



## Solubility-enhancing proteins MBP and NusA play a passive role in the folding of their fusion partners

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### Abstract

It is well established that certain highly soluble proteins have the ability to enhance the solubility of their fusion partners. However, very little is known about how different solubility enhancers compare in terms of their ability to promote the proper folding of their passenger proteins. We compared the ability of two well-known solubility enhancers, *Escherichia coli* maltose-binding protein (MBP) and N utilization substance A (NusA), to improve the solubility and promote the proper folding of a variety of passenger proteins that are difficult to solubilize. We used an intracellular processing system to monitor the solubility of these passenger proteins after they were cleaved from MBP and NusA by tobacco etch virus protease. In addition, the biological activity of some fusion proteins was compared to serve as a more quantitative indicator of native structure. The results indicate that MBP and NusA have comparable solubility-enhancing properties. Little or no difference was observed either in the solubility of passenger proteins after intracellular processing of the MBP and NusA fusion proteins or in the biological activity of solubilized passenger proteins, suggesting that the underlying mechanism of solubility enhancement is likely to be similar for both the proteins, and that they play a passive role rather than an active one in the folding of their fusion partners.

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Insolubility of recombinant proteins is a major bottleneck in structural and functional proteomics projects. Only about half of all recombinant proteins, even those of bacterial origin, are soluble when expressed in *Escherichia coli* [3]. Refolding of proteins is not a high-throughput proposition. Consequently, some means of circumventing the formation of inclusion bodies are highly desirable. Over the last decade, it has emerged that the solubility of recombinant proteins can often be improved by fusing them to a highly soluble carrier protein [16,18,20,23,19,27]. Yet, not all highly soluble proteins are equally effective solubility enhancers [12]. Solubility-enhancing proteins evidently must be highly

soluble, but this property does not entirely account for their ability to promote the solubility of their fusion partners.

It is still uncertain how solubility-enhancing proteins work. Several models have been put forth. One model posits that solubility enhancers exert their effects by forming large micelle-like aggregates with incompletely folded passenger proteins held inside, away from the solvent. Indeed, evidence for large soluble aggregates has been observed in some cases [17,21]. Alternatively, solubility enhancers may function as general molecular chaperones in the context of a fusion protein [12] or as “chaperone magnets” [7]. Irrespective of the mechanism, it is clear that at least some recombinant proteins that would normally accumulate in the form of insoluble aggregates can be recovered in a properly folded conformation after fusing them to a solubility-enhancing

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protein (e.g., [12,2,17,1,24,11]). Consequently, the use of solubility-enhancing fusion partners is an attractive alternative to refolding.

Although it has been firmly established that certain highly soluble proteins can function as general solubility enhancers in the context of a fusion protein, virtually nothing is known about how these proteins compare in terms of their ability to promote the proper folding of their fusion partners. It is possible that solubility enhancers differ markedly in their ability to promote the folding of their fusion partners, with some consistently outperforming others. This would imply that a solubility enhancer plays an active role in the folding of its passenger proteins. Alternatively, the folding efficiency may depend primarily on the passenger protein rather than the solubility enhancer, which would be indicative of a more passive role for the latter. A third possibility is that neither of these trends will hold, and that multiple solubility enhancers will have to be tested to find the optimal partner for each passenger protein.

Although a number of proteins have been touted as solubility enhancers [12,16,23,6,27,4,28], the most effective and thoroughly validated ones are MBP and NusA [12,8,4]. The structures, functions, and biochemical properties of these two proteins are quite different. Hence, it seemed appropriate to begin by comparing the ability of these two proteins to promote the proper folding of their fusion partners.

## Results

### *Construction of MBP and NusA fusion protein expression vectors*

There are two objectives in this study. First, we wanted to compare the ability of MBP and NusA to promote the solubility of a variety of aggregation-prone fusion partners. Although the results of some side-by-side comparisons between MBP and NusA have already been reported [10,24], these studies were flawed because neither the lengths nor the amino acid sequences of the linkers that tethered the solubility enhancers to the passenger proteins were the same. Second, and perhaps more importantly, we wanted to compare the efficiency with which MBP and NusA promote the proper folding of their fusion partners. The passenger proteins, we selected for these experiments, were green fluorescent protein (GFP), glyceraldehyde 3-phosphate dehydrogenase (G3PDH), dihydrofolate reductase (DHFR), rhodanese, luciferase, tissue inhibitor of metalloproteinases-1 (TIMP), YopN, YopJ, YopT, YscK, YscL, and YscO. GFP, G3PDH, DHFR, rhodanese, and luciferase all have biological activities that can be measured and have previously been produced as MBP fusion proteins [8]. The remaining proteins originate from *Yersinia pestis* and are currently the targets for structure

determination by X-ray crystallography. YopN is a negative regulator of type III secretion in *Y. pestis*. YopJ and YopT are cysteine proteases. YscK, YscL, and YscO are either cytosolic or peripheral membrane components of the type III secretion apparatus. These *Y. pestis* proteins were selected primarily because they are only partially soluble as MBP fusion proteins, thereby facilitating a rigorous comparison between the solubility-enhancing activity of NusA and MBP. We included a mixture of passengers that are soluble after they are cleaved from MBP and those that are not, reasoning that this would be the best way to detect differences between the two solubility enhancers if they exist. The NusA and MBP fusion protein expression vectors were assembled by Gateway recombinational cloning to ensure that the interdomain linkers would be identical in all the fusion proteins. A canonical recognition site for tobacco etch virus (TEV) protease (ENLYFQG) was included in all the linkers. Previous experiments established that the attB1-derived polypeptide linker sequence (ITSLYKKAGS) and the TEV protease recognition site do not impede the ability of MBP to promote the solubility of its fusion partners (unpublished observations).

### *Solubility of the MBP and NusA fusion proteins*

We began by comparing the solubility of the MBP and NusA fusion proteins. *E. coli* BL21Pro cells (BD Biosciences Clontech, Palo Alto, CA, USA), containing plasmid expression vectors encoding otherwise identical MBP or NusA fusion proteins under transcriptional control of the *tac* promoter, were grown to mid-log phase and then induced with IPTG for 4 h at 30 °C. The cells were lysed by sonication, after which a sample of total intracellular protein was collected for SDS-PAGE analysis. The sonicated cell suspension was then centrifuged at high speed to pellet insoluble material and a sample of the supernatant, representing the soluble intracellular protein, was also collected for SDS-PAGE analysis. Polyacrylamide gels were stained with Coomassie Brilliant Blue and then scanned with a densitometer to estimate the fraction of each fusion protein that was soluble. Representative SDS-PAGE results for the DHFR, YscO, YopN, and GFP fusion proteins are shown in Figs. 1 and 2. The data for all the fusion proteins are summarized in Table 1.

In general, little difference was observed between the solubility of the MBP fusion proteins and their NusA counterparts. G3PDH was slightly more soluble as an MBP fusion protein, whereas YscL was slightly more soluble as a NusA fusion protein. The greatest difference involved the YscK fusion proteins, where the NusA fusion protein exhibited significantly greater solubility. Overall, however, there were far more similarities than differences between the performance of these two solubility enhancers.

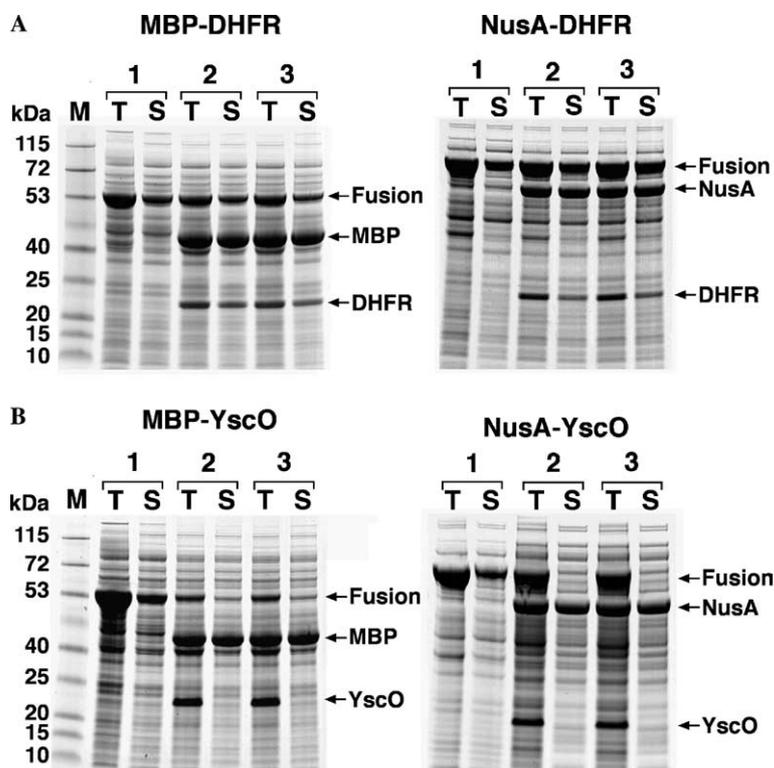


Fig. 1. Examples of intracellular processing of MBP and NusA fusion proteins by TEV protease. (A) MBP–DHFR (left) and NusA–DHFR (right) fusion proteins. (B) MBP–YscO (left) and NusA–YscO (right) fusion proteins. Samples of the total (T) and soluble (S) intracellular protein from BL21Pro cells containing the fusion protein expression vector alone (1) or the fusion protein expression vector and the TEV protease expression vector pRK603 (2 and 3) were prepared as described in Materials and methods and analyzed by SDS–PAGE. All cultures were induced with IPTG at mid-log phase. Cells containing pRK603 were also induced with anhydrotetracycline, either at the same time (2) or 2 h after IPTG induction (3). M, broad-range molecular weight markers (Invitrogen).

#### *Solubility of passenger proteins after release from MBP or NusA*

We have observed that the solubility of a passenger protein after intracellular processing of an MBP fusion protein by TEV protease is a good indicator of its folding status; folded proteins tend to remain soluble, whereas incompletely or improperly folded proteins precipitate when they are cleaved from MBP [13,9]. Therefore, we used this assay to determine, to a first approximation, whether there are any major differences between the ability of MBP and NusA to promote the proper folding of their fusion partners. Representative SDS–PAGE results for the DHFR, YscO, YopN, and GFP fusion proteins are shown in Figs. 1 and 2. The data for all of the fusion proteins are summarized in Table 1.

When the production of the MBP and NusA fusion proteins was induced by IPTG in BL21Pro cells constitutively expressing TEV protease, all the cleaved passenger proteins except DHFR precipitated. The fraction of DHFR that remained soluble after intracellular processing was very similar for the MBP and NusA fusion proteins. Hence, no difference between the ability of MBP and NusA to promote the folding of their fusion partners was detected by this method.

The solubility of cleaved passenger proteins can often be improved by delaying the induction of TEV protease until after the fusion proteins have had an opportunity to accumulate in the cells [15,9]. Accordingly, we next repeated the intracellular processing experiments using a delayed induction protocol. The cells were induced with IPTG when they reached mid-log phase, as usual. However, we waited for two more hours before inducing the production of TEV protease by the addition of anhydrotetracycline. Two hours later, the cells were harvested and samples of the total and intracellular protein were collected and analyzed as described above. Representative SDS–PAGE results for the DHFR, YscO, YopN, and GFP fusion proteins are shown in Figs. 1 and 2. The data for all the fusion proteins are summarized in Table 1.

Several of the passenger proteins showed a substantial improvement in solubility when they were cleaved from MBP or NusA using the delayed induction protocol. These included YopN and GFP (Fig. 2). However, once again the results obtained for MBP and NusA were very similar. YopN and TIMP were slightly more soluble after being released from NusA than from MBP, whereas the opposite was true for G3PDH and YopJ. Hence, the folding efficiency (as assessed indirectly by solubility) appears to depend primarily on the passenger protein rather than the solubility enhancer.

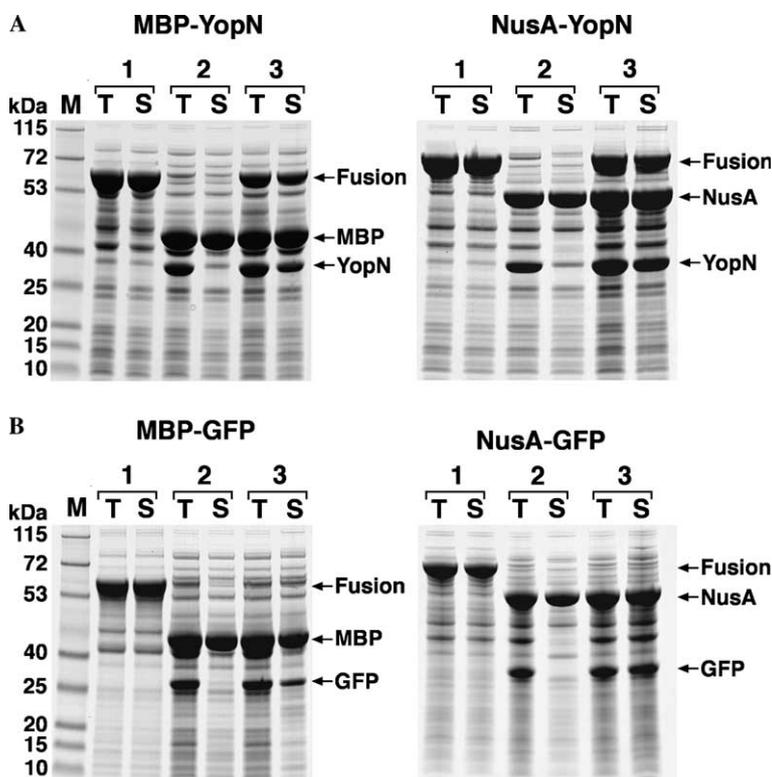


Fig. 2. The solubility of GFP and YopN is affected by the duration of their association with MBP and NusA. (A) MBP–YopN (left) and NusA–YopN (right) fusion proteins. (B) MBP–GFP (left) and NusA–GFP (right) fusion proteins. Samples of the total (T) and soluble (S) intracellular protein from BL21Pro cells containing the fusion protein expression vector alone (1) or the fusion protein expression vector and the TEV protease expression vector pRK603 (2 and 3) were prepared as described in Materials and methods and analyzed by SDS–PAGE. All cultures were induced with IPTG at mid-log phase. Cells containing pRK603 were also induced with anhydrotetracycline, either at the same time (2) or 2 h after IPTG induction (3). *M*, broad-range molecular weight markers (Invitrogen).

Table 1  
Solubility of fusion proteins and cleaved passenger proteins

Passenger	MBP	NusA	Constitutive induction <sup>a</sup>		Delayed induction <sup>b</sup>	
			MBP+TEV	NusA+TEV	MBP+TEV	NusA+TEV
YopN	++++	++++	–	–	++	+++
TIMP	++++	++++	–	–	–	+
GFP	++++	++++	–	–	+++	+++
G3PDH	++	+	–	–	+	–
DHFR	++	++	++	++	++	++
Rhodanese	+	+	–	–	+	+
Luciferase	+	+	–	–	–	–
YopT	+	+	–	–	–	–
YopJ	+	+	–	–	+	–
YscK	+	+++	–	–	–	–
YscL	+	++	–	–	–	–
YscO	+	+	–	–	–	–

The approximate percentage of soluble proteins were estimated as described in Materials and methods, and are reported here as being within the following ranges: +++++, 75–100% soluble; +++, 50–75% soluble; ++, 25–50% soluble; +, 0–25% soluble; –, insoluble.

<sup>a</sup> The expression of TEV protease was induced at the same time as the expression of the fusion protein substrates. The solubility of the cleaved passenger proteins (not the fusion proteins) is reported.

<sup>b</sup> The expression of TEV protease was induced 2 h after the expression of the fusion protein substrates was induced with IPTG. The solubility of the cleaved passenger proteins (not the fusion proteins) is reported.

#### Quantitative evaluation of folding efficiency

Occasionally, a passenger protein may accumulate in a soluble but biologically inactive form after intracellular processing. Exactly how and why this occurs are

unclear, but it is possible that fusion to MBP or NusA may enable certain properties to evolve into kinetically trapped, folding intermediates that are no longer susceptible to aggregation. Therefore, although the solubility after intracellular processing is a useful indicator of

passenger protein's folding state in most cases, it is not absolutely trustworthy.

Two of the passenger proteins that exhibited at least moderate solubility after intracellular processing of the fusion proteins by TEV protease, GFP and DHFR, have biological activities that can be measured. We, therefore, sought to perform a more quantitative assessment of folding efficiency in these cases. To this end, the fluorescence intensity of soluble extracts prepared from an equal number of cells producing MBP–GFP or NusA–GFP fusion proteins was compared. These two fusion proteins accumulate to comparable levels in *E. coli* (Fig. 2B). Whether in the presence or absence of IPTG, the fluorescence intensities were very similar, indicating that there was no substantial difference in the amount of properly folded GFP fused to MBP or NusA (Fig. 3).

We took this one step further in the case of the DHFR fusion proteins. The fusion proteins were purified by immobilized metal affinity chromatography, taking an advantage of the hexahistidine tag on the C-terminus of DHFR (Fig. 4A), and their enzymatic activities were compared in vitro (Fig. 4B). Both the fusion proteins exhibited readily detectable DHFR activity in a spectrophotometric assay, and the specific activity of the two fusion proteins was indistinguishable.

Finally, we compared the ability of MBP and NusA to promote the proper folding of TEV protease. As reported previously, TEV protease is insoluble when it is produced in *E. coli* with an N-terminal polyhistidine tag [12]. However, if the polyhistidine-tagged TEV protease is fused to MBP, a substantial amount of soluble fusion protein is produced. When a TEV protease recognition site is added to the interdomain linker region in the MBP–His<sub>6</sub>–TEV fusion protein, all the fusion proteins are processed intracellularly to yield separate MBP and His<sub>6</sub>–TEV domains. Strikingly, nearly all the His<sub>6</sub>–TEV protease produced in this manner is soluble in the crude cell extract and catalytically active, demonstrating that it

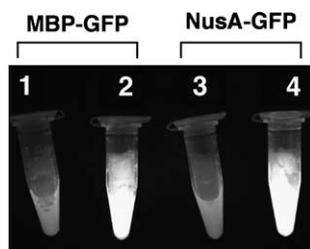


Fig. 3. MBP–GFP and NusA–GFP fusion proteins exhibit comparable fluorescence intensity. BL21Pro cells containing either the MBP–GFP (1 and 2) or NusA–GFP (3 and 4) fusion protein expression vector were grown to mid-log phase at 37 °C and then induced for 4 h with IPTG. Samples of the soluble intracellular protein were prepared from uninduced (1 and 3) and induced (2 and 4) cultures at the same optical density, as described in Materials and methods, and then illuminated with a long wave ultraviolet lamp.

is properly folded [12,14]. The latter result is recapitulated in the experiment shown in Fig. 5, in which the solubility of His<sub>6</sub>–TEV protease is compared after autoproteolytic cleavage from MBP and NusA. It can be

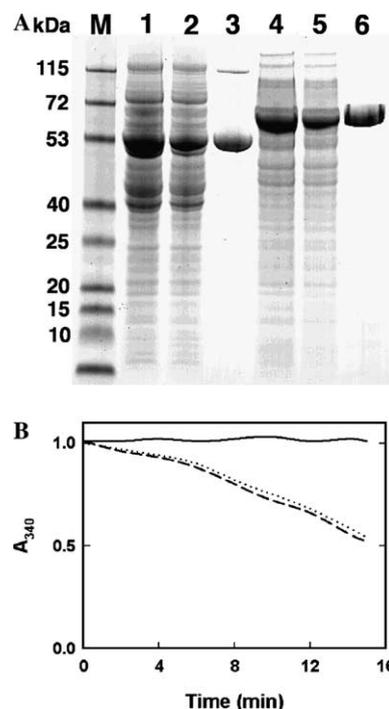


Fig. 4. The MBP–DHFR–His<sub>6</sub> and NusA–DHFR–His<sub>6</sub> fusion proteins have comparable enzymatic activity. (A) Purification of the fusion proteins by immobilized metal affinity chromatography. Coomassie-stained SDS gel (4–12% Nupage) showing samples of lanes: M, broad-range molecular weight standards (kDa); 1, soluble fraction of the MBP–DHFR intracellular protein; 2, flow-through from the Ni–NTA column; 3, eluted protein; 4–6, soluble fraction of the NusA–DHFR intracellular protein; flow-through from the Ni–NTA column; and eluted protein, respectively. (B) Enzymatic activity of the fusion proteins. (–), No enzyme; (—), MBP–DHFR–His<sub>6</sub>; (·····), NusA–DHFR–His<sub>6</sub>.

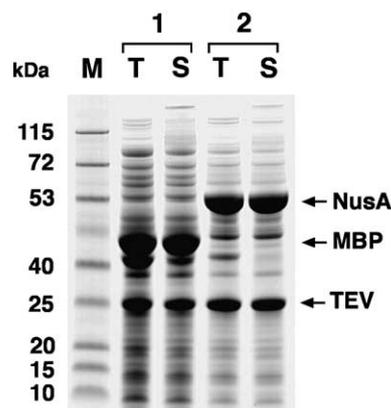


Fig. 5. Both MBP and NusA are capable of promoting the proper folding of TEV protease. Samples of the total (T) and soluble (S) intracellular protein were prepared from *E. coli* BL21Pro cells containing either the MBP–TEV (1) or NusA–TEV (2) expression vectors as described in Materials and methods and then analyzed by SDS–PAGE.

seen that His<sub>6</sub>-TEV protease is also highly soluble after being released from NusA. Moreover, the catalytic activity of His<sub>6</sub>-TEV protease purified after autoprocessing of the NusA fusion protein is indistinguishable from that of His<sub>6</sub>-TEV protease obtained after autoprocessing of the MBP fusion protein (data not shown). Hence, both the solubility enhancers are capable of promoting the proper folding of TEV protease with high efficiency.

## Discussion

Although originally developed to facilitate the detection and purification of recombinant proteins, in recent years it has become clear that affinity tags can have additional benefits. One of these is the ability of some tags to enhance the solubility of their fusion partners. Two of the best studied and well-validated solubility enhancers are MBP and NusA. In the present study, we performed a head-to-head comparison of these two solubility enhancers under rigorously controlled experimental conditions to determine whether there is any substantive difference between them. Our results indicate that, in general, MBP and NusA behave very similarly in terms of their ability to promote the solubility of a diverse set of aggregation-prone passenger proteins. The systematic disparities reported in previous comparisons [10,24] may therefore be due to differences in the lengths and/or sequences of the interdomain linkers in those studies. We also observed minor differences between MBP and NusA in some cases; however, we cannot rule out the possibility that even more substantive differences might turn up in a larger set of passenger proteins. For this reason, we feel it would still be worthwhile trying NusA when an MBP fusion protein exhibits poor solubility and vice versa.

The efficiency of folding, as judged by solubility after release from the solubility-enhancing partner *in vivo*, and, in some cases, by quantitative activity measurements, appears to depend on the passenger protein rather than the solubility enhancer. This implies that the solubility enhancers play a passive role rather than an active one in the folding of their fusion partners and that MBP and NusA probably work by similar mechanisms (see [7] for a detailed discussion of possible mechanisms). Although the experiments reported here do not establish what fraction of each passenger protein was properly folded, the important point is that it was the same fraction, irrespective of which solubility enhancer was used. The structures, physicochemical properties, and biological functions of MBP and NusA are quite different, and yet they perform very similarly as solubility enhancers. This suggests that there may be little to gain by identifying and characterizing additional solubility-enhancing fusion partners unless they operate by an entirely different mechanism or have another distinct advantage. For

instance, one reason to choose MBP instead of NusA is that the former protein is also a natural affinity tag and can therefore be utilized to facilitate the purification of its fusion partners.

## Materials and methods

### Gateway destination vectors

Gateway recombinational cloning (Invitrogen, Carlsbad, CA, USA) was used to facilitate the construction of fusion protein expression vectors. The *E. coli* MBP destination vector, pKM596, was described previously [9]. The NusA destination vector (pDEST543) was obtained from Protein Expression Laboratory, SAIC, Frederick. However, NusA is under transcriptional control of a bacteriophage T7 promoter in pDEST543, whereas MBP is under *tac* promoter control in pKM596. Therefore, a derivative of pDEST543, in which the T7 promoter was replaced by the *tac* promoter (pSN1542), was constructed in the following manner. The *tac* promoter region of pMal-C2x (New England Biolabs, Beverly, MA, USA) was amplified by PCR with primers PE-1526 (5'-GAC CCT CCG CAT GCG ACA GCT TAT CAT CGA CTG CCA-3') and PE-1527 (5'-GAC TTC AGC GAG ACC GTT ATA GCC-3'). The resulting PCR amplicon was cleaved with *Sph*I and *Nde*I, and then inserted between the unique *Sph*I and *Nde*I sites in pDEST543. The nucleotide sequence of the insert was confirmed experimentally.

### Gateway entry clones

To construct Gateway entry clones, the open reading frames (ORFs) encoding each passenger protein were amplified by PCR, using a pair of gene-specific primers with 5' extensions that added an in-frame TEV protease recognition site and a hexahistidine tag to their N- and C-termini, respectively. Next, these PCR amplicons were used as the templates for another PCR with primers PE-277 and PE-278 [5], which are designed to anneal to the sequences encoding the TEV protease recognition site and the His-tag, respectively, and add *att*B1 and *att*B2 recombination sites to the ends of the amplicon. The final PCR amplicons were inserted into pDONR201 (Invitrogen) by recombinational cloning to generate the entry clones. Entry clones encoding rhodanese, luciferase, G3PDH, DHFR, and GFP were described previously [8]. A synthetic gene was the starting point for the construction of the TIMP entry clone. The plasmid pRK793 [14] was the starting point for the construction of the TEV protease entry clone. YopN, YopJ, YopT, YscK, YscL, and YscO were amplified from *Y. pestis* genomic DNA. The nucleotide sequences of all ORFs were verified experimentally.

### Fusion protein expression vectors

The MBP fusion protein expression vectors were constructed by recombining each passenger protein ORF from its entry clone into the MBP destination vector pKM596, using the standard L × R protocol (Invitrogen). The NusA fusion protein expression vectors were constructed in a similar manner, using the destination vector pSN1542. All the fusion proteins had an identical interdomain linker sequence consisting of the translation product of the *attB1* recombination site followed by a TEV protease recognition site (ITSLYKKAGSENLYFQ G).

### Comparison between the solubility of MBP and NusA fusion proteins

*Escherichia coli* BL21Pro cells (BD Biosciences Clontech) containing an MBP or NusA fusion protein expression vector were grown to saturation in Luria broth supplemented with 100 µg/ml ampicillin at 37 °C. The saturated culture was diluted (1:50) in the same medium and grown to mid-log phase ( $A_{600\text{nm}} = 0.4\text{--}0.5$ ) at 37 °C, at which time the temperature was shifted to 30 °C and IPTG was added to a final concentration of 1 mM to initiate production of the fusion protein. After 4 h at 30 °C, the cells from 10 ml of each culture were recovered by centrifugation and resuspended in 1 ml of 50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 200 mM NaCl. The cell suspensions were lysed by sonication, after which aliquots of the cell lysates were mixed with an equal volume of 2× SDS sample buffer [22] to produce samples of the total intracellular protein for SDS–PAGE. The disrupted cell suspensions were then centrifuged at 14,000g for 10 min to pellet the insoluble material. Aliquots of the supernatant fractions were removed and mixed with an equal volume of 2× SDS sample buffer to produce samples of the soluble intracellular protein for SDS–PAGE. All samples were heated at 90 °C for 2 min and then centrifuged at 14,000g for 5 min prior to SDS–PAGE. Samples were analyzed on 4–12% Bis–Tris NuPage gels (Invitrogen) and visualized by staining with Coomassie brilliant blue. Coomassie-stained gels were scanned with a Molecular Dynamics Personal Densitometer and the pixel densities of the bands corresponding to the fusion proteins or the cleaved passenger proteins were obtained directly by volumetric integration. The percentage of soluble protein was estimated by dividing the soluble amount by the total amount after subtracting normalized background values obtained from the pixel densities of bands corresponding to endogenous proteins.

### Intracellular processing of fusion proteins by TEV protease

*Escherichia coli* BL21Pro cells (BD Biosciences Clontech) containing an MBP or NusA fusion protein

expression vector and pRK603, a TEV protease expression vector [13], were grown to saturation in Luria broth supplemented with 100 µg/ml ampicillin and 30 µg/ml kanamycin at 37 °C. The saturated culture was diluted in the same medium (1:50) and grown to mid-log phase ( $A_{600\text{nm}} = 0.4\text{--}0.5$ ) at 37 °C, at which time the temperature was shifted to 30 °C and IPTG was added to a final concentration of 1 mM to initiate the production of the fusion protein. In addition, to induce the production of TEV protease, anhydrotetracycline was added to a final concentration of 100 ng/ml either at the same time as the addition of IPTG or 2 h later. Four hours after the addition of IPTG, samples of the total and soluble intracellular protein were prepared and analyzed by SDS–PAGE and densitometry as described above.

### Overproduction and purification of DHFR fusion proteins

*Escherichia coli* BL21(DE3) cells (Novagen, Madison, WI, USA) containing the MBP–DHFR fusion protein expression vector were grown to saturation at 37 °C in Luria broth supplemented with 100 µg/ml ampicillin. The saturated culture was diluted in 1 L of the same medium (1:50) and grown in a shake-flask to mid-log phase ( $A_{600\text{nm}} = 0.4\text{--}0.5$ ). At this point, IPTG was added to a final concentration of 1 mM to initiate production of the MBP–DHFR fusion protein and the culture was grown for an additional 4 h at 30 °C. The cells were then recovered by centrifugation and the cell pellet was stored at –80 °C.

*Escherichia coli* cell paste was suspended in ice-cold 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 25 mM imidazole (buffer A). The cells were lysed with an APV Gaulin Model G1000 homogenizer at 10,000 psi and centrifuged at 30,000g for 30 min at 4 °C. The supernatant was filtered through a 0.44-µm polyethersulfone membrane and applied to a 25-ml Ni–NTA Superflow affinity column (Qiagen, Valencia, CA, USA) equilibrated in buffer A. The column was washed with 10 column volumes of buffer A and then eluted with a linear gradient from 25 to 250 mM imidazole in buffer A. Fractions containing recombinant MBP–DHFR were pooled and the sample was concentrated using an Amicon YM10 membrane (Millipore, Billerica, MA, USA). Aliquots were flash-frozen with liquid nitrogen and stored at –80 °C until use. The NusA–DHFR fusion protein was overproduced and purified in exactly the same manner.

### Spectrophotometric assay of dihydrofolate reductase activity

The DHFR enzyme assay was conducted in a quartz cuvette with 1 cm path length according to the method of Viitanen et al. [26]. Enzyme activity was assayed in the direction of NADPH oxidation in the presence of dihydrofolate. Oxidation of NADPH was monitored at

340 nm using a Beckman DU 640 spectrophotometer. The reactions (final volume, 600  $\mu$ l) were performed in 1.5-ml microcentrifuge tubes containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 3.3 mM KCl, 10 mM dithiothreitol, 0.1 mM dihydrofolate, and 0.1 mM NADPH. The reactions were initiated by the rapid addition of protein (MBP-DHFR-His<sub>6</sub> or NusA-DHFR-His<sub>6</sub>) to a final concentration of 0.4  $\mu$ M. After vigorous mixing, the mixtures were immediately transferred to cuvettes and the  $A_{340\text{nm}}$  was monitored as a function of time. The starting  $A_{340\text{nm}}$  reading was normalized to a value of 1.0.

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