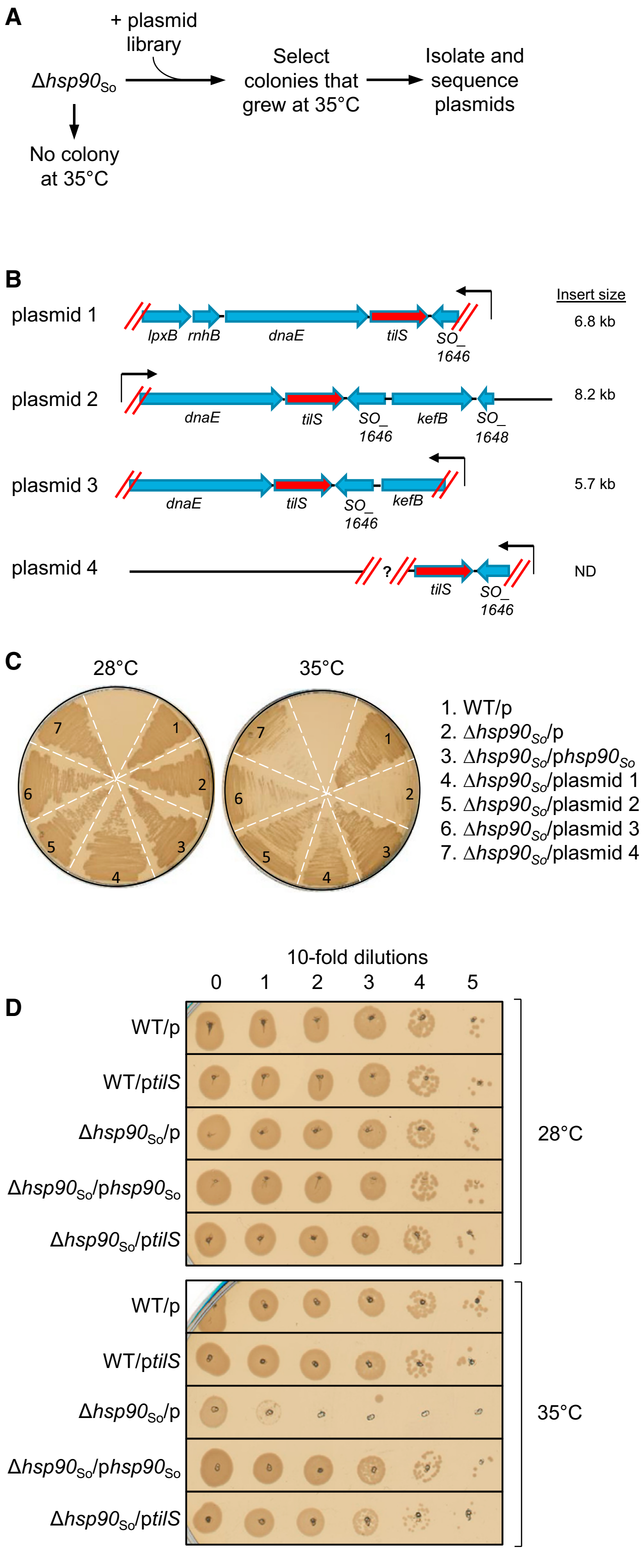

Figure 2. TilS Overproduction Rescues the Growth Defect of the Δ*hsp90*$_{So}$ Strain

(A) Experimental design to identify proteins that rescue the growth defect of the Δ*hsp90*$_{So}$ strain at high temperature using a plasmid library.

(B) Schematic of the genes present in the plasmids identified with the plasmid library. The *tilS* gene (colored red) was identified in the plasmids from four independent clones. Black arrows show the orientation of the pBad promoter. When present, double red lines indicate that genes are interrupted. For plasmid 4, the DNA sequence was non-continuous, probably due to the ligation of two fragments.

(C) Plasmids identified from the library were isolated and reintroduced into the Δ*hsp90*$_{So}$ strain. Strains were streaked on LB plates containing 0.2% arabinose and the plates were incubated at 28°C and 35°C.

(D) The *tilS* gene was cloned into the pBad33 vector (p). The resulting plasmid, p*tilS*, was introduced into the WT and the Δ*hsp90*$_{So}$ strains. Strains grown at 28°C were diluted to OD_{600} = 1. 10-fold serial dilutions were spotted on LB plates containing 0.2% arabinose that were incubated for 24 hr at 28°C or 35°C as indicated. In (C) and (D), plates are representative of three experiments. See also Figure S2.

L563 residues corresponding to the conserved residues W467 and L553 from Hsp90$_{Ec}$ were simultaneously mutated (double mutant W476R-L563A) in Hsp90$_{So}$ (Figure 1A). At 28°C, the mutation did not reduce *S. oneidensis* growth (Figure S1C) and western blots show that the mutant protein was also produced (Figure S1E). In contrast, the Δ*hsp90*$_{So}$ strain producing Hsp90$_{So}$ W476R-L563A was affected for growth at high temperature, although less drastically than the strain producing the E34A mutant (Figure 1C). This result suggests that this region is important for client binding in Hsp90$_{So}$, but that other residues in this region or in another region of Hsp90 might also participate in client binding as proposed by other groups (Karagöz and Rüdiger, 2015; Röhl et al., 2013). Because Hsp90$_{So}$ is essential in *S. oneidensis*, the identification of such regions will be facilitated.

Altogether, these results indicate that ATPase activity and client binding play key roles for chaperone function of Hsp90$_{So}$ at high temperature.

Overproduction of TilS Suppresses the Growth Defect of the Δ*hsp90*$_{So}$ Strain at High Temperature

We used the growth phenotype of the Δ*hsp90*$_{So}$ strain at high temperature to identify proteins that, when overproduced, can suppress the growth defect of the mutated strain. Such proteins could either be other chaperones that compensate for the absence of Hsp90$_{So}$ or clients of Hsp90$_{So}$. Indeed, a high amount of an essential client protein could bypass the need of Hsp90$_{So}$, because a fraction of the client protein pool could spontaneously be folded without the chaperone. We constructed a library of plasmids that contained partially digested genomic DNA fragments (5–8 kb) of *S. oneidensis* (Figure 2A). The library was introduced into the Δ*hsp90*$_{So}$ strain. Sixteen clones out of 2×10^8 cells were recovered at high temperature (Figure S2A), and the plasmids were partially sequenced. Because the chromosomal fragments we cloned when we constructed the library were rather large, several genes were present in the plasmids. Strikingly, among the 16 plasmids we partially sequenced, the entire *tilS* gene was identified ten times in four independent plasmids (Figure 2B). To confirm that growth restoration of the Δ*hsp90*$_{So}$ strain at 35°C was due to the presence of these plasmids, we extracted and reintroduced them into the Δ*hsp90*$_{So}$ strain. The presence of these plasmids partially rescued the growth defect of the Δ*hsp90*$_{So}$ strain at 35°C (Figure 2C). Finally, the *tilS* gene was cloned into a vector under an arabinose-inducible promoter to yield p*tilS*. Overproduction of TilS from the p*tilS* plasmid greatly improved the growth of the Δ*hsp90*$_{So}$ strain at 35°C (Figure 2D). As a control, we found that the growth of the Δ*hsp90*$_{So}$ strain at 35°C was not improved in the absence of the arabinose inducer (Figure S2B). In addition, the overproduction of TilS did not modify the growth of the WT and the Δ*hsp90*$_{So}$ strains at 28°C (Figure 2D) and did not induce a heat-shock response at 28°C or 34°C (Figure S2C).

Altogether, we identified a protein, TilS, that when overproduced bypasses the requirement of the Hsp90$_{So}$ chaperone at high temperature, and we therefore propose that TilS is a client of Hsp90$_{So}$. However, overproduction of TilS did not entirely suppress the growth defect of the Δ*hsp90*$_{So}$ strain at 35°C (Figures 2C and 2D). It suggests that either TilS is not the only essential client affected at this temperature in the absence of Hsp90$_{So}$, or that TilS always requires Hsp90$_{So}$ to be fully functional.

TilS Activity Is Reduced In Vivo in the Absence of Hsp90$_{So}$ at High Temperature

According to the literature, TilS is a tRNA^{Ile}-lysidine synthetase (Soma et al., 2003; Suzuki and Miyauchi, 2010). Essential in *E. coli*, this enzyme specifically modifies the tRNA^{Met} that originally allows the translation of the AUG codon into methionine, in the tRNA^{Ile} that allows the translation of the AUA codon into isoleucine. For that, TilS catalyzes the addition of a lysine on the cytidine 34 of the tRNA^{Met}, resulting in a lysidine (Ikeuchi et al., 2005). As expected, we obtained *S. oneidensis tilS* deletion strains only when *tilS* was expressed in *trans* from a plasmid, strongly suggesting that, as shown in *E. coli*, TilS is also essential in *S. oneidensis*. If TilS is a client of Hsp90$_{So}$, its activity should be lower in the absence of Hsp90$_{So}$ than in its presence at high temperature. To follow the activity of TilS in *E. coli*, Soma et al. (2003) added after the start codon of the *lacZ* reporter gene, a sequence containing four ATA codons, and they showed that the level of β-galactosidase activity became dependent on TilS activity. We used the same approach in *S. oneidensis* and a plasmid with four ATA codons fused to the sequence of *lacZ*, p(AUA)*lacZ*, was introduced into the WT and the Δ*hsp90*$_{So}$ strains (Figure 3A). The resulting strains were grown in liquid media at 28°C and 34°C, a sub-lethal temperature, and expression of the reporter gene was induced for 2 hr before β-galactosidase activity was measured. At both temperatures, we observed that in liquid media growth of the WT and Δ*hsp90*$_{So}$ strains was similar.

At 28°C, β-galactosidase activity was similar in the WT and in the Δ*hsp90*$_{So}$ strains containing the control plasmid, indicating that the production of the β-galactosidase was not affected by the absence of Hsp90$_{So}$ (Figure 3B). The WT and Δ*hsp90*$_{So}$ strains containing the p(AUA)*lacZ* plasmid had also a similar level of β-galactosidase activity although this level was slightly higher than that with the control plasmid for an unknown reason. When the strains were grown at high temperature (34°C), the WT strain containing either the p*lacZ* control plasmid or the p(AUA)*lacZ* plasmid had the same level of β-galactosidase activity (Figure 3B). In contrast, in the Δ*hsp90*$_{So}$ background, a significant decrease in β-galactosidase activity was measured in the strain containing the p(AUA)*lacZ* plasmid compared to the one with the control plasmid (Figure 3B). These results indicate that in the absence of Hsp90$_{So}$ and at high temperature, the activity of TilS is reduced, leading to a reduced translation of the β-galactosidase protein from the p(AUA)*lacZ* plasmid.

To confirm these results, we hypothesized that the overproduction of TilS should increase the overall TilS activity and consequently the level of β-galactosidase activity measured at high temperature in the Δ*hsp90*$_{So}$ strain. To do that, the gene coding for TilS was cloned in operon downstream of *lacZ* into p(AUA)*lacZ* plasmid, and a similar experiment as described above was performed. At high temperature, we observed that when TilS was overproduced, there was an increase of β-galactosidase activity in the Δ*hsp90*$_{So}$ strain containing the p(AUA)*lacZ-tilS* plasmid (Figure 3C), and the level of β-galactosidase activity was similar to the one measured in the WT strain. At lower temperature, the overproduction of TilS did not affect β-galactosidase activity. Therefore, the overproduction of TilS rescues TilS activity in the absence of Hsp90$_{So}$ at high temperature.

Altogether, these experiments support the idea that in physiological conditions at high temperature, the activity of TilS depends on Hsp90$_{So}$. Moreover, they strongly suggest that TilS is a client of Hsp90$_{So}$.

The Amount of TilS Is Reduced in the Absence of Hsp90$_{So}$ at High Temperature

When Hsp90 clients are not correctly folded, they can be recognized and degraded by proteases (Sato et al., 2010; Yosef et al., 2011). We therefore decided to follow the amount of the TilS protein in the presence or absence of Hsp90$_{So}$ at high temperature. To do that, a sequence coding for a 6His tag was added at the 3′ extremity of *tilS* in the p*lacZ-tilS* plasmid (Figure S3A), and the resulting plasmid was introduced into the WT and Δ*hsp90*$_{So}$ strains. Cells were then grown at 28°C and 34°C, and 0.02% or 0.2% arabinose was added to induce expression of the plasmid genes. After 2 hr, total protein extracts were loaded on a SDS-PAGE and TilS was detected by western blot with a 6His antibody. When the strains were grown at 28°C, similar levels of TilS were observed in the WT and Δ*hsp90*$_{So}$ strains, and the amount of TilS increases with increasing arabinose concentration (Figure 3D). However, when the strains were grown at high temperature with 0.02% and 0.2% arabinose, we observed a strong decrease of TilS amount in the absence of Hsp90$_{So}$ compared to the WT strain (Figure 3D). As a control, we showed that the decrease in TilS measured in the Δ*hsp90*$_{So}$ strain grown at high temperature was not due to a decrease in gene expression from the p*lacZ-tilS*$_{6His}$ plasmid. Indeed, we found that β-galactosidase activity was similar in the two strains grown at high temperature at each arabinose concentration (Figure S3B). As expected, addition of the Hsp90 inhibitor radicicol in the WT strain also led to a significant decrease in the amount of TilS at high temperature (Figure S3C).

Together, these results show that the amount of the TilS protein is strongly reduced at high temperature in the absence of Hsp90$_{So}$. Moreover, they suggest that in the absence of Hsp90$_{So}$, TilS is not correctly folded and is therefore degraded by proteases.

Hsp90$_{So}$ Interacts with TilS and Protects TilS from Aggregation In Vitro

Our results indicate that TilS is a client of Hsp90$_{So}$, implying that the two proteins interact. To demonstrate this, we first used an in vivo bacterial two-hybrid assay (Battesti and Bouveret, 2012).

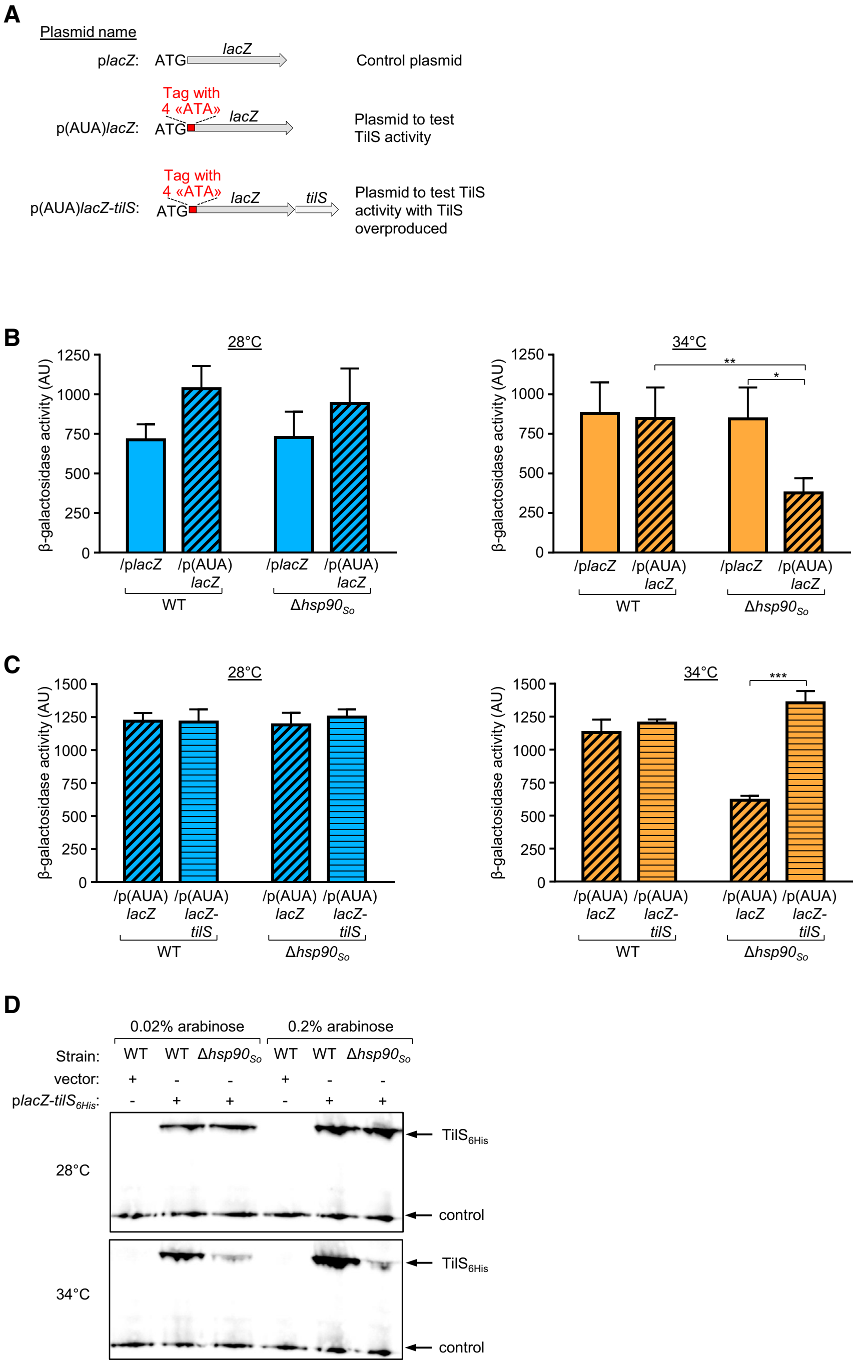

Figure 3. TilS Activity and Amount Are Reduced at High Temperature in the Absence of $Hsp90_{So}$

(A) Schematic of the plasmids used to follow TilS activity in vivo. A DNA sequence containing 4 “ATA” codons coding for Ile was inserted at the 5′ extremity (after the ATG start codon) of the *lacZ* reporter gene, leading to the p(AUA)*lacZ* plasmid. As a control, the *lacZ* reporter gene was cloned into the pBad33 vector (control plasmid). The *tilS* gene was cloned in operon downstream of the *lacZ* coding sequence into p(AUA)*lacZ*, leading to p(AUA)*lacZ-tilS*. In these plasmids, *lacZ* and *tilS* are under the transcriptional control of the pBad promoter.

(B) Cultures of WT and Δ*hsp90*$_{So}$ strains containing p*lacZ* or p(AUA)*lacZ* were diluted at OD_{600} = 0.1 and were grown at 28°C or 34°C for 3 hr before addition of 0.2% arabinose. β-galactosidase activity was measured after 2 hr.

(C) Same as (B) using WT and Δ*hsp90*$_{So}$ strains containing p(AUA)*lacZ* or p(AUA)*lacZ-tilS*. In (B) and (C), data from at least three replicates are shown as mean ± SEM. Where indicated, t test analyses show that the differences measured are significant (*p = 0.0112; **p = 0.0107; ***p = 0.0026).

(D) Cultures of WT and Δ*hsp90*$_{So}$ strains containing pBad33 (vector) or p*lacZ-tilS*$_{6His}$ were grown at 28°C or 34°C for 3 hr before addition of 0.02% or 0.2% arabinose. After 2 hr, protein extracts were analyzed by western blot using a 6His antibody. The band corresponding to TilS is shown by the arrow. The band labeled “control” corresponds to a protein that is nonspecifically detected by the antibody and shows that same amount of protein was loaded in the different wells. These western blots are representative of three independent experiments.

See also Figure S3.

By measuring β-galactosidase activity, we followed the activity of the adenylate cyclase reporter protein that is split in the T18 and T25 domains fused to TilS and $Hsp90_{So}$, respectively. When T18-TilS and T25-$Hsp90_{So}$ proteins were produced together, a high level of β-galactosidase activity was measured indicating that TilS and $Hsp90_{So}$ interact (Figure 4A). The interaction between TilS and $Hsp90_{So}$ is specific because background levels of β-galactosidase activity were observed when T18-TilS and T25, or T18 and T25-$Hsp90_{So}$ were produced together. As expected, we also showed that $Hsp90_{So}$ dimerizes (Figure 4A).

A pull-down experiment confirmed that $Hsp90_{So}$ co-purified with TilS that contains a calmodulin binding peptide (CBP) tag (Figure 4B). Interestingly, the client binding defective mutant $Hsp90_{So}$W476R-L563A did not copurify with TilS (Figure 4B), confirming that TilS is recognized as a client by $Hsp90_{So}$.

Finally, we showed that $Hsp90_{So}$ protects TilS from aggregation at high temperature. Indeed, using light scattering, we observed that purified TilS aggregated when incubated at 42°C (Figure 4C). However, the aggregation of TilS decreased when it was mixed with increasing concentrations of purified $Hsp90_{So}$. Nearly no aggregation of TilS occurs with four times more $Hsp90_{So}$ than TilS. This experiment demonstrates that $Hsp90_{So}$ protects TilS against aggregation in vitro. As a control, we observed that BSA or the $Hsp90_{So}$W476R-L563A mutant did not prevent aggregation of TilS (Figures 4C and S4A). Altogether, our results clearly show that TilS is a client of Hsp90 in *S. oneidensis.*

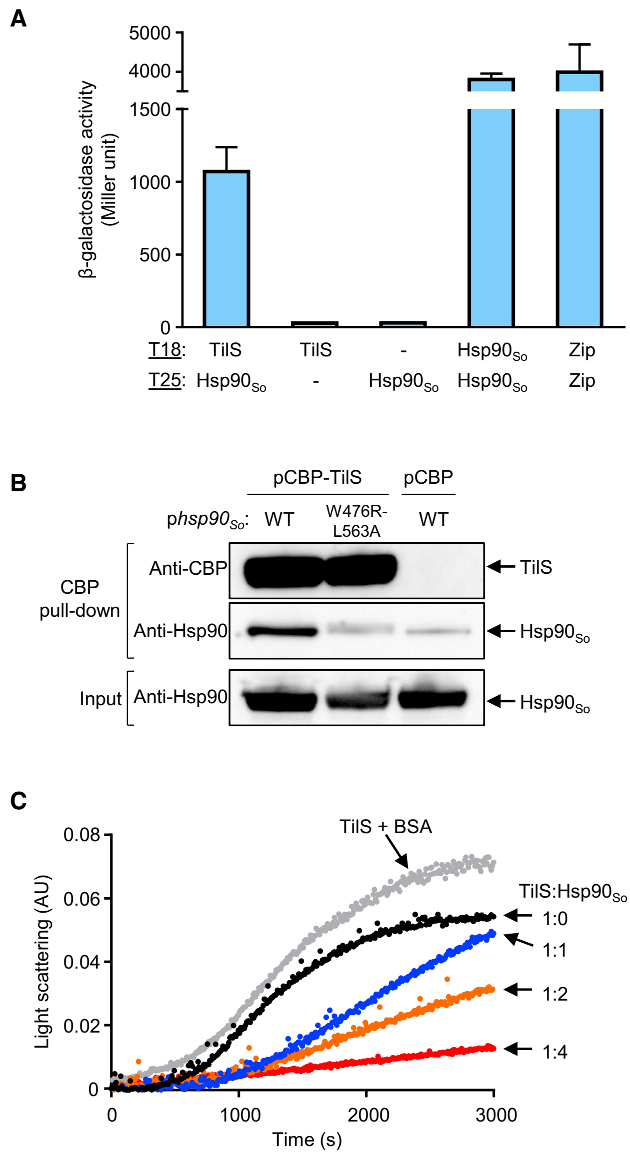

Figure 4. $Hsp90_{So}$ Binds to TilS and Protects It from Aggregation

(A) Two-hybrid method showing that $Hsp90_{So}$ and TilS interact. *E. coli* Bth101Δ*hsp90* strains transformed with T18-TilS and T25-$Hsp90_{So}$, T18-TilS and T25, T18 and T25-$Hsp90_{So}$, T18-$Hsp90_{So}$ and T25-$Hsp90_{So}$, or T18-Zip and T25-Zip were grown at 28°C overnight and β-galactosidase activity was measured. Data from three replicates are shown as mean ± SEM.

(B) Pull-down assay. TilS with a CBP tag was produced with or without $Hsp90_{So}$ WT or mutant in the *E. coli* MG1655Δ*htpG* strain. CBP-TilS was purified on CBP affinity resin, and after several washing steps, proteins were analyzed by western blot with anti-CBP and anti-Hsp90 antibodies (CBP pull-down panels). The same amount of Hsp90 WT or mutant was present in the protein extracts before purification (input panel). These western blots are representative of three independent experiments.

(C) Light scattering experiment showing that $Hsp90_{So}$ prevents aggregation of TilS. Purified TilS (5.4 μM) with or without purified $Hsp90_{So}$ (5 μM, 10 μM, 20 μM as indicated) were incubated at 42°C and OD_{360} was measured with time. An increase in absorbance indicates that TilS aggregates. As a control, $Hsp90_{So}$ was replaced by BSA (20 μM). The graph shown is representative of three independent experiments.

See also Figure S4.

In conclusion, we found that the Hsp90 chaperone is essential for growth at high temperature in *S. oneidensis* proteobacteria. We used this phenotype to screen a plasmid library and identified a first client, TilS, involved in tRNA modification in *S. oneidensis*. The strength of our genetic screen allowed the discovery of a client that had not been identified in a global study looking at $Hsp90_{So}$ clients (García-Descalzo et al., 2011). Because TilS is conserved in bacteria (Suzuki and Miyauchi, 2010), we question whether TilS from other bacteria also needs Hsp90 for folding in stress conditions. Future work will lead to the identification of other $Hsp90_{So}$ clients, and the fact that $Hsp90_{So}$ is essential will greatly facilitate the study of the molecular mechanism of action of this chaperone. In turn, because bacterial Hsp90 is also involved in virulence, its inhibition could represent a new strategy to combat infections.

EXPERIMENTAL PROCEDURES

Growth Conditions, Strains, and Plasmids

Strains were grown aerobically with shaking in rich lysogeny broth (LB) medium at the temperatures indicated in the figures. When necessary, chloramphenicol (25 μg/mL), kanamycin (50 μg/mL), ampicillin (50 μg/mL), or streptomycin (100 μg/mL) was added. The WT *S. oneidensis* strain used in this study is MR1-R (Bordi et al., 2003). Constructions of the *hsp90$_{So}$* deletion strain, plasmids, and the plasmid library are described in the Supplemental Experimental Procedures.

Protein Purification

To purify $Hsp90_{So}$ WT or mutants, pET-$Hsp90_{So}$, pET*hsp90$_{So}$*E34A, or pET*hsp90$_{So}$*W476R-L563A was introduced into the BL21(DE3) strain by transformation. The resulting strains were grown in LB at 37°C. At OD_{600} = 0.8, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. After 3 hr at 37°C, cells were collected by centrifugation, lysed by French Press and centrifuged at 13,000 rpm, 4°C, 15 min. After a second centrifugation step at 45,000 rpm, 4°C, 45 min, the supernatant was loaded onto a HiTrap FF resin (GE Healthcare), and purification was performed as described by the manufacturer. Limited proteolysis experiments with trypsin suggest that the W476R-L563A mutations do not modify the global folding of $Hsp90_{So}$ (Figure S4B). The same protocol was used to produce and purify TilS-6His from the pET-$TilS_{So}$ plasmid, except that cells were grown at 28°C, and 0.1 mM IPTG was added at OD_{600} = 0.8 for 2 hr. Purified proteins were stored at −80°C in 50 mM Tris-HCl pH = 7.5, 100 mM KCl, 1 mM DTT, and 10% glycerol. BSA was purchased from ID Bio. Concentrations were measured by Bradford assays and are given for monomeric TilS and BSA and dimeric $Hsp90_{So}$.

Reporter Assay to Follow TilS Activity In Vivo

WT and Δ*hsp90$_{So}$* strains containing p*lacZ*, p(AUA)*lacZ*, or p(AUA)*lacZ-tilS* plasmids were grown at 28°C overnight with chloramphenicol. Cells were then diluted to OD_{600} = 0.1 in LB and were grown at 28°C or 34°C for 3 hr, before addition of 0.2% arabinose. After 2 hr at 28°C or 34°C, β-galactosidase activity was measured using a Tecan Spark 10M microplate reader.

Experiments to Assess TilS Amount

The p*lacZ-tilS$_{6His}$* plasmid was introduced by conjugation into the WT and Δ*hsp90$_{So}$* strains. The resulting strains as well as the WT strain containing an empty vector were grown at 28°C overnight with chloramphenicol. Cells were then diluted to OD_{600} = 0.1, grown at 28°C or 34°C, and after 3 hr, 0.02% or 0.2% arabinose was added. Two hours later, the same amount of cells from the different cultures was heat-treated, loaded on SDS-PAGE, and transferred by western blot. TilS was visualized with a 6His antibody (Thermo, MA1-80218). In parallel, β-galactosidase activity was measured on the same cultures using a Tecan Spark 10M microplate reader. When indicated, radicicol (100 μM) or DMSO was added during both the preculture and the culture.

Two-Hybrid Experiments

Bacterial two-hybrid experiments were performed as described (Battesti and Bouveret, 2012). In this method, the catalytic domain of the adenylate cyclase (encoded by the *cya* gene) is split in the T18 and T25 subdomains that are each fused to the proteins to be tested for interaction. If the two proteins interact, the catalytic domain of the adenylate cyclase is reconstituted, therefore leading to the production of cAMP and in turn to the transcription of the *lacZ* reporter gene. Two-hybrid plasmids were transformed in the Bth101Δ*htpG* strain (Genest et al., 2015). The resulting strains were incubated for 3 days at 28°C. After inoculation of the strains in LB with ampicillin, kanamycin, and 0.5 mM IPTG, β-galactosidase activity was measured on overnight cultures.

Statistical Analysis

Results are given as mean ± SEM and are calculated with at least three replicates. In Figure 3B (six replicates) and Figure 3C (three replicates), paired t test analysis using parametric test was performed using GraphPad Prism Software version 6.05 to show that the differences between the samples are significant (p value <0.05).

Pull-Down Experiments

These experiments were adapted from a previous study (Battesti et al., 2013). MG1655Δ*htpG E. coli* strain (Genest et al., 2013) containing plasmids pCBP-TilS with p$hsp90_{So}$ WT or mutant and pCBP with p$hsp90_{So}$ WT were grown at 37°C. At OD_{600} = 0.8, 0.05% arabinose was added. After 1 hr, cells were pelleted, resuspended in CBP buffer (10 mM Tris HCl pH = 8, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% Triton, 70 μL βME), lysed with French Press, and centrifuged for 10 min at 8,000 rpm. The supernatant (1.4 mL) was incubated with Calmodulin binding peptide (CBP) affinity resin (Agilent) (50 μL) for 1 hr. The resin was then centrifuged 1 min at 2,000 rpm, washed four times with 1 mL of CBP buffer, resuspended in 40 μL of loading buffer, and heat-denatured at 95°C for 10 min. Proteins were loaded on SDS-PAGE and transferred by western blot. Samples of protein extracts before purification were also loaded on the gel as a control (input). $Hsp90_{So}$ was visualized with an anti-$Hsp90_{Ec}$ antibody (Genest et al., 2013), and TilS was visualized with an anti-CBP antibody (Millipore 07-482).

Light Scattering Experiments

TilS (20 μL, 5.4 μM final) was incubated at 42°C in 25 mM HEPES buffer, pH = 7.8, 200 μL final volume, and aggregation was followed spectrophotometrically at 360 nm using a Tecan Spark 10M microplate reader. When indicated, $Hsp90_{So}$ WT or mutant (5 μM, 10 μM, or 20 μM) dialyzed in 25 mM HEPES buffer, pH = 7.8, or BSA (20 μM) dialyzed in the same buffer, was incubated with TilS, and aggregation was monitored with time.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.082.

AUTHOR CONTRIBUTIONS

F.A.H., V.M., and O.G. designed experiments, interpreted data, and wrote the paper. F.A.H. and O.G. performed experiments.

ACKNOWLEDGMENTS

We thank Aurélia Battesti, Marianne Ilbert, Sue Wickner, Pierre Genevaux, Carsten Zeilinger, and members of the team for fruitful discussions. We also thank Chantal Iobbi-Nivol and Cécile Jourlin-Castelli for proof reading and Ahmed Zellat for technical assistance. This work was supported by the Centre National de la Recherche Scientifique and Aix-Marseille Université (AMU). O.G. was supported by a grant from the Agence Nationale de la Recherche (ANR-16-CE11-0002-01). F.A.H. was supported by the Ecole Pratique des Hautes Etudes.

Received: December 21, 2016
Revised: March 13, 2017
Accepted: March 31, 2017
Published: April 25, 2017

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