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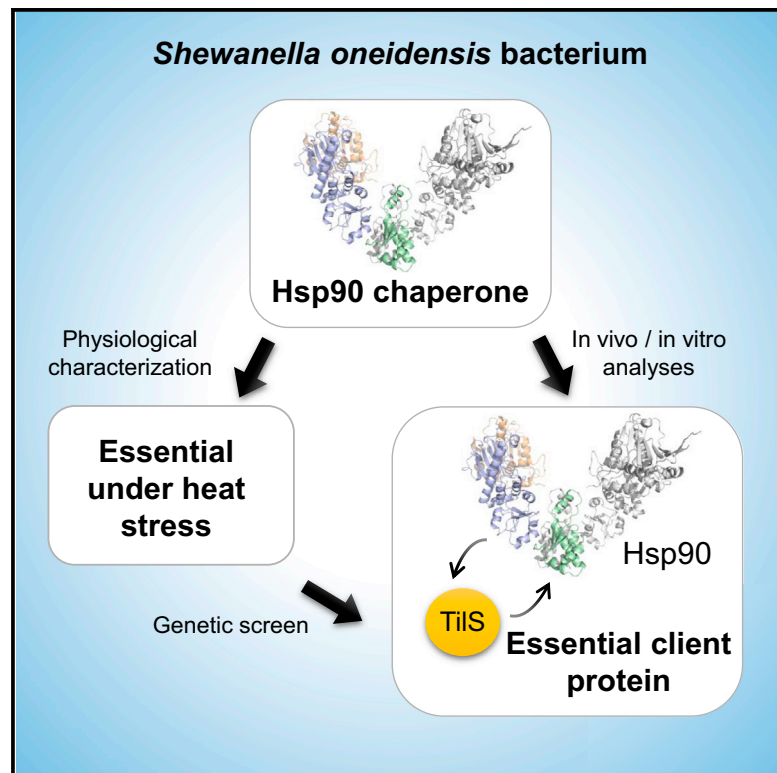
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## Hsp90 Is Essential under Heat Stress in the Bacterium *Shewanella oneidensis*

### Graphical Abstract



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### 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 TiIS 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 (TiIS) involved in tRNA maturation
- Hsp90 increases the amount and activity of the TiIS client under heat stress
- Hsp90 interacts with TiIS and protects it from aggregation in vitro



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

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## 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 TiIS, an essential protein involved in tRNA maturation. Overexpression of TiIS rescued the growth defect of the *hsp90* deletion strain under heat stress. In vivo, the activity and the amount of TiIS were significantly reduced in the absence of Hsp90 at high temperature. Furthermore, we showed that Hsp90 interacts with TiIS, and Hsp90 prevents TiIS aggregation in vitro at high temperature. Together, our results indicate that TiIS 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

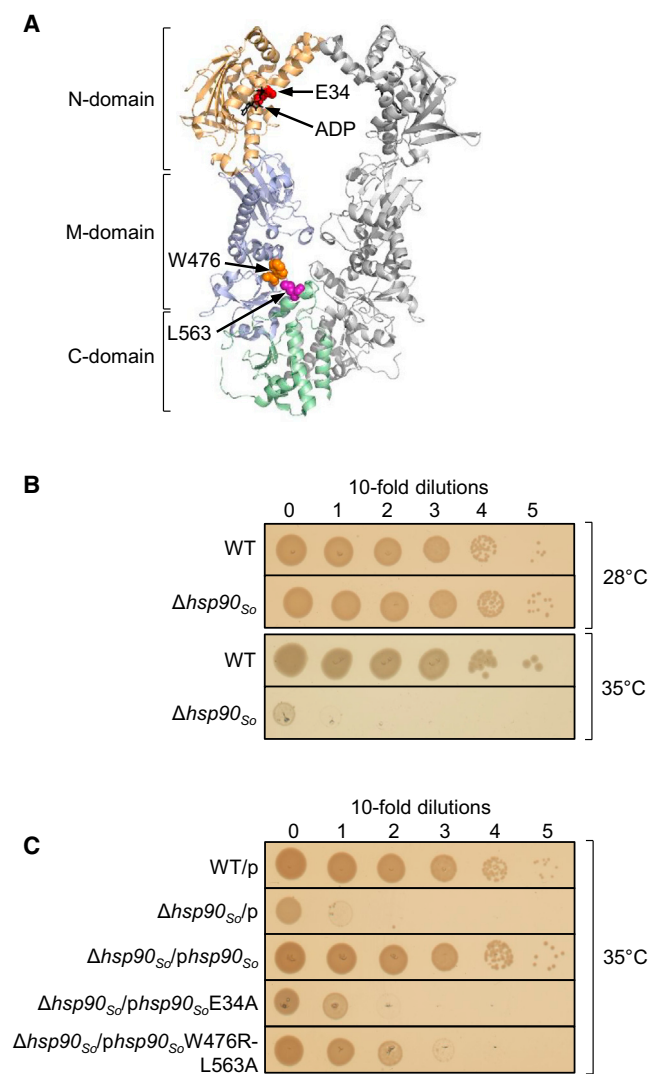
All living organisms have developed several response mechanisms to survive in stress conditions. One of them involves a class of proteins, the chaperones, that are essential to maintain cellular proteostasis (Balchin et al., 2016). The Hsp90 chaperone is conserved in bacteria and in eukaryotes and participates, 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  $\gamma$ -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<sub>So</sub>) is essential for growth at high temperature. We also identified an essential client of Hsp90<sub>So</sub> involved in tRNA maturation. When overproduced, this client restored the growth defect of the Hsp90<sub>So</sub> 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<sub>So</sub> Is Essential for *S. oneidensis* Growth at High Temperature**

(A) Model of the structure of Hsp90 from *E. coli* (Hsp90<sub>Ec</sub>) crystalized in the presence of ADP (PDB: 2iop) (Shiau et al., 2006). Hsp90<sub>Ec</sub> 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<sub>So</sub> numbering) important for client binding are colored and shown as CPK models.

(B) WT and  $\Delta hsp90_{So}$  strains grown at 28°C were diluted to OD<sub>600</sub> = 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  $\Delta hsp90_{So}$  strain containing either pBad33 ( $\Delta hsp90_{So}/p$ ) or the *phsp90<sub>So</sub>* WT or mutant plasmids grown at 28°C with chloramphenicol were diluted to OD<sub>600</sub> = 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<sub>So</sub> Is Essential at High Temperature in *S. oneidensis*

To evaluate the role of Hsp90 in *S. oneidensis*, we deleted the gene coding for Hsp90<sub>So</sub>. The wild-type (WT) and  $\Delta hsp90_{So}$  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  $\Delta hsp90_{So}$  strains (Figures 1B and S1A). However, when the plates were incubated at a high temperature for *S. oneidensis* (35°C), the growth of the  $\Delta hsp90_{So}$  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).

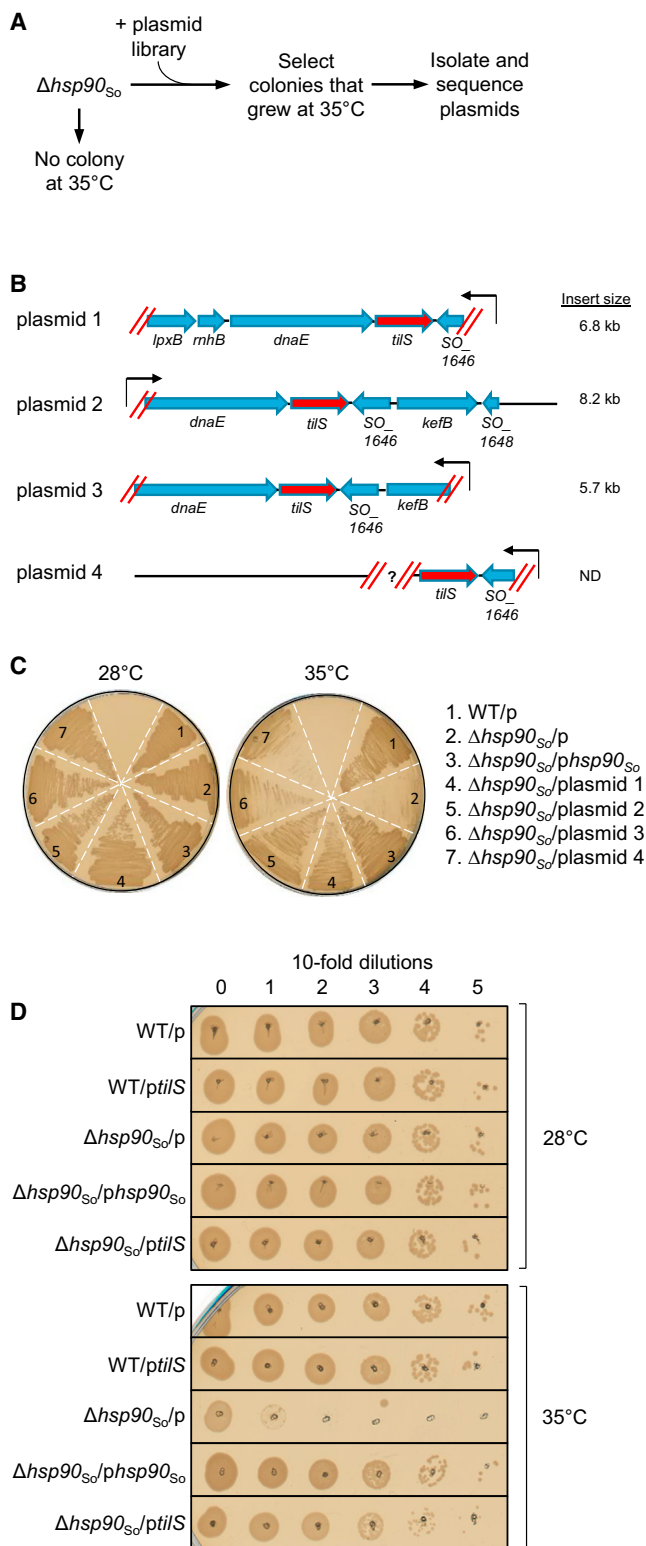
To confirm that the growth defect was due to the absence of Hsp90<sub>So</sub>, we performed a complementation assay. The  $\Delta hsp90_{So}$  strain containing an empty vector grew very poorly at 35°C on plate (Figure 1C). In contrast, in the presence of the *phsp90<sub>So</sub>* plasmid producing Hsp90<sub>So</sub>, 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<sub>So</sub> 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  $\gamma$ -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<sub>So</sub> 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<sub>So</sub> at high temperature, we tested whether an Hsp90<sub>So</sub> mutant that is affected for ATPase activity could complement the  $\Delta hsp90_{So}$  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<sub>So</sub> 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<sub>So</sub>* mutant gene, *phsp90<sub>So</sub>E34A*, was introduced into the  $\Delta hsp90_{So}$  strain. We found that the mutant protein Hsp90<sub>So</sub>E34A did not complement the growth defect of the  $\Delta hsp90_{So}$  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<sub>So</sub> that is known to participate in client binding in Hsp90<sub>Ec</sub> and yeast Hsp82 (Genest et al., 2013; Street et al., 2012). The W476 and



**Figure 2. TiiS Overproduction Rescues the Growth Defect of the  $\Delta hsp90_{So}$  Strain**

(A) Experimental design to identify proteins that rescue the growth defect of the  $\Delta hsp90_{So}$  strain at high temperature using a plasmid library.

L563 residues corresponding to the conserved residues W467 and L553 from Hsp90<sub>Ec</sub> were simultaneously mutated (double mutant W476R-L563A) in Hsp90<sub>So</sub> (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  $\Delta hsp90_{So}$  strain producing Hsp90<sub>So</sub> 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<sub>So</sub>, 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<sub>So</sub> 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<sub>So</sub> at high temperature.

### Overproduction of TiiS Suppresses the Growth Defect of the $\Delta hsp90_{So}$ Strain at High Temperature

We used the growth phenotype of the  $\Delta hsp90_{So}$  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<sub>So</sub> or clients of Hsp90<sub>So</sub>. Indeed, a high amount of an essential client protein could bypass the need of Hsp90<sub>So</sub>, 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  $\Delta hsp90_{So}$  strain. Sixteen clones out of  $2 \times 10^8$  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 *tiiS* gene was identified ten times in four independent plasmids (Figure 2B). To confirm that growth restoration of the  $\Delta hsp90_{So}$  strain at 35°C was due to the presence of these plasmids, we extracted and reintroduced them into the  $\Delta hsp90_{So}$  strain. The presence of these plasmids partially rescued the growth defect of the  $\Delta hsp90_{So}$  strain at 35°C (Figure 2C). Finally, the *tiiS* gene was cloned into a vector under an

(B) Schematic of the genes present in the plasmids identified with the plasmid library. The *tiiS* 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  $\Delta hsp90_{So}$  strain. Strains were streaked on LB plates containing 0.2% arabinose and the plates were incubated at 28°C and 35°C.

(D) The *tiiS* gene was cloned into the pBad33 vector (p). The resulting plasmid, *ptiiS*, was introduced into the WT and the  $\Delta hsp90_{So}$  strains. Strains grown at 28°C were diluted to OD<sub>600</sub> = 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.



arabinose-inducible promoter to yield *ptiIS*. Overproduction of TiIS from the *ptiIS* plasmid greatly improved the growth of the  $\Delta hsp90_{So}$  strain at 35°C (Figure 2D). As a control, we found that the growth of the  $\Delta hsp90_{So}$  strain at 35°C was not improved in the absence of the arabinose inducer (Figure S2B). In addition, the overproduction of TiIS did not modify the growth of the WT and the  $\Delta hsp90_{So}$  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, TiIS, that when overproduced bypasses the requirement of the Hsp90<sub>So</sub> chaperone at high temperature, and we therefore propose that TiIS is a client of Hsp90<sub>So</sub>. However, overproduction of TiIS did not entirely suppress the growth defect of the  $\Delta hsp90_{So}$  strain at 35°C (Figures 2C and 2D). It suggests that either TiIS is not the only essential client affected at this temperature in the absence of Hsp90<sub>So</sub>, or that TiIS always requires Hsp90<sub>So</sub> to be fully functional.

### TiIS Activity Is Reduced In Vivo in the Absence of Hsp90<sub>So</sub> at High Temperature

According to the literature, TiIS is a tRNA<sup>Ile</sup>-lysine synthetase (Soma et al., 2003; Suzuki and Miyauchi, 2010). Essential in *E. coli*, this enzyme specifically modifies the tRNA<sup>Met</sup> that originally allows the translation of the AUG codon into methionine, in the tRNA<sup>Ile</sup> that allows the translation of the AUA codon into isoleucine. For that, TiIS catalyzes the addition of a lysine on the cytidine 34 of the tRNA<sup>Met</sup>, resulting in a lysidine (Ikeuchi et al., 2005). As expected, we obtained *S. oneidensis* *tiIS* deletion strains only when *tiIS* was expressed in *trans* from a plasmid, strongly suggesting that, as shown in *E. coli*, TiIS is also essential in *S. oneidensis*. If TiIS is a client of Hsp90<sub>So</sub>, its activity should be lower in the absence of Hsp90<sub>So</sub> than in its presence at high temperature. To follow the activity of TiIS 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  $\beta$ -galactosidase activity became dependent on TiIS 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  $\Delta hsp90_{So}$  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  $\beta$ -galactosidase activity was measured. At both temperatures, we observed that in liquid media growth of the WT and  $\Delta hsp90_{So}$  strains was similar.

At 28°C,  $\beta$ -galactosidase activity was similar in the WT and in the  $\Delta hsp90_{So}$  strains containing the control plasmid, indicating that the production of the  $\beta$ -galactosidase was not affected by the absence of Hsp90<sub>So</sub> (Figure 3B). The WT and  $\Delta hsp90_{So}$  strains containing the p(AUA)*lacZ* plasmid had also a similar level of  $\beta$ -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 *placZ* control plasmid or the p(AUA)*lacZ* plasmid had the same level of  $\beta$ -galactosidase activity (Figure 3B). In contrast, in the  $\Delta hsp90_{So}$  background, a significant decrease in  $\beta$ -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<sub>So</sub> and at high temperature, the activity of

TiIS is reduced, leading to a reduced translation of the  $\beta$ -galactosidase protein from the p(AUA)*lacZ* plasmid.

To confirm these results, we hypothesized that the overproduction of TiIS should increase the overall TiIS activity and consequently the level of  $\beta$ -galactosidase activity measured at high temperature in the  $\Delta hsp90_{So}$  strain. To do that, the gene coding for TiIS 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 TiIS was overproduced, there was an increase of  $\beta$ -galactosidase activity in the  $\Delta hsp90_{So}$  strain containing the p(AUA)*lacZ-tiIS* plasmid (Figure 3C), and the level of  $\beta$ -galactosidase activity was similar to the one measured in the WT strain. At lower temperature, the overproduction of TiIS did not affect  $\beta$ -galactosidase activity. Therefore, the overproduction of TiIS rescues TiIS activity in the absence of Hsp90<sub>So</sub> at high temperature.

Altogether, these experiments support the idea that in physiological conditions at high temperature, the activity of TiIS depends on Hsp90<sub>So</sub>. Moreover, they strongly suggest that TiIS is a client of Hsp90<sub>So</sub>.

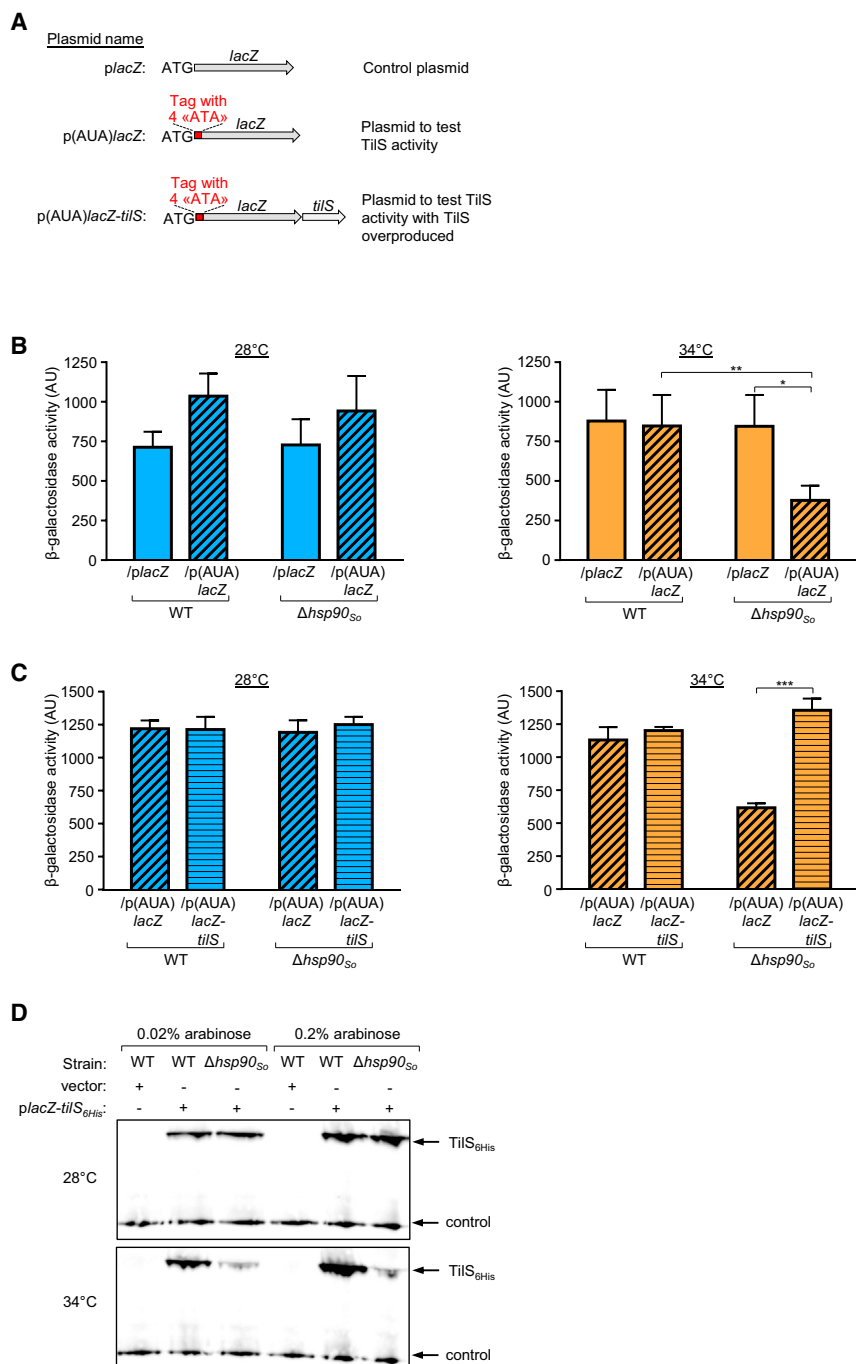
### The Amount of TiIS Is Reduced in the Absence of Hsp90<sub>So</sub> at High Temperature

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 TiIS protein in the presence or absence of Hsp90<sub>So</sub> at high temperature. To do that, a sequence coding for a 6His tag was added at the 3' extremity of *tiIS* in the *placZ-tiIS* plasmid (Figure S3A), and the resulting plasmid was introduced into the WT and  $\Delta hsp90_{So}$  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 TiIS was detected by western blot with a 6His antibody. When the strains were grown at 28°C, similar levels of TiIS were observed in the WT and  $\Delta hsp90_{So}$  strains, and the amount of TiIS 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 TiIS amount in the absence of Hsp90<sub>So</sub> compared to the WT strain (Figure 3D). As a control, we showed that the decrease in TiIS measured in the  $\Delta hsp90_{So}$  strain grown at high temperature was not due to a decrease in gene expression from the *placZ-tiIS*<sub>6His</sub> plasmid. Indeed, we found that  $\beta$ -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 TiIS at high temperature (Figure S3C).

Together, these results show that the amount of the TiIS protein is strongly reduced at high temperature in the absence of Hsp90<sub>So</sub>. Moreover, they suggest that in the absence of Hsp90<sub>So</sub>, TiIS is not correctly folded and is therefore degraded by proteases.

### Hsp90<sub>So</sub> Interacts with TiIS and Protects TiIS from Aggregation In Vitro

Our results indicate that TiIS is a client of Hsp90<sub>So</sub>, 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. TiIS Activity and Amount Are Reduced at High Temperature in the Absence of Hsp90<sub>So</sub>**

(A) Schematic of the plasmids used to follow TiIS 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 *tiIS* gene was cloned in operon downstream of the *lacZ* coding sequence into *p(AUA)lacZ*, leading to *p(AUA)lacZ-tiIS*. In these plasmids, *lacZ* and *tiIS* are under the transcriptional control of the *pBad* promoter.

(B) Cultures of WT and  $\Delta hsp90_{So}$  strains containing *placZ* 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.  $\beta$ -galactosidase activity was measured after 2 hr.

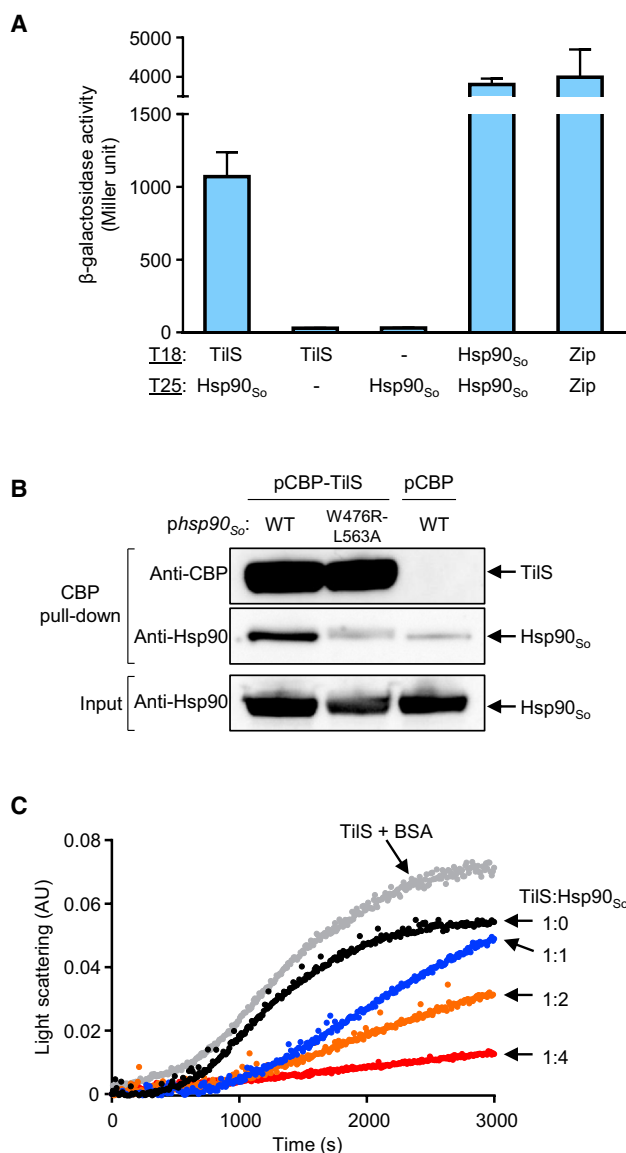
(C) Same as (B) using WT and  $\Delta hsp90_{So}$  strains containing *p(AUA)lacZ* or *p(AUA)lacZ-tiIS*. In (B) and (C), data from at least three replicates are shown as mean  $\pm$  SEM. Where indicated, t test analyses show that the differences measured are significant (\* $p = 0.0112$ ; \*\* $p = 0.0107$ ; \*\*\* $p = 0.0026$ ).

(D) Cultures of WT and  $\Delta hsp90_{So}$  strains containing *pBad33* (vector) or *placZ-tiIS<sub>6His</sub>* 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 TiIS 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  $\beta$ -galactosidase activity, we followed the activity of the adenylate cyclase reporter protein that is split in the T18 and T25 domains fused to TiIS and Hsp90<sub>So</sub>, respectively. When T18-TiIS and T25-Hsp90<sub>So</sub> proteins were produced together, a high level of  $\beta$ -galactosidase activity was measured indicating that TiIS and Hsp90<sub>So</sub> interact (Figure 4A). The interaction between TiIS and Hsp90<sub>So</sub> is specific because background levels of  $\beta$ -galactosidase activity were observed when T18-TiIS and T25, or T18 and T25-Hsp90<sub>So</sub> were produced

together. As expected, we also showed that Hsp90<sub>So</sub> dimerizes (Figure 4A). A pull-down experiment confirmed that Hsp90<sub>So</sub> co-purified with TiIS that contains a calmodulin binding peptide (CBP) tag (Figure 4B). Interestingly, the client binding defective mutant Hsp90<sub>So</sub>W476R-L563A did not copurify with TiIS (Figure 4B), confirming that TiIS is recognized as a client by Hsp90<sub>So</sub>. Finally, we showed that Hsp90<sub>So</sub> protects TiIS from aggregation at high temperature. Indeed, using light scattering, we observed that purified TiIS aggregated when incubated at 42°C (Figure 4C). However, the aggregation of TiIS decreased when it was mixed with increasing concentrations of purified Hsp90<sub>So</sub>. Nearly no aggregation of TiIS occurs with four times more Hsp90<sub>So</sub> than TiIS. This experiment demonstrates that Hsp90<sub>So</sub> protects TiIS against aggregation in vitro. As a control, we observed that BSA or the Hsp90<sub>So</sub>W476R-L563A mutant did not prevent aggregation of TiIS (Figures 4C and S4A). Altogether, our results clearly show that TiIS is a client of Hsp90 in *S. oneidensis*.



**Figure 4. Hsp90<sub>So</sub> Binds to TiIS and Protects It from Aggregation**

(A) Two-hybrid method showing that Hsp90<sub>So</sub> and TiIS interact. *E. coli* Bth101Δ*hsp90* strains transformed with T18-TiIS and T25-Hsp90<sub>So</sub>, T18-TiIS and T25, T18 and T25-Hsp90<sub>So</sub>, T18-Hsp90<sub>So</sub> and T25-Hsp90<sub>So</sub>, 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. TiIS with a CBP tag was produced with or without Hsp90<sub>So</sub> WT or mutant in the *E. coli* MG1655Δ*htpG* strain. CBP-TiIS 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<sub>So</sub> prevents aggregation of TiIS. Purified TiIS (5.4 μM) with or without purified Hsp90<sub>So</sub> (5 μM, 10 μM, 20 μM as indicated) were incubated at 42°C and OD<sub>360</sub> was measured with time. An increase in absorbance indicates that TiIS aggregates. As a control, Hsp90<sub>So</sub> 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, TiIS, 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<sub>So</sub> clients (García-Descalzo et al., 2011). Because TiIS is conserved in bacteria (Suzuki and Miyachi, 2010), we question whether TiIS from other bacteria also needs Hsp90 for folding in stress conditions. Future work will lead to the identification of other Hsp90<sub>So</sub> clients, and the fact that Hsp90<sub>So</sub> 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<sub>So</sub>* deletion strain, plasmids, and the plasmid library are described in the Supplemental Experimental Procedures.

### Protein Purification

To purify Hsp90<sub>So</sub> WT or mutants, pET-Hsp90<sub>So</sub>, pET*hsp90<sub>So</sub>*E34A, or pET*hsp90<sub>So</sub>*W476R-L563A was introduced into the BL21(DE3) strain by transformation. The resulting strains were grown in LB at 37°C. At OD<sub>600</sub> = 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<sub>So</sub> (Figure S4B). The same protocol was used to produce and purify TiIS-6His from the pET-TiIS<sub>So</sub> plasmid, except that cells were grown at 28°C, and 0.1 mM IPTG was added at OD<sub>600</sub> = 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 TiIS and BSA and dimeric Hsp90<sub>So</sub>.

### Reporter Assay to Follow TiIS Activity In Vivo

WT and Δ*hsp90<sub>So</sub>* strains containing *placZ*, *p(AUA)lacZ*, or *p(AUA)lacZ-tiIS* plasmids were grown at 28°C overnight with chloramphenicol. Cells were then diluted to OD<sub>600</sub> = 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 TiIS Amount

The *placZ-tiIS*<sub>6His</sub> plasmid was introduced by conjugation into the WT and Δ*hsp90<sub>So</sub>* 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<sub>600</sub> = 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. TiIS 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-TiIS with *phsp90<sub>So</sub>* WT or mutant and pCBP with *phsp90<sub>So</sub>* WT were grown at 37°C. At OD<sub>600</sub> = 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<sub>2</sub>, 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<sub>So</sub> was visualized with an anti-Hsp90<sub>Ec</sub> antibody (Genest et al., 2013), and TiIS was visualized with an anti-CBP antibody (Millipore 07-482).

### Light Scattering Experiments

TiIS (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<sub>So</sub> 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 TiIS, 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.

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