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Escherichia coli transcription termination factor NusA: heat-induced oligomerization and chaperone activity

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Escherichia coli **NusA, an essential component of the RNA polymerase elongation complex, is involved in transcriptional elongation, termination, anti-termination, cold shock and stress-induced mutagenesis. In this study, we demonstrated that NusA can self-assemble into oligomers under heat shock conditions and that this property is largely determined by the C-terminal domain. In parallel with the self-assembly process, NusA also acquires chaperone activity. Furthermore, NusA overexpression results in the enhanced heat shock resistance of host cells, which may be due to the chaperone activity of NusA. Our results suggest that *E. coli* NusA can act as a protector to prevent protein aggregation under heat stress conditions *in vitro* and in the NusA-overexpressing strain. We propose a new hypothesis that NusA could serve as a molecular chaperone in addition to its functions as a transcription factor. However, it remains to be further investigated whether NusA has the same function under normal physiological conditions.**

Bacterial RNA synthesis that is mediated by DNA-dependent RNA polymerase (RNAP) is assisted and regulated by a host of transcription factors. The N-utilizing substance A protein (NusA) is a major transcription factor in prokaryotes and archaea, and it has been extensively studied for many years. As a highly conserved multifunctional protein, it plays essential roles in transcriptional elongation, pausing, termination and anti-termination. Gill *et al.* reported that NusA and the Sigma factor bind competitively with core RNA polymerase[1]. As the RNAP complex moves into the elongation phase, NusA replaces σ^{70} and binds to the polymerase, interacting with the core enzyme and the nascent RNA. In *Bacillus subtilis*, NusA can stimulate hairpin-dependent pausing, which is crucial for the synchronization of transcription and translation[2]. As a termination factor, NusA mediates Rho-independent termination, depending on the DNA/RNA sequence[3]. NusA can also modulate Rho activity to stimulate or inhibit Rho-dependent termination[4–6]. By interacting with the intrinsically unstructured phage λ N protein, NusA modulates highly efficient anti-termination[7].

NusA protein generally exists in a monomeric form in solution[8]. The crystal structures of NusA from *Mycobacterium tuberculosis* and *Thermotoga maritima* have been determined. The protein was found to be composed of an N-terminal domain that contains an α3/β3 structure, an RNA-binding domain consisting of an S1 region, and two K homolog domains[9,10] that are flexibly linked by a short chain. This characteristic is closely related to the simultaneous interaction of this protein with RNAP and nascent RNA transcripts. NusA proteins from *Escherichia coli* and other γ-proteobacteria contain an additional C-terminal extension with a dual repeated acidic domain, which has been shown to act as a versatile protein-protein interaction region[11]. Interestingly, the acidic domains also show structural similarity to the sterile alpha motif (SAM) domain of the human EphB2 receptor, for which an oligomeric structure has been reported[12]. However, such an oligomeric structure has not previously been observed for NusA proteins.

The structure of the *M. tuberculosis* NusA-RNA complex indicates that NusA can bind to nascent RNA structures and function as an RNA chaperone[13], which is similar to the function of most other cold shock proteins. Furthermore, together with two other transcription factors, Rho and NusG, NusA also suppresses the expression of foreign genes, some of which could be detrimental to the host[14]. Unexpectedly, in addition to its role as a transcription factor, NusA is required for stress-induced mutagenesis through its interactions with DinB[15]. Recently, it was also found to promote a novel mechanism of transcription-coupled repair[16], indicating that NusA is closely related to multi-stress resistance.

Molecular chaperones, such as DnaK and GroEL, can be used as fusions in expression plasmid vectors to improve the solubility of recombinant proteins[17]. Interestingly, NusA has also been used for many years as a favorable solubility partner in heterologous expression[18]. Thus, we investigated whether NusA can also act as a molecular chaperone. Molecular chaperones are a set of protective proteins that can recognize and bind to the hydrophobic surfaces of denatured substrates and form defined complexes to inhibit protein aggregation under stressful conditions such as high temperature, extreme pH, osmotic pressure, or the presence of toxic chemicals. Additionally, these chaperones are also implicated in protein refolding and degradation after stress[19]. In this study, we demonstrated that the *E. coli* transcription termination factor NusA forms high molecular weight (HMW) oligomers under heat shock conditions *in vitro* and in the NusA-overexpressing strain. This structural change is associated with molecular chaperone activity of NusA. We also demonstrated that NusA overexpression results in the enhanced heat resistance of *E. coli*. These data lead to a hypothesis that in addition to its main functions in transcription, NusA could serve as a molecular chaperone to control protein quality under heat shock conditions.

Results

Structural switch of NusA during heat shock. *E. coli* NusA is a member of the family of thermostable proteins, many of which can form oligomeric structures. The high temperature stability of *E. coli* NusA under physiological conditions has previously been demonstrated[20]. Herein, we overexpressed and purified *E. coli* NusA. A hexa-histidine tag was added to the N-terminus of NusA to simplify the purification procedure, and the recombinant NusA was used in all of the experiments performed in our work. We tested the heat stability of NusA *in vitro* at different levels of pH by monitoring its optical density at 360 nm over a range of temperatures from 30 to 80°C. As shown in Fig. S1, in pH 7.5 Tris-HCl buffer, NusA remained stable at 80°C. However, at pH 6.5, the protein precipitated at temperatures as low as 50°C, as evidenced by a dramatic increase in OD_{360}, indicating that the heat stability of NusA is affected by environmental pH. Therefore, we chose a moderate pH of 7.2 for further tests of its properties.

We further characterized the changes in NusA using size exclusion chromatography (SEC) and non-denaturing gel electrophoresis under a range of heat shock conditions. SEC (Fig. 1a) and non-denaturing gel electrophoresis (Fig. 1b) revealed that under heat shock conditions, NusA self-assembles into soluble HMW oligomers, rather than forming aggregates, as do most other proteins. This temperature-dependent transformation commenced at temperatures as low as 45°C, and almost all NusA molecules were detected in a soluble oligomeric state after heat treatment at 55°C for 60 min (Fig. 1a). When the pre-heated samples were cooled to room temperature, the oligomers remained stable (Fig. 1c). These results indicate that similar to other heat-stable proteins[20], NusA can form stable HMW oligomers.

We further examined the structural changes in the native and 55°C-pretreated forms of NusA using dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS revealed an obvious increase of the mean effective diameter of NusA after heat treatment (Fig. 1d). The mean effective diameter of untreated NusA was estimated to be 1.6 ± 0.3 nm, which is consistent with a monomeric state. However, after treatment at 55°C for 60 min, the diameter of NusA increased to 16.2 ± 1.8 nm. Moreover, TEM images revealed that NusA molecules appeared as small particles under normal conditions but that after heat treatment at 55°C for 60 min, HMW oligomers of NusA were present in the form of large spherical particles (Fig. 1e). This result corresponds well with the SEC and DLS data.

For comparison, we examined the NusA proteins from several other bacterial strains. The NusA proteins from the ubiquitous pathogen *Pseudomonas aeruginosa* PAO1[21] and the aromatic compound-degrading strain *Pseudomonas putida* KT2440[22], which are homologous to *E. coli* NusA, form oligomers under conditions of heat shock (Fig. S2). Interestingly, we found that NusA purified from the Gram-positive, non-γ-proteobacteria strains *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842[23] and *Bacillus coagulans* 2–6[24] also formed oligomers (Fig. S2) despite their sequence heterology. These findings suggest that the NusA proteins from many bacteria, not just *E. coli*, have the ability to oligomerize.

NusA acquires chaperone activity under heat shock conditions. The function of NusA to improve solubility of recombinant proteins[18], along with its ability to oligomerize, prompted us to hypothesize that oligomeric NusA could have chaperone activity. The suppression of stress-induced aggregation is the most common characteristic of molecular chaperones. We used 1,4-dithiothreitol (DTT)-induced non-thermal aggregation of insulin to measure the ability of oligomeric NusA to inhibit aggregation. Because we had shown that oligomerization was almost complete after treatment at 55°C, we tested for chaperone activity in the native and 55°C-pretreated samples. As shown in Fig. 2a, the addition of 2 μM native NusA produced a very weak effect on DTT-induced insulin aggregation. However, in the presence of as little as 0.5 μM heat-treated NusA, there was a clear reduction in insulin aggregation, as indicated by decreased red light scattering. This inhibitory effect increased with increasing concentrations of heat-pretreated NusA. At a concentration of 2 μM NusA, insulin aggregation was largely suppressed, indicating that NusA self-assembles and acquires chaperone activity after heat shock.

To detect the influence of NusA on heat-induced aggregation, we used lactate dehydrogenase (LDH). LDH was incubated at 48°C in the absence or presence of NusA. As shown in Fig. 2b, LDH slowly aggregated at 48°C without the protection of NusA. However, when this experiment was performed in the presence of NusA, aggregation was visibly reduced. These results suggest that NusA also protects unfolded proteins from irreversible aggregation during thermal stress.

Many chaperones, e.g., small heat shock proteins (sHSPs), can bind preferentially to partially denatured proteins and form large complexes, which is a characteristic that is indispensable for their holdase functions[25]. In this study, we performed an *in vitro* binding assay using SEC to determine whether NusA can act as a classical chaperone. LDH was used as the substrate. In the absence of NusA, almost all heat-treated LDH formed insoluble aggregates, and no obvious peak was detected. However, after the co-incubation of LDH with NusA at 55°C for 60 min, an HMW peak was detected that was much larger than the elution peak of NusA alone (Fig. 2c). Furthermore, SDS-PAGE demonstrated that this peak contained NusA and LDH (Fig. S3), which is consistent with the function of NusA as a chaperone.

Collectively, these data indicate that similar to many other chaperones, NusA interacts with partially denatured proteins under stress conditions *in vitro*. NusA can prevent stress-induced aggregation by binding to non-native proteins and forming soluble HMW complexes.

Hydrophobic interactions play important roles in many chaperones. To obtain a measurement of the hydrophobic surfaces of NusA, we used the fluorescent probe 8-anilino-1-naphthalene sulfonic acid (ANS). As self-assembly progressed, ANS binding to NusA increased, and it was as indicated by increased fluorescence intensity, accompanied by a blue shift (Fig. 2d). This finding indicates that in the oligomeric state, hydrophobic patches of NusA were largely exposed on the surface. Indeed, a similar phenomenon has been reported for HdeA and some sHSPs[26,27] that acquire chaperone activity in parallel with structural changes and increased hydrophobicity.

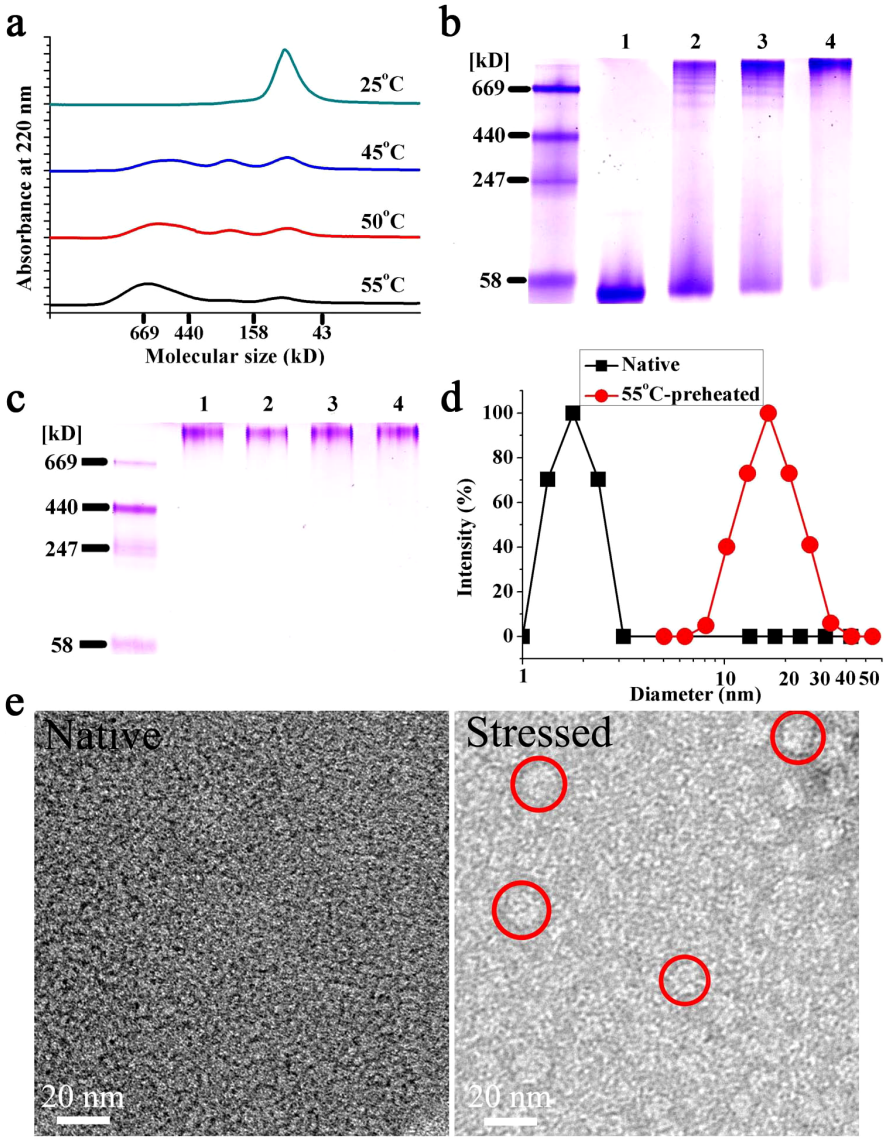


Figure 1 | Heat-induced oligomerization of NusA. (a) Changes in the oligomeric state of NusA in response to various heat treatments for 60 min, as determined by SEC. 25°C (green), 45°C (blue), 50°C (red) and 55°C (black). (b) Non-denaturing pore gradient gel electrophoresis. NusA was diluted to 5 μM and incubated at room temperature (1) or 55°C for 10 min (2), 30 min (3) or 60 min (4) and then loaded onto a 5–12% non-denaturing gel. (c) NusA oligomers remain stable under room temperature. NusA proteins were incubated at 55°C for 60 min, and the oligomers were isolated by SEC. The samples were then concentrated to 5 μM and incubated at room temperature for 0 h (1), 6 h (2), 12 h (3) or 24 h (4), respectively, and loaded onto 5–12% non-denaturing gels. (d) DLS showed an obvious increase of mean effective diameter of NusA after incubation at 55°C for 60 min, which is consistent with oligomerization. (e) Electron micrographs showing the structures of NusA in the native state or after pretreatment at 55°C for 60 min. After 55°C heat treatment, particles of approximately 15 nm were observed, which is consistent with DLS data (circles indicate individual particles).

In addition, we tested the chaperone activity of oligomeric *P. aeruginosa*, *P. putida*, *L. bulgaricus* and *B. coagulans* NusA. As expected, NusA proteins from these bacteria were also effective in suppressing DTT-induced insulin aggregation (Fig. S4), which was in agreement with the results of our protein oligomerization experiment.

Effect of oligomeric NusA on protein refolding. When binding to partially denatured proteins, most chaperones release their substrates either unaided or in the presence of ATP or other cofactors to accelerate refolding. Therefore, we examined the chaperone activity of NusA by analyzing its effect on the refolding of LDH. As shown in Fig. S5, when oligomeric NusA was added, the spontaneous refolding of LDH from the denatured state was noticeably inhibited. Furthermore, this suppressive effect increased as the concentration of NusA increased. These data suggest that in the absence of other chaperones, NusA can only bind to substrates in a stable and inactive state but cannot release the substrates or promote refolding. Oligomeric NusA could be similar to sHSPs, most of which bind to denatured proteins to maintain them in a folding-competent model, acting as effective ATP-independent holdases with high capacity but not facilitating refolding themselves. However, in the presence of some ATP-dependent chaperones, the sHSPs-bound substrates can be released and returned to their native state[28]. A similar mechanism could exist that enables NusA to act as part of the chaperone machinery. As shown in Fig. 2e, our results indicate that in the presence of DnaK, DnaJ, GrpE and ATP, which resemble the functional entity of the bacterial Hsp70 system[29], the LDH molecules were partially released by NusA. Therefore, we propose that NusA could mediate substrate refolding by a mechanism which is similar to that of sHSPs.

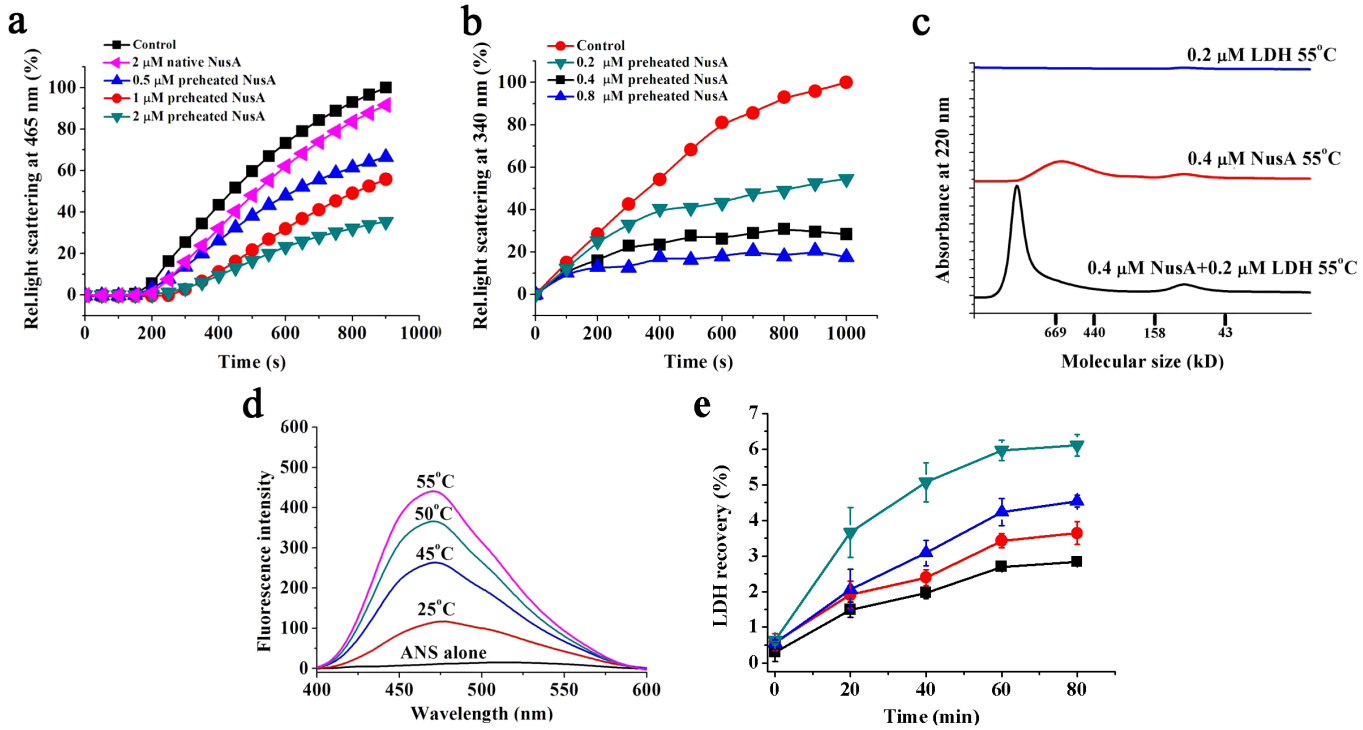


Figure 2 | Chaperone activity of *E. coli* NusA. (a) NusA inhibits DTT-induced insulin aggregation. The DTT-induced aggregation of 40 μM insulin was measured by light scattering at 25°C. The insulin mixture was observed alone (control) (■), or mixed with 2 μM native NusA (◀), or 0.5 μM (▲), 1 μM (●) and 2 μM (▼) preheated NusA. (b) NusA inhibits thermal-induced LDH aggregation. LDH was diluted to 0.4 μM and incubated at 48°C. The aggregation was measured by light scattering after adding 0 μM (control) (●), 0.2 μM (▼), 0.4 μM (■) or 0.8 μM (▲) NusA. (c) *In vitro* binding to denatured LDH, as determined by SEC. NusA (0.4 μM) and LDH (0.2 μM) were incubated at 55°C for 60 min either individually (red and blue, respectively) or together (black), and the soluble complexes that were formed were analyzed by SEC. (d) The heat shock-induced increase in hydrophobicity was measured based on 8-anilino-1-naphthalene sulfonic acid (ANS) binding. NusA was incubated at 25°C (line *b*), 45°C (line *c*), 50°C (line *d*), or 55°C (line *e*). The incubation of ANS alone is shown by line *a*. (e) Dissociation of NusA-LDH complexes. LDH activity is recorded for NusA-LDH only (control) (■), NusA-LDH with DnaK, DnaJ and GrpE only (▲), NusA-LDH with DnaK, DnaJ, GrpE and ATP (▼), or LDH alone with DnaK, DnaJ, GrpE and ATP (●). Values are the mean ± SD of 3 separate determinations.

Enhanced stress resistance of a NusA-overexpressing strain. An NusA-overexpressing strain was used to determine whether NusA exhibits chaperone activity *in vivo*. We performed Western blotting analysis and measured the survival rate of this strain after heat shock. The temperature-dependent oligomeric status of NusA was confirmed using an anti-His tag antibody that could specifically bind to recombinant NusA on a polyvinylidene fluoride (PVDF) membrane. After heat treatment for 40 min at 48°C, most NusA formed HMW oligomers (Fig. 3a), suggesting that the overexpressed NusA undergoes a structural change in the cell, which is similar to what is observed *in vitro*.

It has been shown that *nus*A-deleted *E. coli* strains acquire a temperature-sensitive phenotype[30]; however, this finding could be attributed to deficiencies in the transcription of certain vital proteins. As reported earlier, the overexpression of certain chaperones endows the host cell with enhanced stress resistance due to the holdase function of these chaperones, which protects proteins from aggregation[31–35].

Here, we also tested the effect of overexpressed NusA on the high temperature resistance of host cell. The *E. coli* BL21 (DE3) strain containing an empty vector was used as a control. After induction with IPTG, both strains were challenged by treatment at 48°C for various time periods. As shown in Fig. 3b, after 60 min, the NusA-overexpressing strain had a survival rate of 76.0%, compared to the control strain's survival rate of 23.4%. We also overexpressed another cytoplasmic protein, NusG, as a control to further validate our results. A very moderate change of cellular viability was observed with the NusG-overexpressing strain. These results suggest that the presence of excess NusA molecules (approximately 16.8% of the total protein as shown in Fig. S6) could prevent the heat-induced loss of cell viability. The cellular aggregates after heat shock were also analyzed. As shown in Fig. 3c, the control strain showed more extensive aggregation than the NusA-overexpressing strain, which suggests that such enhanced resistance is related to the chaperone activity of overexpressed NusA.

Roles of the C-terminal domain in oligomerization and chaperone activity. To elucidate the mechanism of NusA oligomerization, we used circular dichroism (CD) to detect the change in secondary structure at various temperatures. As shown in Figs. 4a and 4b, when the temperature increased from 25°C to as high as 60°C, the band shifted slightly. No secondary structural change was observed during this process, as analyzed by CDNN. When the temperature was elevated to 70°C and 80°C, the ratio of β sheets increased, indicating that denaturation had begun. The mean residue ellipticity (MRE) data also show that when the temperature increased from 25°C to 45°C, the value (at 220 nm) increased moderately. Interestingly, a plateau was observed from 45°C to 60°C, indicating that the secondary structure underwent little change with the temperature increase. However, when the temperature rose to 70°C, a dramatic increase in MRE was observed (Fig. 4c). This result can be explained as the severe denaturation and loss of secondary structure of the NusA molecules. Accordingly, NusA preheated to 70°C or 80°C also showed a loss of chaperone activity, while 60°C-pretreated NusA retained its chaperone activity (Fig. S7). Therefore, we propose that in the oligomeric state (45°C–55°C), NusA protein

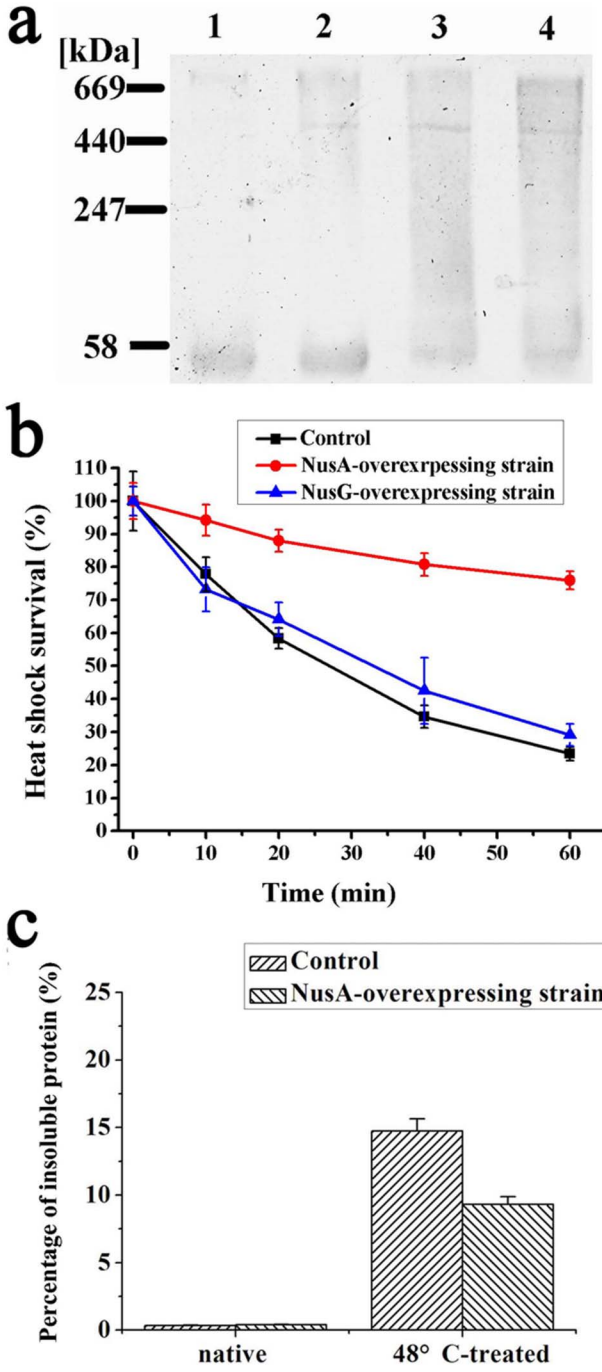
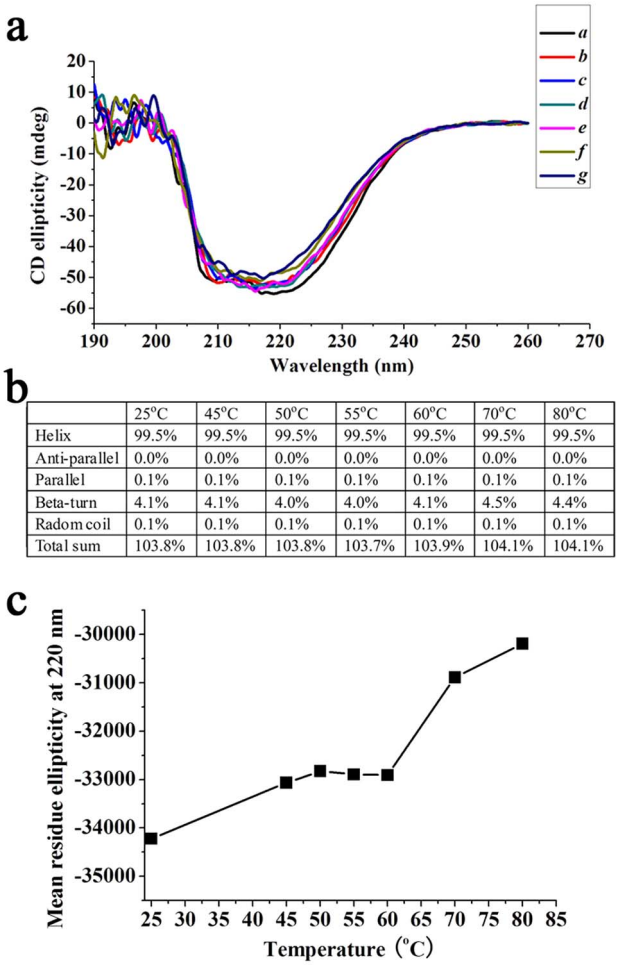


Figure 3 | Enhanced heat-shock resistance of the NusA-overexpressing strain. (a) The heat shock-dependent structural switch of *E. coli* NusA *in vivo*. A Western blot analysis showed that under normal conditions, most NusA molecules exist as monomers in the cell. After heat shock at 48°C for 40 min, most NusA molecules were transformed into HMW oligomers. The following treatments are shown: Untreated sample (lane 1); 48°C, 10 min (lane 2); 48°C, 20 min (lane 3); 48°C, 40 min (lane 4). (b) NusA overexpression resulted in enhanced heat shock survival. The control strain (black), NusA-overexpressing strain (red) and the NusG-overexpressing strain (blue) were induced by IPTG and challenged by 48°C incubation. The survival rates were estimated by counting the colonies on the agar plates. Values are the mean ± SD of 3 separate determinations. (c) Quantification of the aggregates relative to total protein. The aggregated cellular proteins in the heat-treated cells were isolated and quantified as described in the Methods. Values are the mean ± SD of 3 separate determinations.



	25°C	45°C	50°C	55°C	60°C	70°C	80°C
Helix	99.5%	99.5%	99.5%	99.5%	99.5%	99.5%	99.5%
Anti-parallel	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Parallel	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
Beta-turn	4.1%	4.1%	4.0%	4.0%	4.1%	4.5%	4.4%
Radom coil	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%
Total sum	103.8%	103.8%	103.8%	103.7%	103.9%	104.1%	104.1%

Figure 4 | Circular dichroism (CD) shows the secondary structure change of NusA during heat shock. (a) CD shows the secondary structure change of NusA during heat shock. The bands of the 25°C–60°C pretreated samples shifted slightly, while the 70°C and 80°C bands shifted dramatically. (*a*) 25°C, (*b*) 45°C, (*c*) 50°C, (*d*) 55°C, (*e*) 60°C, (*f*) 70°C, (*g*) and 80°C. (b) CDNN analysis of the CD data. As indicated in the figure, almost no change was observed in the secondary structures of NusA from 25°C to 60°C. When the temperature was elevated to 70°C, the ratio of β sheets increased moderately, indicating that denaturation had begun. (c) MRE analysis of the CD data. The change in the MRE value at 220 nm indicates that the secondary structure of NusA underwent very moderate change as the temperature increased from 25°C to 60°C. When the temperature increased to 70°C and 80°C, the secondary structure changed dramatically.

largely preserves its secondary structure and is not in an unfolded state as aggregates.

As shown in Fig. 5a, the *E. coli* NusA consists of five domains, including two acidic repeat domains (residues 353–416 composing repeat 1 and residues 431–490 composing repeat 2) that constitute a C-terminal domain (CTD). Because both acidic repeats contain helix-hairpin-helix (HhH) motifs and show structural similarity to the SAM domain[36], whose oligomeric structure has been reported[12], we speculated that this domain could be essential for oligomerization. To test this hypothesis, we deleted either repeat 2 or both repeats. The resulting two truncated proteins NusA(1–417) and NusA(1–343) were purified and tested by SEC. As shown in Figs. 5b and 5c, after heat treatment at 55°C, NusA(1–417) remained stable and was still able to form large oligomers, indicating that this domain might not be essential for NusA oligomerization. However, when both repeats were deleted, NusA(1–343) displayed sensitivity

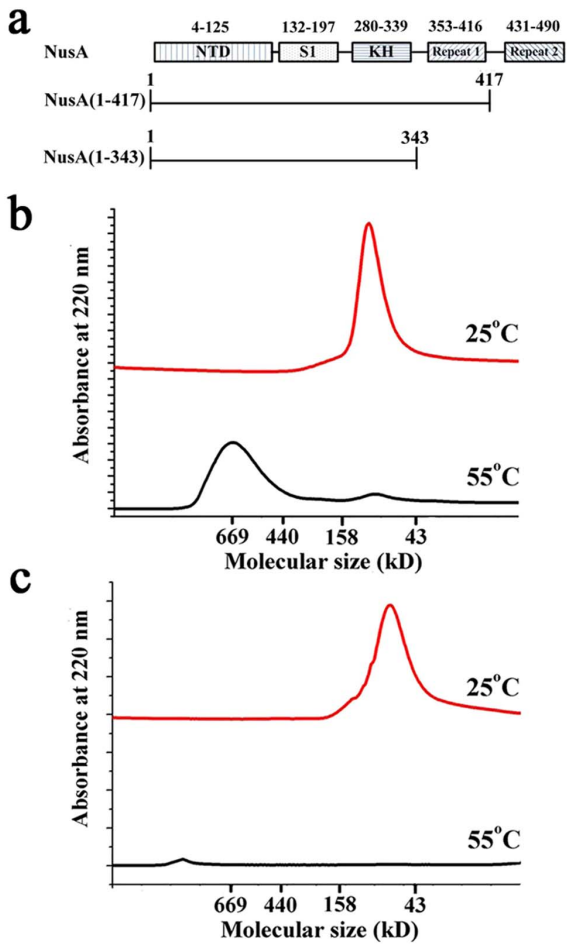


Figure 5 | The C-terminal domain (CTD) of NusA mediates oligomerization. (a) The scheme for the deletion mutants of NusA, with either repeat 2 or both repeats deleted, resulting in the two mutants, NusA(1–417) and NusA(1–343). (b) The deletion of repeat 2 does not affect the oligomerization of NusA(1–417). NusA(1–417) can still form oligomers after heat treatment at 55°C for 60 min, as determined by SEC. (c) With both repeats deleted, NusA(1–343) can no longer oligomerize after heat shock, as determined by SEC.

to heat shock and failed to oligomerize, instead forming insoluble aggregates after heat treatment (Fig. S8). This result suggests that the CTD of NusA plays an indispensable role in oligomerization. Given that the two repeats display clear differences in charge distribution and the recognition of binding partners[37,38], we hypothesize that the acidic repeat 1 could be more important in this process despite the sequence homology and structural similarity between the repeat domains. Furthermore, we also tested the chaperone activity of pre-heated NusA(1–417) and NusA(1–343). While the NusA(1–417) could still inhibit the aggregation of substrates, NusA(1–343) lost this function (Fig. S9), suggesting that the chaperone activity is tightly associated with oligomerization.

The function of the C-terminus of *L. bulgaricus* NusA was also tested. Although it contains only a short extension that is heterologous to *E. coli* NusA (Fig. S10), when this domain was deleted, the NusA(1–343) also became labile to aggregation under heat shock (Fig. S11). Comparatively, the *Rhodococcus erythropolis*[39] NusA that inherently lacks a C-terminal extension (Fig. S10) showed temperature sensitivity and deficiency in oligomerization (Fig. S11). These results suggest that NusA from various bacteria could share a similar mechanism of oligomerization and that the C-terminus could play a role in their oligomerization and stability.

In addition, we tested whether the absence of the C-terminus would affect the chaperone activity of overexpressed NusA *in vivo*. NusA(1–343) could retain its function as a transcription factor (as indicated by the *R. erythropolis* NusA that does not have a C-terminal domain) but not a molecular chaperone (as demonstrated in Fig. S9). As shown in Fig. S12, the presence of excess NusA(1–343) molecules (approximately 30.7% of the total protein as shown in Fig. S6) caused a very moderate change of cellular viability. This result implies that the C-terminus is also important for the *in vivo* chaperone activity of overexpressed NusA. This result further indicates that the enhanced heat resistance of the NusA-overexpressing strain results from the chaperone activity but not the transcriptional function of NusA.

Discussion

NusA is a transcription factor that is involved in elongation, termination and anti-termination. It has also been reported to participate in stress-induced mutagenesis[15]. Recently, the transcription elongation factor GreA was reported to have chaperone activity[40]. In this work, we investigated whether NusA can also act as a molecular chaperone because it can be used as a solubility partner in heterologous expression, which is similar to some well-studied chaperones[17,18]. Protein oligomerization has been shown to enhance the thermostability of thermophilic archaeal proteins[41]. In mesophilic *E. coli*, a large proportion of heat stable proteins can also form high oligomers[20], implying that oligomerization could also contribute to protein stability in mesophiles. As one of these heat stable proteins, the *E. coli* NusA, which exists in monomeric form in its native state[8], was found in this study to form HMW oligomers *in vitro* and in the cells of the NusA-overexpressing strain. However, it remains to be determined whether the oligomerization will occur with the endogenous NusA at physiological concentrations that, although currently unknown, are undoubtedly lower than those in conditions of overexpression. We also showed that the C-terminal acidic repeat, which structurally resembles the SAM domain, plays a role in oligomerization. The NusA(1–343), which lacks the two repeats at its C-terminal domain, was found to display deficiency in oligomerization and sensitivity to heat shock. Interestingly, although NusA proteins from other bacteria have short and heterologous C-terminal domains, they can also form oligomers under heat shock. Secondary structure analysis was performed for the NusA proteins from different bacteria. To assure the accuracy of prediction, the predicted secondary structure of *E. coli* NusA was compared with the resolved structure in the PDB database. Our predicted secondary structure of *E. coli* NusA contains a total of 9 α-helices but no β-strand in the C-terminal domain, which is mainly consistent with the PDB structure 1WCL that has 10 α-helices (Fig. S10). The prediction result indicates that as observed for *E. coli* NusA, the C-terminal domains of NusA proteins from certain other bacteria that can form oligomers are also rich in α-helices (Fig. S10), suggesting that there could be connections between the oligomerization and this special characteristic of secondary structure.

Oligomerization has been reported for many chaperones such as GroES/GroEL (7-mer/14-mer)[42] and for almost all sHSPs (large oligomers)[19]. Thus, the ability to form oligomers of NusA provides further evidence that NusA could act as a molecular chaperone. The substrate-binding ability and chaperone functions of these molecules are tightly regulated by their state of self-association. In this study, we found that in parallel with its temperature-dependent structural changes, the *E. coli* NusA suppresses stress-induced aggregation and binds to denatured proteins, suggesting that it acquires chaperone activity. In addition, the oligomeric NusA from various bacteria also possesses such chaperone activity (Fig. S4). Thus, we speculate that while NusA acts as a transcription factor that mediates RNA synthesis under normal conditions, it could play another role of a molecular chaperone after heat shock treatment. Similar stress-induced functional switches have been previously

reported for yeast peroxiredoxins[43], Arabidopsis thioredoxin-like protein AtTDX[44], serine/threonine protein phosphatase 5[45] and alkylhydroperoxide reductase[46], although little sequence homology was found among these proteins. In the NusA-overexpressing strain, the chaperone activity of the protein caused enhanced resistance to heat shock of the host cells by reducing protein aggregation, which is the same as what has been reported for many other molecular chaperones[31–35]. Nevertheless, further demonstration is needed to clarify whether this function exists for endogenous NusA at its physiological level of expression.

Interestingly, these oligomeric chaperones could share a similar mechanism. When oligomerization occurs, some hydrophobic residues are exposed at the surface. Such hydrophobic patches act as a binding surface for unfolded substrates, thereby conferring chaperone activity to these proteins. This property allows the proteins to serve as latent chaperone reservoirs in the cell. If the cells are challenged by severe heat shock, such proteins are rapidly activated to protect unfolded proteins from aggregation in the absence of newly translated chaperones. The increased surface hydrophobicity of NusA was also observed, which positively correlated with the progression of self-assembly. However, as the oligomerization of NusA apparently does not require a readily observable change of secondary structure, the exact structural change of NusA leading to the exposure of hydrophobic residues requires further investigation (e.g., the protein structure data in the two states). In contrast to traditional chaperones, oligomeric NusA captures denatured proteins to form stable and soluble complexes in a folding-competent model, but it does not facilitate refolding in the absence of other chaperones. This activity has been attributed to the absorption of unfolded proteins on exposed hydrophobic patches of the oligomers' surface[47]. Furthermore, similar to the behavior of sHSPs, the NusA-bound substrates can be released in the presence of the Hsp70 system (DnaK, DnaJ, GrpE and ATP). Thus, we speculate that these similarities could be intrinsically connected and that NusA could only play a buffer role *in vivo* against heat-induced aggregation, which is similar to the function of sHSPs[19].

RNAP-mediated transcription is a fundamental process that is conserved in all kingdoms of life. The activity of RNAP could be affected by various environmental factors, including high temperatures, extreme pH and nutritional deficiency. In *E. coli*, RpoA and RpoB, the major components of RNAP, are prone to aggregation under heat stress *in vivo*. They were reported to interact with GroEL and DnaK, mainly for the protection from aggregation or refolding into a nonnative state[48,49]. However, based on the strong interaction between NusA and RNAP, we propose that the chaperone activity of NusA could provide more direct and concentrated protection for RNAP under stress, i.e., NusA could simultaneously act as a latent protector of RNAP during transcription elongation and termination.

Furthermore, it has been reported that the mutation of *nusA* had a similar effect as the inactivation of the ribosomal protein S10, affecting both transcription and translation[50,51]. Therefore, it is also possible that the chaperone activity of NusA is related to ribosomes and translation, which is similar to some translation factors with chaperone activity (e.g., EF-G, EF-Tu, IF2)[52,53].

In conclusion, we have demonstrated that NusA, a well-investigated transcription factor, forms HMW oligomers and acquires chaperone activity under heat shock *in vitro* and in the NusA-overexpressing strain. These properties could allow NusA to serve as a latent protector of RNAP and cellular proteins. Although without investigation of endogenous NusA, our results raise the possibility that this protein could be related to protein quality control in addition to acting as a transcription factor, which implies more connections between transcription machinery and chaperone systems. Nevertheless, further research on NusA at a restricted expression level or directly on endogenous NusA, as well as the protein structural data at different states, will provide more valuable information to support this hypothesis.

Methods

Protein expression and purification. The *nusA, nusA(1–343), nusA(1–417), nusG, dnaK, dnaJ* and *grpE* genes were amplified from *E. coli* genomic DNA (strain DH10B) by PCR and ligated to the pET-28a expression vector. The recombinant plasmids were transformed into the *E. coli* BL21 (DE3) strain. The *nusA* genes from *P. aeruginosa* PAO1[21], *P. putida* KT2440[22], *L. bulgaricus* ATCC 11842[23], *B. coagulans* 2–6[24] and *R. erythropolis* XP[39] as well as *nusA(1–343)* from strain ATCC 11842 were also cloned and ligated to the pET-28a vector for expression.

The overexpressing strains were cultured to an OD_{600} of 0.4 and induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37°C for 5 h. The cells were harvested and resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) containing 10% glycerol, 1 mM dithiothreitol (DTT) and 1 mM phenylmethanesulfonyl fluoride (PMSF), and subsequently lysed by sonication. After centrifugation at 15,000 × *g* for 10 min, the supernatant was loaded on a 5 mL HisTrap column (GE Healthcare Life Sciences, USA) and eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The elute was loaded onto a Superdex 200 column to remove excess salts and other proteins. DnaK, DnaJ and GrpE were purified using the same procedure.

Size exclusion chromatography (SEC). The NusA protein was diluted to 0.5 μM in 50 mM Tris-HCl buffer (pH 7.2) and incubated at 25°C, 45°C, 50°C or 55°C for 60 min. After cooling, the samples were subjected to SEC using a Superdex 200 HR column. The samples were detected by absorbance at 220 nm. The markers used were thyroglobin (660 kD), ferritin (440 kD), aldolase (158 kD), and ovalbumin (43 kD).

The purified NusA(1–343) and NusA(1–417) proteins were diluted to 0.5 μM in 50 mM Tris-HCl buffer (pH 7.2), incubated at 25°C or 55°C for 60 min and then subjected to SEC.

***In vitro* stability in different pH.** The NusA protein was diluted in 50 mM Tris-HCl buffer of different pH values (6.5, 6.8, 7.0, 7.2, 7.5) to a final concentration of 2 μM. The samples were incubated at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C for 60 min. The optical density was detected at 360 nm using an Ultrospec 2100 spectrometer.

Electron microscopy. Native or heat-pretreated (55°C, 60 min) NusA samples were applied onto glow-discharged carbon-coated copper grids that were rinsed with deionized water and stained with phosphotungstic acid. Images were recorded by TEM.

Non-denaturing gradient gel electrophoresis. The *E. coli* NusA protein was diluted to 2 μM in 50 mM Tris-HCl buffer (pH 7.2) and incubated at 55°C for 10, 30 or 60 min. Loading buffer (bromophenol blue in 10% glycerol, 10 mM DTT) was added to the samples, which then were loaded onto a 5–12% non-denaturing gel. The running buffer contained 25 mM Tris-base and 192 mM glycine. The markers used were thyroglobin (660 kD), ferritin (440 kD), catalase (247 kD) and cellulase (58 kD).

To determine the reversibility of oligomeric NusA, NusA oligomers were separated by SEC. Following concentration to 2 μM and incubation at room temperature for 0, 6, 12 or 24 h, the samples were loaded onto 5%–12% non-denaturing gels.

The NusA proteins from *P. aeruginosa*, *P. putida*, *L. bulgaricus, B. coagulans* and *R. erythropolis* strains, as well as NusA(1-343) from the strain of *L. bulgaricus*, were diluted to 4 μM and tested for their ability to oligomerize as mentioned above, with the exception that *B. coagulans* NusA was treated at 45°C.

DLS analysis. The average particle sizes were determined using a Research Goniometer and Laser Light Scattering System (Brookhaven, BI-200SM). The native or 55°C pretreated NusA samples were diluted to 2 μM in Tris-HCl buffer and loaded onto the analyzer. Measurements were taken with a material refraction index of 1.33 and a viscosity of 0.89 cP.

Chaperone activity of NusA. Chaperone activity was determined by monitoring the aggregation of insulin in the presence or absence of NusA. DTT-induced aggregation was examined in 50 mM Tris-HCl buffer (pH 7.2) at 25°C. A total of 50 mM DTT was added to 40 μM insulin containing native NusA or different concentrations of 55°C-pretreated NusA (0.5 μM, 1 μM, or 2 μM). Light scattering was monitored using a JASCO FP-6500 fluorescence spectrometer. The emission and excitation wavelengths were set at 465 nm.

LDH was used to detect the effect of NusA on heat-induced aggregation. LDH was diluted to 0.4 μM in 50 mM Tris-HCl buffer (pH 7.2), and different concentrations of pre-heated NusA were added (0.2 μM, 0.4 μM, and 0.8 μM). Following incubation at 48°C, the aggregation was monitored using a Hitachi 4500 fluorescence spectrometer. The emission and excitation wavelengths were both set at 360 nm.

We also tested the chaperone activity of 55°C treated NusA(1–417) and NusA(1–343) (0.5 μM, 1 μM). The experiments were performed using insulin as mentioned above. The NusA proteins preheated at 60°C, 70°C or 80°C were tested at 2 μM. In addition, the NusA proteins from *P. aeruginosa* PAO1, *P. putida* KT2440, *L. bulgaricus* ATCC 11842 and *B. coagulans* 2–6 were tested for their chaperone activity as described above.

***In vitro* binding assay.** LDH (0.2 μM) and NusA (0.4 μM) in 50 mM Tris-HCl buffer (pH 7.2) were incubated at 55°C either together or individually for 60 min. The samples were analyzed by SEC on a Superdex 200 HR column with a mobile phase of 80 mM sodium phosphate containing 300 mM NaCl (pH 7.4). The elution peak was collected and detected by SDS-PAGE.

Effect on protein refolding. LDH (15 μM) was denatured in 6 M GnHCl containing 10 mM DTT at 25°C for 30 min. It was then diluted 100-fold (0.15 μM) in refolding buffer (50 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, and 10 mM KCl) in the presence of 55°C-pretreated NusA (0.1 μM, 0.2 μM, or 0.5 μM). After incubation at 25°C for 30 min, LDH activity was measured as previously described[54].

Dissociation of NusA-LDH complexes in the presence of DnaK, DnaJ, GrpE and ATP. Complexes were formed by incubating LDH (0.2 μM) in the presence of 0.4 μM NusA for 60 min at 55°C, followed by incubation at 25°C for 30 min. Next, $MgCl_2$ (100 μM), DnaK, DnaJ, GrpE (2 μM each) and ATP (100 μM) were added. LDH activity was measured as described above. The same experiments were also performed using DnaK, DnaJ and GrpE or ATP only. Heat-denatured LDH alone (0.2 μM) with the addition of DnaK, DnaJ, GrpE and ATP was also used as a control.

ANS binding assay. NusA protein (4 μM) was incubated at 25°C, 45°C, 50°C, or 55°C for 60 min and then mixed with 40 μM ANS. Following incubation at 25°C for 20 min, the samples were scanned using a JASCO FP-6500 fluorescence spectrometer. The excitation wavelength was set at 370 nm, and the emission wavelength ranged between 400 nm and 600 nm.

Western blot analysis of NusA. The NusA-overexpressing strain was cultured at 37°C and induced with 1 mM IPTG when the OD_{600} reached 0.4. After 2 h, cells were harvested and suspended in Tris-HCl buffer. Following heat shock at 48°C for 0, 10, 20, or 40 min, the soluble proteins that were extracted by sonication were adjusted to a concentration of 0.8 μg/μL and subjected to 5–12% non-denaturing gel electrophoresis. The proteins were transferred onto a PVDF membrane at a voltage of 24 V for 100 min. Subsequently, the membrane was saturated with 5% non-fat milk (wt/vol) in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) at room temperature for 1 h, followed by overnight incubation at 4°C with monoclonal antibodies against the His-tag (1 : 500 dilution). After two washes with TBS/0.1% Tween 20 and one wash with TBS, the membrane was incubated with peroxidase-conjugated anti-rabbit IgG (1 : 5000 dilution) at room temperature for 1 h. After washing with TBS/0.1% Tween 20 and TBS, the membrane blots were developed using a DAB substrate kit.

Heat shock survival experiments. The NusA-overexpressing strain and the control strain containing the empty pET28a plasmid were cultured in LB medium to an OD_{600} of 0.4 and induced with 1 mM IPTG. After induction for 1 h, bacterial cultures (10 μL) were diluted 1000-fold in pre-warmed LB medium (pH 7.2) to 500 μL and then incubated at 48°C in a water bath for 0, 10, 20, 40, or 60 min. A 10 μL aliquot was plated on triplicate LB agar plates and incubated at 37°C for 1 d. Cell viability was estimated by counting the number of surviving cells. The heat shock survival of the NusA(1–343)-overexpressing strain was estimated using the same procedure.

To examine the *in vivo* mechanism of NusA function, the induced cells were harvested by centrifugation and resuspended in LB medium. After incubation at 48°C for 0 or 40 min, the bacterial cultures were cooled to 0°C in an ice water bath. Cells were collected by 5,000 × *g* centrifugation at 4°C and then resuspended in buffer A (10 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA, 20% sucrose, 1 mg/mL lysozyme) according to the optical density (40 μL buffer A for 8 mL cultures had an OD_{600} = 1.0). After incubation on ice for 30 min, 360 μL buffer B (10 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA) was added and mixed. Cells were lysed by sonication. After centrifugation at 2,000 *g* for 15 min at 4°C, the supernatants were centrifuged at 15,000 × *g* for 20 min at 4°C. The pellet fractions were resuspended in buffer B and centrifuged (15,000 × *g*, 20 min, 4°C). The washed pellet fractions were again resuspended in 320 μL buffer B. Afterwards, 80 μL 10% NP40 was added, and the aggregated proteins were isolated by centrifugation (15,000 × *g*, 30 min, 4°C). The NP40-insoluble pellets were washed with 400 μL buffer B, resuspended in 200 μL buffer B and qualified.

CD analysis. The NusA protein was diluted to 3 μM in 50 mM Tris-HCl buffer and incubated at various temperatures (25°C, 45°C, 50°C, 55°C, 60°C, 70°C, or 80°C) for 60 min. After cooling, the samples were loaded onto a Jasco J-810 spectrometer. Data were collected between 190 nm and 260 nm. We then used the CDNN program to analyze the ratio of secondary structures. The data at 220 nm were also collected and are represented in MRE.

Secondary structure analysis. The NusA sequences from the strains of *E. coli*, *P. aeruginosa*, *P. putida*, *L. bulgaricus*, *B. coagulans* and *R. erythropolis* were analyzed using the PROFsec program on the PredictProtein server (http://www.predictprotein.org/)[55].

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Author contributions

The experiments in this work were conceived and designed by P.X. and K.L. Most of the experiments were performed by K.L. for his Ph.D. study, and some of the experiments for the revised manuscript were performed by T.J. The data were analyzed by K.L., C.G. and T.J. The reagents and materials were contributed by P.X., Y.M., B.Y., L.W. and C.M. The manuscript was prepared by P.X., K.L. and T.J.

Additional information

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