**Escherichia coli** small heat shock protein IbpA is an aggregation-sensor that self-regulates its own expression at posttranscriptional levels

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**Abstract**
Aggregation is an inherent characteristic of proteins. Risk management strategies to reduce aggregation are critical for cells to survive upon stresses that induce aggregation. Cells cope with protein aggregation by utilizing a variety of chaperones, as exemplified by heat-shock proteins (Hsps). The heat stress-induced expression of IbpA and IbpB, small Hsps in *Escherichia coli*, is regulated by the σ^{32} heat-shock transcriptional regulator and the temperature-dependent translational regulation via mRNA heat fluctuation. We found that, even without heat stress, either the expression of aggregation-prone proteins or the *ibpA* gene deletion profoundly increases the expression of IbpA. Combined with other evidence, we propose novel mechanisms for the regulation of the small Hsps expression. Oligomeric IbpA self-represses the *ibpA*/IbpB translation, and mediates its own mRNA degradation, but the self-repression is relieved by sequestration of IbpA into the protein aggregates. Thus, the function of IbpA as a chaperone to form co-aggregates is harnessed as an aggregation sensor to tightly regulate the IbpA level. Since the excessive preemptive supply of IbpA in advance of stress is harmful, the prodigious and rapid expression of IbpA/IbpB on demand is necessary for IbpA to function as a first line of defense against acute protein aggregation.

**KEYWORDS**
gene expression regulation, bacterial, IbpA protein, *E. coli*, molecular chaperones, protein aggregates, protein biosynthesis, small heat-shock proteins

1 | **INTRODUCTION**

Since proteins tend to form aggregates, cellular maintenance by keeping proteins in their native states and removing denatured proteins is crucial for all organisms. Multilayered quality control systems are essential to maintain such cellular protein homeostasis (proteostasis) (Hipp et al., 2019; Mogk et al., 2018). Refolding and degradation of denatured proteins caused by stresses are two primary strategies to prevent the accumulation of protein aggregates. Sequestration of denatured proteins is a third strategy, to keep misfolded proteins in a state that is easy to restore or degrade after stresses (Hipp et al., 2019; Mogk et al., 2018). Small heat shock proteins (sHsps) participate in the third strategy as “sequestrases,” constituting a first line of stress defense against irreversible protein aggregation (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Liberek et al., 2008; Mogk et al., 2019).

sHsps, defined as having low subunit molecular weights (12–43 kDa), are widely conserved chaperones from bacteria to mammals (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Liberek et al., 2008; Mogk et al., 2019). sHsps protect denatured proteins from forming irreversible aggregates by co-aggregating with the denatured proteins, in an ATP-independent manner (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Liberek et al., 2008; Mogk et al., 2019).
Vierling, 2015; Haslbeck et al., 2019; Liberek et al., 2008; Mogk et al., 2018; Mogk et al., 2019). The denatured proteins co-aggregated with sHsps can then be efficiently processed by other chaperones (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Liberek et al., 2008; Mogk et al., 2018; Mogk et al., 2019). Although the minimum physiological unit of sHSPs is a dimer, sHsps usually form various types of oligomers, which are required for the energy-independent sequestration activity (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Mogk et al., 2019). sHsps are composed of three domains, a flexible N-terminal domain, a highly conserved α-crystallin domain, and a C-terminal domain with an oligomerization motif, containing the characteristic three-residue IX(I/V) motif (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Mogk et al., 2019). The C-terminal IX(I/V) motif functions as a cross-linker for intermolecular binding among dimeric sHsps (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Mogk et al., 2019).

sHsps are among the most upregulated Hsps upon stress (Haslbeck and Vierling, 2015; Kortmann and Narberhaus, 2012; Mogk et al., 2019). The gene expression of the α- and γ-proteobacterial sHsps is regulated by two mechanisms (Kortmann and Narberhaus, 2012). One is the heat-shock transcriptional regulator σ32, an RNA polymerase subunit, which regulates the transcription of many Hsp genes (Guisbert et al., 2008; Guo and Gross, 2014). At normal growth temperatures, σ32, which is an extremely unstable protein, is rapidly degraded via a DnaK/DnaJ-mediated pathway. However, σ32 is stabilized to allow the transcription of many Hsp genes upon heat shock, since DnaK/J is sequestered to rescue the emerging heat-denatured proteins (Guisbert et al., 2008; Guo and Gross, 2014). The other is the thermo-responsive mRNA structures in the 5′ untranslated region (UTR), called RNA thermometers (RNATs), which mask the Shine–Dalgarno (SD) sequence in their stem-loop structures at normal or low temperatures (Kortmann and Narberhaus, 2012; Krajewski and Narberhaus, 2014). The heat fluctuation by a temperature up-shift melts the stem loops in RNATs and allows the ribosome to initiate translation, using the exposed SD sequence (Kortmann and Narberhaus, 2012; Krajewski and Narberhaus, 2014). The thermo-responsivity of many bacterial RNATs has been established, and the RNATs of sHsps have conserved shapes harboring two to four stem loops (Kortmann and Narberhaus, 2012; Krajewski and Narberhaus, 2014). Thus, the expression of sHsps is controlled at both the transcriptional level using heat-shock transcriptional factors, and the translational level using RNATs, in contrast with other Hsps, which are only controlled at transcriptional levels (Kortmann and Narberhaus, 2012; Krajewski and Narberhaus, 2014).

Inclusion body-associated protein A (IbpA) and B (IbpB) are Escherichia coli sHsps, and were originally identified as proteins induced in response to heterologous protein expression in the cell (Allen et al., 1992; Laskowska et al., 1996). IbpA/IbpB function as a holer of denatured proteins, to facilitate the initiation step of the refolding pathway via DnaK/DnaJ and ClpB (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Mogk et al., 2019). IbpA/IbpB mediate the efficient transfer of denatured proteins from the sHsp co-aggregation to the DnaK/DnaJ system (Matuszewska et al., 2005; Mogk et al., 2003; Veinger et al., 1998; Żwirowski et al., 2017). IbpA and IbpB are highly homologous proteins with ~50% of amino acid sequence identity (Allen et al., 1992), and form hetero-oligomers in E. coli (Matuszewska et al., 2005; Ratajczak et al., 2009; Ratajczak et al., 2010). Although most γ-proteobacteria only have a single sHsp (IbpA), a two-sHsp (IbpA and IbpB) system has evolved in a subset of Enterobacteriales, including E. coli, from a single gene duplication event (Obuchowski et al., 2019). Previous in vitro studies using recombinant IbpA and IbpB proteins revealed the distinct features of the two sHsps: IbpA is more efficient in binding denatured proteins to form the coaggregates (Obuchowski et al., 2019; Ratajczak et al., 2009), and even self-forms fibril-like aggregates (Ratajczak et al., 2010). However, the coaggregates with IbpA are inefficient substrates for the disaggregation assisted by DnaK/DnaJ and ClpB (Obuchowski et al., 2019; Ratajczak et al., 2009). The additional presence of IbpB in the coaggregates is required for the disaggregation process and facilitates IbpA degradation by Lon protease, suggesting that the interplay between IbpA and IbpB is important to modulate the interactions with substrates in the mixed oligomer states (Bissonnette et al., 2010; Haslbeck and Vierling, 2015; Mogk et al., 2019; Obuchowski et al., 2019; Ratajczak et al., 2009; Żwirowski et al., 2017).

The mRNA encoding the ibpA-ibpB operon has RNATs in the 5′ UTRs of both the ibpA and ibpB ORFs, as revealed by RNA structure probing and reporter assays (Gaubig et al., 2011; Kortmann and Narberhaus, 2012; Waldminghaus et al., 2009). Previous analyses of the RNAT in ibpB using a reporter revealed the possible influence of the IbpA protein on ibpB expression (Gaubig et al., 2011; Kortmann and Narberhaus, 2012). In addition to the heat stress, the expression level of ibpA/IbpB was profoundly upregulated, by 10–50-fold, under non-heat stressed conditions such as 30–37°C in the dnaK-dnaJ deleted strain (Calloni et al., 2012; Zhao et al., 2019) or upon oxidative stress induced by copper (Matuszewska et al., 2008). Since the RNAT regulation would not be effective at normal growth temperatures, the mechanism for the massive upregulation under the non-heat stressed conditions remains to be elucidated.

Here, we addressed why IbpA is upregulated in non-heat stressed cells. We found that the accumulation of protein aggregates was sufficient for the upregulation. Intriguingly, a reporter assay using the 5′ UTR of the ibpA mRNA revealed that the deletion of the ibpA gene increased the reporter translation, which was repressed by the overexpression of oligomeric IbpA. Combined with other evidence including the requirement of oligomerization of IbpA on the repression and the specific interaction of IbpA with 5′ UTR of ibpA mRNA, we propose that the IbpA oligomers self-repress the ibpA translation and are involved in its own mRNA degradation, which are relieved by the sequestration of IbpA by co-aggregation with protein aggregates. The role of the aggregation sensor is specific to IbpA, since the homologous IbpB lacks this self-repression function. The significance of the self-repression by IbpA at the translational level is discussed in relation to the unique role of IbpA in protecting cells from acute heat stress.
2 | RESULTS

2.1 | *ibpA* translation is upregulated in response to protein aggregation

Although the thermometer in the mRNA (RNAT) and the transcriptional control by σ32 are known mechanisms to upregulate the expression of *ibpA*, previous studies have reported that *ibpA* expression is also upregulated under non-heat-stressed conditions, such as in the *dnaKJ* deletion strain or upon copper stress (Calloni et al., 2012; Matuszewska et al., 2008; Zhao et al., 2019). The absence of DnaK/DnaJ leads to the production of protein aggregates (Calloni et al., 2012; Mogk et al., 1999). The addition of copper disturbs protein homeostasis in cells with oxidative stress (Matuszewska et al., 2008; Mogk et al., 1999; Yang et al., 2015). Therefore, we hypothesized that protein aggregation might somehow be involved in the upregulation of *ibpA* under the non-heat-stressed conditions.

To investigate whether the expression of *ibpA* is upregulated not only by heat shock but also by protein aggregation under non-heat-stress conditions, we expressed aggregation-prone proteins in a wild-type *E. coli* (BW25113). To do so, we overexpressed rhodanese, a bovine mitochondrial protein, which is known to aggregate in *E. coli* at 37°C (Ewalt et al., 1997) (Figure S1A). Strikingly, the expression of *ibpA* increased upon the rhodanese expression (agg++, Figure 1A). Overexpression of another aggregation-prone protein, SerA of *E. coli* (Mogk et al., 1999), (Figure S1A), also massively induced the *ibpA* expression in the wild-type *E. coli* strain (Figure S1B). The rhodanese expression did not increase the levels of DnaK and GroEL, two of the representative Hsps in *E. coli* (Figure S1C). The rhodanese expression did not affect the *ibpA* degradation rate in cells (Figure S1D), suggesting that the increased amount of *ibpA* is not due to an inhibition of *ibpA* degradation, at least partly mediated by Lon protease. These results support the idea that the accumulation of aggregated proteins in cells increases the *ibpA* expression.

We suspected that the aggregates might induce *ibpA* via upregulated σ32-mediated transcriptional control at 37°C, since the aggregation-prone proteins could sequester DnaK/J, thus, protecting σ32 from DnaK/J-mediated degradation and eventually stabilizing σ32 to promote the expression of Hsps. If so, then, the overexpression of σ32 would increase the mRNA levels to upregulate *ibpA* as well as other Hsps. After we confirmed the ~10-fold induction of *ibpA* mRNAs in the σ32-overexpressing cells, using quantitative real-time PCR (qRT-PCR) (Figure 1B), we compared the protein expression levels. Upon the σ32 overexpression, the expression level of *ibpA* did not obviously increase (Figure 1A), even though the *ibpA* mRNA increased. In contrast, the DnaK and GroEL expression levels increased (Figure S1C), confirming that the excess σ32 is effective in increasing the Hsps under the non-heat stress. The results indicate that the *ibpA* induction in the presence of aggregates is not explained by the σ32-mediated transcriptional upregulation.

![FIGURE 1](https://example.com/figure1.png)

**FIGURE 1** Accumulation of protein aggregates upregulates *ibpA* expression. (a) Western blotting to evaluate the endogenous *ibpA* expression in *E. coli* wild-type strain (BW25113) under various conditions. *E. coli* cells were grown at 37°C or shifted to 42°C for 10 min. agg++, *E. coli* wild-type BW25113 expressing rhodanese; σ32++, *E. coli* wild-type BW25113 expressing σ32. Unless otherwise indicated, *E. coli* cells were grown at 37°C. Expression of FtsZ was also examined as a control for a constitutively expressed protein. (b) Ratios of the *ibpA* mRNA amounts in cells under conditions corresponding to (a). Error bars represent SD; *n = 3* biological replicates. Student’s *t* test was used to assess the statistical significance of differences (*p < 0.01*). (c) Evaluation of *ibpA* translation by GFP reporters. The reporters harboring the 5′ UTR of *ibpA* or the 5′ UTR from a plasmid without the *ibpA* sequence were expressed under various conditions. Western blotting was performed using anti-GFP and anti-FtsZ antibodies [Colour figure can be viewed at wileyonlinelibrary.com]

To demonstrate that the *ibpA* upregulation by the accumulation of protein aggregates does not occur at the transcriptional level, we investigated the efficiency of the *ibpA* translational initiation in a reporter assay. Since the 5′ UTR in the *ibpA* mRNA is critical for the translational control using the stem loops in the mRNA, we constructed a plasmid harboring the 5′ UTR of *ibpA* fused with the *gfp* gene, under the control of an arabinose-inducible promoter (Figure 1C). We observed a substantial induction of GFP upon the rhodanese overexpression (Figure 1C). The effect is specific for the 5′ UTR of *ibpA*, since there was no upregulation of GFP upon the rhodanese overexpression when the 5′ UTR was substituted with 5′ UTRs derived from the parent plasmid (Figure 1C) or *dnaK* (Figure S1E). In addition, the overexpression of another aggregation-prone protein, SerA, also induced the GFP reporter with the 5′ UTR of *ibpA* (Figure S1F). Combined with the results that the
rhodanese overexpression did not change the mRNA level and the GFP degradation rate of the reporter (Figure S1G,H), the results using the reporter further confirmed that the accumulation of protein aggregates upregulates the \textit{ibpA} expression at the posttranscriptional level.

### 2.2 | IbpA self-represses \textit{ibpA} expression level

Next, we investigated the connection between protein aggregation and IbpA expression induction. The well-known physiological function of IbpA as a chaperone is the co-aggregation with denatured proteins (Haslbeck and Vierling, 2015; Haslbeck et al., 2019; Liberek et al., 2008; Mogk et al., 2018; Mogk et al., 2019). The recruitment of IbpA to protein aggregates might cause the entrapment of the free IbpA in the cytosol. Indeed, the localization of IbpA fused with GFP shifted to the cell poles in the rhodanese-overexpressing cells (Figure 2A), consistent with previous observations that IbpA and denatured proteins accumulate as inclusions at the cell poles (Govers et al., 2018; Lindner et al., 2008; Pu et al., 2019). Taking this into consideration, we hypothesized that the entrapment of free IbpA from the cytosol, due to the sequestration of denatured proteins, would induce the \textit{ibpA} translation (Figure 2B). If this model is correct, then, the deletion of IbpA, which is nonessential for \textit{E. coli} growth, would...
upregulated the translation of the reporter harboring the 5' UTR of ibpA, used in Figure 1C.

We deleted the operon including ibpA-ibpB. After we confirmed that the growth of the ∆ibpAB cells was similar to that of the wild-type E. coli BW25113 (Figure S2A), we evaluated the translation initiation of the reporter. Strikingly, the reporter expression was strongly promoted by the ibpA deletion, even in the absence of induced protein aggregation (Figure 2C, lane 2), suggesting that ibpA suppresses ibpA translation. The rhodanese overexpression did not further increase the expression level of the reporter in the ∆ibpAB cells (Figure 2C, lane 4). The lack of additional effects by the aggregates on the ibpA induction suggests that the shortage of ibpA in cells governs the ibpA expression. We further examined the effect of ibpA overexpression, which caused a slower growth rate in wild-type E. coli (Figure S2B). The overexpression of ibpA in wild-type cells completely suppressed the expression of the reporter (Figure 2D, lane 3). More importantly, the replenishment of ibpA by the overexpression in the ∆ibpAB cells almost completely repressed the upregulated expression of the GFP reporter (Figure 2D, lane 4). These results indicate that the amount of ibpA governs its own expression.

2.3 | mRNA degradation is partially involved in the ibpA expression regulation

qRT-PCR analysis revealed that the mRNA level of the reporter harboring the ibpA 5' UTR in the ∆ibpAB cells was ~6-fold higher than that in wild-type cells (Figure 3A). Kinetic analysis of the mRNA degradation showed that the higher abundance was attributed to the stabilization of the mRNA in the ∆ibpAB cells (Figure 3B). The stabilization was reverted by the overexpression of ibpA (Figure 3B), suggesting that ibpA might be involved in the mRNA turnover to regulate its own expression. We also found that the aggregation induced by rhodanese expression stabilized the mRNAs with the ibpA 5’ UTR (Figure S3A) and endogenous ibpA (Figure S3B) in wild-type cells, further supporting the involvement of ibpA in the process by the sequestration of ibpA into the aggregates.

Previous in vivo ibpA crosslinking experiment in E. coli identified polynucleotide phosphorylase (PNPase) and enolase, both of which are important components of an mRNA-degrading machine, called the RNA degradosome (Applied et al., 2005; Carpousis, 2007), raising the possibility that PNPase might be associated with the degradation of the ibpA mRNA to prevent a constitutive expression of ibpA (Figure 3C). This is indeed the case since the both the reporter and the endogenous ibpA mRNA was remarkably stabilized in PNPase-deleted (∆pnp) cells (Figures 3D, S3B), showing an involvement of PNPase in the ibpA mRNA turnover.

We subsequently evaluated the expression of the reporter in the ∆pnp cells. GFP translated from the reporter was upregulated in the ∆pnp cells (Figure 3E, lane 3), reflecting the higher abundance of the mRNA. However, the increasing level in the ∆pnp cells was significantly weaker than that in the ∆ibpAB cells (Figure 3E, lane 2), suggesting that the effect of PNPase is not a major mechanism to regulate the ibpA expression.

2.4 | Ibpa directly suppresses its own translation

In addition to the involvement of ibpA to its own mRNA degradation, we tested whether ibpA affects its own translation in a direct approach. We translated the GFP reporter harboring the 5' UTR of ibpA using a reconstituted cell-free translation system of E. coli (the PURE system), which only contains essential factors for the translation, where there is no chaperone including ibpA (Shimizu et al., 2001). Strikingly, the ibpA translation was repressed by almost half in the presence of recombinant ibpA (Figure 4A). The repression by the recombinant ibpA was not observed in the control reporter without 5’ UTR of ibpA (Figure 4A). The effect was specific to ibpA since the addition of purified GFP instead of ibpA did not repress the reporter translation (Figure S4).

Since ibpA specifically repressed the translation of the reporter gene with the 5' UTR of ibpA in the reconstituted cell-free translation, we anticipated that the self-repression of the ibpA might be through a direct recognition of ibpA with the 5' UTR of ibpA mRNA. We next performed an electrophoretic mobility shift assay (EMSA) to investigate whether ibpA interacts with the ibpA mRNA. In the presence of ibpA at 500 nM, mRNA with the 5’ UTR of ibpA shifted upward, whereas none of mRNA without the 5’ UTR of ibpA moved (Figure 4B). Western blotting showed an accumulation of ibpA at the position of the upshifted mRNA, suggesting that an association of ibpA with the mRNA with the 5’ UTR of ibpA. Taken together, these in vitro experiments show that ibpA directly self-represses the ibpA translation.

2.5 | Oligomeric Ibpa is critical for the self-repression of translation

What region of ibpA is critical for the self-regulation? At first, we deleted the N- or C-terminal domain of ibpA and examined the effect on expression repression (Figure 5A). The GFP reporter assay revealed that the C-terminal truncation eliminated the ability to suppress the expression in the ∆ibpAB cells (Figure 5A), although the expression level of the C-terminal truncated mutant was comparable to that of wild-type ibpA (Figure S5A). In contrast, the suppression by the N-terminal truncation was almost the same as that by wild-type ibpA (Figure 5A), showing that the C-terminal domain is responsible for the self-repression.

The C-terminal domain of ibpA contains a universally conserved motif for oligomerization, IX(I/V) (Jaya et al., 2009; Stróżecka et al., 2012). We substituted the motif, IEI in ibpA, with AEA, and confirmed the impaired oligomerization of the AEA mutant (Figure S5B), as reported previously (Stróżecka et al., 2012). Although the expression level of the ibpA (AEA) mutant was almost the same in the ∆ibpAB cells, co-expression of the AEA mutant
**FIGURE 3** PNPase is partially involved in the regulation of the IbpA expression. (a) Ratios of mRNA amounts of the reporter harboring the 5′ UTR of *ibpA* or the 5′ UTR from a plasmid in cells under the conditions corresponding to Figure 2D. Error bars represent SD; *n* = 3 biological replicates. Student’s *t* test was used to assess the statistical significance of differences (*p* < .01). (b) Degradation kinetics of the mRNAs shown in (a). Time shows the minutes from the addition of Rifampicin. Error bars correspond to the SD. (c) Model of the IbpA expression regulation via PNPase-mediated mRNA degradation. PNPase (yellow) degrades the *ibpA* mRNA with the aid of IbpA (circles). (d) Degradation kinetics of the *ibpA* mRNA in the PNPase-deleted BW25113 strain (Δpnp). Time shows the minutes from the addition of Rifampicin. Error bars correspond to the SD. (e) Evaluation of the *ibpA* translation initiation by the reporters used in Figure 1C. *E. coli* lysates from wild-type BW25113 strain (WT), the *ibpAB* operon-deleted strain (ΔAB), and the *pnp* operon-deleted strain (Δpnp) were analyzed. Western blotting using anti-GFP and anti-FtsZ antibodies is shown [Colour figure can be viewed at wileyonlinelibrary.com]
with the reporter did not repress the *ibpA* expression (Figures 5B, S5A). The AEA mutant also did not affect the *ibpA* translation in the PURE system and the mobility of mRNA with the 5′ UTR of *ibpA* (Figure 5C,D), indicating that the oligomeric state of IbpA is critical for the IbpA self-regulation.

### 2.6 IbpA also suppresses the expression of *ibpB*

*E. coli* possesses IbpB, a paralog of IbpA, and its expression is also regulated by RNAT in the 5′ UTR of the *ibpB* mRNA (Gaubig et al., 2011; Kortmann and Narberhaus, 2012; Waldminghaus et al., 2009). Gaubig et al. observed that IbpA suppresses the *ibpB* translation (Gaubig et al., 2011), but the reporter system used in the study could not reveal the influence of IbpB on the *ibpB* translation. Therefore, we tested the effect of IbpB on the *ibpB* translation, after we replaced the 5′ UTR of *ibpA* with that of *ibpB* in the GFP reporter system. Although the induction level of IbpA and IbpB were almost the same (Figure S6), IbpA, but not IbpB, suppressed the reporter for the *ibpB* expression in the Δ*ibpAB* strain (Figure 6A). The translation of the *ibpB* reporter was also repressed by the recombinant IbpA in the PURE system (Figure 6B). These results show the specific function of IbpA as a repressor of the small Hsps, IbpA and IbpB, in *E. coli*.

In contrast, the overexpression of IbpB did not change the *ibpA* reporter upregulation in the Δ*ibpAB* strain (Figure 6C, lane 4), indicating that IbpB cannot substitute for IbpA in the IbpA repression mechanism. In the wild-type strain, the IbpB overexpression
increased the amount of the GFP reporter (Figure 6C, lane 3), probably reflecting the hetero-oligomerization of IbpB with endogenous IbpA to reduce the amount of free IbpA for the self-repression.

2.7 | Stem loops in the 5′ UTR of ibpA mRNA mediate the self-repression of ibpA expression

Previous studies revealed that the secondary mRNA structures of the ibpA 5′ UTR regulate the ibpA translation (Waldminghaus et al., 2009). The ibpA 5′ UTR contains three stem loops, and the two upstream stem loops (SL1 and SL2) are thought to stabilize the downstream thermo-responsive stem loop (SL3) to mask the SD sequence (Figure 7) or protect the mRNA from degradation (Krajewski and Narberhaus, 2014; Waldminghaus et al., 2009). Since the contribution of these stem loops to the regulation revealed here remains unclear, we constructed a series of stem loop variants in which the stem loop stabilities were weakened or strengthened (Figure S7), and evaluated the effects on the GFP reporter. For all three weakened stem loops, the GFP expressions were upregulated in wild-type E. coli as compared to those of the unchanged stem loops (Figure 7A-C, compare lanes 1 and 4). The up-regulation levels in wild-type E. coli were almost the same as those in the ∆ibpAB cells (compare lanes 4 and 5), but were largely suppressed when IbpA was overexpressed (lane 6). These results suggest that the suppression by the endogenous level of IbpA was compromised in the reporters harboring the weak stem loops in SL1-3.

The expression patterns of the reporters using the strong SL1 and SL2 stem loops were almost the same as those using the weak variants (Figure 7A,B). In contrast, the expression of the reporter using the strong SL3 was not observed under all conditions examined (Figure 7C), probably because the strong stem loop containing the SD motif is too tight to expose the SD motif for the translation initiation.
3 | DISCUSSION

IbpA and IbpB, small Hsps in *E. coli*, are chaperones that sequester aggregation-prone proteins by co-aggregation during stress. This study unveiled a previously unknown function of IbpA, as a mediator for negative feedback regulation at the post-transcriptional levels. Our experiments revealed that the IbpA oligomers serve as their own suppressor for the expression. The titration of IbpA by co-aggregation with denatured proteins relieves the suppression.

We propose a novel mechanism of the sHsps regulation, where IbpA-mediated aggregation sensing self-regulates the expression of *ibpA* and *ibpB* (Figure 8). Without protein aggregation, IbpA oligomers suppress sHsps translation initiation and stimulate degradation of the *ibpA-ibpB* mRNA. In contrast, under stress conditions involving protein aggregate accumulation, IbpA recruits aggregation-prone proteins to form co-aggregates. This titrates the free IbpA away to relieve the suppression, leading to the massive increase in IbpA expression to maintain cellular proteostasis.

Translation repression by a direct binding of IbpA is unexpected, since so far there is no known RNA-binding motif in IbpA. In addition to the translation repression, we found that the increased IbpA expression is caused by a relief of IbpA mRNA degradation mediated by PNPase and IbpA. Although the involvement of PNPase in the *ibpA* mRNA degradation was previously reported (Carpousis, 2007), our finding adds IbpA as a critical player in this mRNA degradation pathway for the self-regulation. It has been known that PNPase usually functions in a second step in the RNA degradosome pathway after the first step mediated by the RNaseE. However, it is also reported that PNPase participates in post-transcriptional regulations depending on 5´UTR (Chen et al., 2019; Jarrige et al., 2001). In this manner, it would be plausible that PNPase would participate in the repression of the *ibpA* translation. How the interaction between IbpA and PNPase, as reported previously (Applied et al., 2005), regulates the *ibpA* mRNA degradation via the 5´UTR of *ibpA* is not known, but would be worth pursuing in the future.

Thus, we revealed two mechanisms for the IbpA-mediated self-regulation. Then, what is the relation between the translation suppression and stimulated mRNA degradation? One immediate explanation would be a synergistic effect to complement each other. Alternatively, these two mechanisms might be not necessarily mutually exclusive. Translation suppression by direct binding of IbpA to its own mRNA might be just an intermediate step to deliver the mRNA to the degradation pathway.

This model of IbpA expression regulation resembles the σ^{32}-mediated transcriptional regulation of Hsps (Guisbert et al., 2008; Guo and Gross, 2014), since the chaperones are titrated away by denatured...
proteins under stress conditions in both mechanisms. However, the IbpA-mediated self-regulation, which takes place at the posttranscriptional levels, would have an advantage for a rapid response to the emergence of aggregation-prone proteins. This self-regulation mechanism enables IbpA to serve as a first line of defense against protein aggregation.

Previous studies revealed the layered regulation of IbpA expression: σ32-mediated transcriptional control and RNA thermometer (RNAT) translational control (Gaubig et al., 2011; Kortmann and Narberhaus, 2012). Since the stem loops in the RNAT system influence the IbpA-mediated expression repression (Figure 7), RNAT and the

**FIGURE 7** Effect of stem loops in the ibpA mRNA on the IbpA-mediated suppression. The stem loops in the ibpA 5′ UTR of the GFP reporter used in Figure 1C were mutated and evaluated in the wild-type BW25113 (WT), and ΔibpAB (ΔAB) strains. Mutations to weaken or strengthen the stem-loop structures were introduced in SL1 (a), SL2 (b), and SL3 (c). Schematic representations of stem-loop mutations are shown (see also Figure S7). Where indicated, IbpA was overexpressed (+). The ibpA 5′ UTRs with no (WT), weak and strong mutations in the stem loops were tested using the GFP reporter. Western blotting with anti-GFP and anti-FtsZ antibodies is shown [Colour figure can be viewed at wileyonlinelibrary.com]
self-repression control are not independent. In the RNAT mechanism, the stem loops fluctuate and melt to expose the SD region, depending on the temperature. As reported previously, higher temperatures cause more melting of the stem loops, like a “thermometer.” In other words, the temperature responsiveness is not an all-or-none fashion (Kortmann and Narberhaus, 2012; Krajewski and Narberhaus, 2014). This inherent property would allow the stem loops to partly open even under mild conditions such as 37°C (Waldminghaus et al., 2009), leading to a potential leaky expression of certain amounts of IbpA. Thus, the IbpA-mediated self-repression would function to tightly shut off the IbpA expression as a “safety catch” in the leaky RNAT system. Taken together, the stringent repression mechanism, combining RNAT and the IbpA-mediated negative feedback control, has evolved to fulfill the following requirements: tight repression under unstressed conditions, and acute upregulation upon aggregation-stress. This mechanism enables IbpA to be one of the most upregulated chaperones upon aggregation inducing stresses.

IbpA serves as a first line of defense against protein aggregation, where oligomerized IbpA co-aggregates with aggregation-prone proteins for sequestration. Why does IbpA employ such a feedback control mechanism in addition to the known regulation controls including σ32 and RNATs? Considering that IbpA is an ATP-independent oligomeric chaperone, a greater than stoichiometric amount of IbpA would be necessary to sequester the aggregation-prone proteins. One strategy for risk management is to prepare an abundance of IbpA protein even under unstressed conditions. However, this might not be appropriate, since IbpA overexpression had detrimental effects under the normal conditions (Figure S2B), probably due to the self-formation of fibril aggregates (Ratajczak et al., 2010), which could perturb proteostasis and compromise the sequestration activity. Thus, the expression of IbpA should be tightly repressed under normal conditions, since IbpA can be regarded as a “double-edged sword.” The self-regulation mechanism proposed here can overcome the dilemma that the high abundance of IbpA is necessary in cases of aggregation stress, but an excessive preemptive supply could be detrimental to the cell.

Our analysis of the stem loops in the 5’ UTR of the ibpA mRNA revealed that the secondary structures of the mRNA are critical to regulate the expression. How does IbpA couple to the mRNA structure in RNAT for the suppression? One possibility is that the oligomeric states of IbpA bind to their own mRNA to suppress the expression.
Experiments on the stem loop variants revealed that SL1/SL2 affect SL3 for the expression, suggesting that SL1/SL2 interact with SL3 to control the regulation. In this case, the ibpA oligomers might associate with the RNAT system to suppress the translation. Alternatively, a trans-acting modulator might contribute to the link between RNAT and ibpA.

E. coli has two small Hsps, ibpA and its highly homologous paralog ibpB, which are encoded in the ibpAB operon (Allen et al., 1992). Several lines of evidence have shown the distinct roles of ibpA and ibpB, where ibpA and ibpB function as a canonical binder and its noncanonical paralog that enhances the dissociation of sHsps from the co-aggregates, respectively (Obuchowski et al., 2019; Rajaczak et al., 2009). In addition to this distinction, our findings demonstrated another aspect of the difference between the two sHsps in the expression regulation. Ibpa suppressed the ibpB reporter translation in the ΔibpAB strain (Figure 6) (Gaubig et al., 2011). In contrast, ibpB could not suppress the ibpA translation. Thus, ibpA plays a pivotal role as a master regulator of the expression of sHsps at the posttranscriptional levels, ensuring that ibpA and ibpB cooperate to cope with protein aggregation.

The overexpression of ibpB in the wild-type strain increased the ibpA level (Figure 6). This ibpA induction is interpreted to be due to the ibpA deprivation by hetero-oligomer formation between ibpA and ibpB, implying that, in addition to the aggregation-prone proteins, the factors that can associate with ibpA could trigger its upregulation. Therefore, we suggest the possibility that ibpA plays a pivotal role as a trans-regulator for the expression of other proteins. Indeed, the translation level of ibpB is decreased upon ibpA co-expression (Gaubig et al., 2011). The fact that the ibpB mRNA also has an RNAT in the 5′ UTR (Gaubig et al., 2011; Waldminghaus et al., 2009) implies that ibpA recognizes a particular structure of stem loops in 5′ UTRs, such as RNAT.

4 | MATERIALS AND METHODS

4.1 | E. coli strains

The E. coli strains are listed in Table S1. DH5α strain was used for cloning. The BW25113 strain was used for each assay. Deletion of the chromosomal ibpA-ibpB operon or dnaK-dnaA operon was accomplished by the procedures described previously (Datsenko and Wanner, 2000). BW25113Δpnp was generated with a P1 transduction using the Keio collection JW5851 (Δpnp::FRT-Km-FRT) (Baba et al., 2006). The DNA fragment amplified from JW3664 (ΔibpA::FRT-Km-FRT) (Baba et al., 2006), using the primers PT0456 and PM0195, and that from JW3663 (ΔibpB::FRT-Km-FRT) (Baba et al., 2006), using the primers PT0457 and PM0196, were mutually annealed and amplified using PT0456 and PT0457. The purified DNA was electroporated into the E. coli strain BW25113 harboring pKD46, and the transformant resistant to 40 μg/ml kanamycin was stored as BW25113ΔibpAB. Primers are listed in Table S2.

4.2 | Plasmids

Plasmids were constructed using standard cloning procedures and Gibson assembly. Plasmids for reporter or microscopy assays: pBAD30-ibpA 5′ UTR-gfp, pBAD30-gfp, pBAD30-ibpB 5′ UTR-gfp, and pBAD30-ibpA 5′ UTR-ibpA-gfp, pBAD30-dnaK 5′ UTR-gfp were constructed using DNA fragments amplified from pBAD30 (Guzman et al., 1995). DNA fragments amplified from superfolder GFP (Pédelacq et al., 2006), derived from a plasmid constructed previously (Ishimoto et al., 2014), and DNA fragments amplified from E. coli genomic DNA. Plasmids for overexpression: pCA24N-rodanes, pCA24N-rpoH, pCA24N-serA, pCA24N-gfp, pCA24N-ibpA, pCA24N-ibpB 5′ UTR-gfp, pCA24N-ibpAΔN, pCA24N-ibpAΔC, pCA24N-ibpA_AEA, and pCA24N-ibpB were constructed using DNA fragments amplified from pCA24N (Kitagawa et al., 2005), DNA fragments amplified from superfolder GFP (Ishimoto et al., 2014; Pédelacq et al., 2006) or DNA fragments amplified from E. coli genomic DNA. Primers used for cloning are listed in Table S2.

4.3 | SDS-PAGE and western blotting

E. coli BW25113 cells harboring a plasmid carrying the reporter were precultured at 30°C for 16 h in LB medium. In RNase deletion assay, E. coli BW25113Δpnp strain harboring a pMW118 plasmid carrying the reporter was used. The precultured cells were grown to an OD600 of 0.4–0.6 at 37°C in LB medium. The reporter carried in pBAD30 plasmid was induced with 2 × 10⁻⁶% arabinose. The reporter in pMW118 or pCA24N, the induction was performed with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), added 2 h after starting the culture. For the co-expression assay, E. coli BW25113 cells harboring plasmids carrying the reporter genes and pCA24N plasmids were used. The induction of protein co-expression was performed with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), added 2 h after starting the culture. In the case of using the reporter in pCA24N, the induction was performed with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), added 2 h after starting the culture. Cell cultures were sampled and mixed with an equal volume of 10% TCA, to stop the biological reactions and precipitate the macromolecules. After standing on ice for at least 15 min, the samples were centrifuged at 20,000 xg for 3 min at 4°C, and the supernatant was removed by aspiration. Precipitates were washed with 1 ml of acetone by vigorous mixing, centrifuged again, and dissolved in 1× SDS sample buffer (62.5 mM of Tris-HCl, pH 6.8, 5% of 2-mercaptoethanol, 2% of SDS, 5% of sucrose, and 0.005% of bromophenol blue) by vortexing for 15 min at 37°C. The samples were separated by SDS-PAGE. The separated samples were transferred to PVDF membranes. Membranes were blocked by 5% non-fat milk in Tris-Buffered Saline, with 0.002% Tween-20. Mouse anti-sera against GFP (mFx75, Wako), rabbit anti-sera against ibpA (Eurofin), and rabbit anti-sera against PtsZ (a gift from Dr. Shinya Sugimoto at Jikei Medical University) were used as primary antibodies at a 1:10,000 dilution. HRP conjugated anti-mouse IgG (Sigma-Aldrich) and HRP-conjugated anti-rabbit IgG (Sigma-Aldrich) were used as secondary antibodies. Blots were visualized using HRP-conjugated anti-mouse IgG (Sigma-Aldrich) and HRP-conjugated anti-rabbit IgG (Sigma-Aldrich) as secondary antibodies.

Table S2.
secondary antibodies. Blotted membranes were detected with an LAS 4,000 mini imager (Fujifilm).

4.4 | Quantitative RT-PCR

To quantify mRNA relative amounts, *E. coli* BW25113 cells harboring a plasmid carrying the reporter were precultured at 37°C for 16 h in LB medium. The precultured cells were grown to an OD$_{660}$ of 0.4 – 0.6 at 37°C in 5 ml of LB medium. The reporter carried in pBAD30 plasmid was induced with 2 × 10$^{-6}$% arabinose. The induction of protein co-expression was performed with 0.1 mM of isopropyl-$\beta$-D-thiogalactopyranoside (IPTG), added 2 h after starting the culture. The cultures were pelleted at 10,000 × g for 3 min at 4°C.

In the case of the quantification of mRNA degradation, *E. coli* BW25113 cells harboring a plasmid carrying the reporter were precultured at 37°C for 16 h in LB medium. The precultured cells were grown to an OD$_{660}$ of 0.4 – 0.6 at 37°C in 20 ml of LB medium. The reporter carried in pBAD30 plasmid was induced with 2 × 10$^{-6}$% arabinose. In RNase deletion assay, *E. coli* BW25113Δpnp strain was used. The reporter in pMW118 was induced with 0.1 mM of IPTG, added 2 h after starting the culture. After 2.5 h from the starting culture, cells were aliquoted 5 ml and treated with 250 µM of Rifampicin. The cultures were sampled 5 ml in each timepoints 2, 5 min from rifampicin treatment and pelleted at 10,000 × g for 3 min at 4°C.

Total mRNA was extracted using Tripure Isolation Reagent (Merck) and treated with recombinant DNase I (Takara). The treated RNA was purified with a RNeasy Mini kit (Qiagen). The samples were prepared using a Luna Universal One-Step RT-qPCR Kit (New England Biolabs). Quantitative RT-PCR was performed with an Mx3000P qPCR system (Agilent) and analyzed by the MxPro QPCR software (Agilent). The amount of target mRNA was normalized with ftsZ mRNA by the $\Delta \Delta$Ct method (Livak and Schmittgen, 2001). Primers used for PCR are listed in Table S2.

4.5 | Microscopy

To observe the IbpA localization in cells, we used the *E. coli* BW25113 wild-type strain carrying pCA24N-rhodanese and the dnaKJ deletion strain. Each strain carrying pBAD30-ibpA 5’ UTR-ibpA-gfp vector was grown to an OD$_{660}$ of ~0.4 at 37°C in LB medium. Cells were observed with an inverted microscope IX71 (Olympus) and a mercury lamp with a GFP filter. Fluorescent images were recorded with an iXon DV897 electron multiplying CCD camera (Andor) and the Andor SOLIS software (Andor).

4.6 | Protein purification

To purify IbpA, we used the BL21 (DE3) strain carrying pCA24N-ibpA or pCA24N-ibpA_AEA. Cells were grown in LB media at 37°C to an OD$_{660}$ of 1.0, and ibpA production was induced with 1 mM IPTG for 2 h. The cells overexpressing IbpA were lysed by sonication (Branson) in buffer A (50 mM of Tris-HCl, pH 7.4, 10% of glycerol, 1 mM of dithiothreitol, and 100 mM of KCl). After the sonication, we followed the established methods using anion exchange chromatography for the purification of the wild-type IbpA (Matuszewska et al., 2005) and the AEA mutant (Strózecka et al., 2012).

4.7 | Sucrose density gradient assay

The purified ibpA (10 µM) was incubated for 30 min at 48°C in buffer B (50 mM of Tris-HCl, pH 7.4, 150 of mM KCl, 20 mM of magnesium acetate, and 5 mM of dithiothreitol). After the incubation, the samples were applied onto an 11 ml gradient of 10%-50% (w/v) sucrose in buffer B and centrifuged, using a Beckman SW41Ti rotor at 150,000 × g at 4°C for 80 min. The samples were collected as 20 separate fractions, using a fractionator (BioComp). The fractions were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

4.8 | Cell-free translation

The transcription–translation-coupled PURE system (PUREfrex®, GeneFrontier) reaction, including Cy5-labeled tRNA$	ext{Met}$, was performed at 37°C for 2 h in the presence or the absence of 1 µM IbpA. After the protein synthesis, the SDS-sample buffer (0.125 M of Tris-HCl (pH 6.8), 10% (v/v) of 2-mercaptoethanol, 4% (w/v) of SDS, 10% (w/v) of sucrose, and 0.01% (w/v) of bromophenol blue) was added and incubated at 95°C for 5 min. The samples were separated by SDS-PAGE and detected with fluorescence imager (FLA2000, FUJI FILM) at the 633 nm wavelength. The band intensity was quantified with an analysis software (Multi gauge, FUJI FILM).

4.9 | Electrophoretic mobility shift assay

The reporter mRNA harboring 5’ UTR of ibpA vector was synthesized by in vitro transcription with CUGA7 in vitro transcription kit (Nippon Gene), and purified with RNeasy Mini kit (Qiagen). The mRNA at 100 nM and purified IbpA were incubated for 30 min at 37°C. The samples were loaded on 1 × TBE 5% of acrylamide gel. The samples were electrophoresed for 80 min at 200 V. After the electrophoresis, the gel was immersed in 1 × TBE with 1/50000 SYBR Gold (Thermo Fisher) for 5 min. Fluorescence was detected with a fluorescence imager IN-6W-CAM (Natural Immunity).

4.10 | Growth assay

The *E. coli* wild-type strain (BW25113), the ibpAB deleted strain, and the wild-type strain harboring pCA24N-ibpA or pCA24N-gfp were
preincubated at 30°C for 16 h in LB medium. The precultured cells were incubated at 37°C with shaking at 70 rpm, using a TVS062CA incubator (Advantec).

### 4.11 Statistical analysis

Student’s t test was used for calculating statistical significance, with a two-tailed distribution with unequal variance. All experiments represent a minimum of three independent experiments, with the bars showing the mean values ± SD.

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### AUTHOR CONTRIBUTIONS

T. Miwa performed experiments; T. Miwa, Y. Chadani, and H. Taguchi conceived the study, designed experiments, and analyzed the results; H. Taguchi supervised the entire project; T. Miwa and H. Taguchi wrote the manuscript.

### DATA AVAILABILITY STATEMENT

Data in this manuscript have been uploaded on the Mendeley Data set public repository (http://dx.doi.org/10.17632/fnjyyyvrxd.1).

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SUPPORTING INFORMATION

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