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Folding and aggregation of export-defective mutants of the maltose-binding protein

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Abstract

We previously characterized a defective-folding variant of the periplasmic maltose-binding protein, MalE31. To examine the alternative folding pathways open to the MalE31 precursor, we have analyzed the cellular fates of this aggregation-prone protein carrying altered signal sequences. Our results are most easily interpreted by a kinetic competition between exportation, folding, and degradation. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Heat-shock proteins; Maltose; Protein folding; Protein precursors

1. Introduction

Exported proteins with an ultimate destination of the periplasm or outer membrane in *Escherichia coli* are synthesized as precursor proteins with an amino-terminal signal sequence. Interaction with cellular chaperones maintains these precursor proteins in a translocation-competent conformation until they are transported through the inner membrane by the translocation machinery [9]. As the precursor proteins enter the periplasm, the signal sequence is removed by signal peptidase, an integral membrane protein with the active site facing the periplasm. After translocation and signal sequence cleavage, the newly exported mature proteins are subsequently sorted, folded and assembled in the periplasm and outer membrane [20]. In contrast to the folding and targeting of nascent precursors emerging from the ribosome, little is known about these processes after release from the translocation machinery in the periplasm. Recently, studies of outer membrane protein biogenesis have permitted characterization of two regulatory pathways, the σ^E regulatory system and the CpxRA two-component signal transduction pathway, which are both activated by the presence of misfolded proteins in the periplasm [22].

To study the competitive processes between export, folding and aggregation, we use a defective folding mutant of the maltose-binding protein MalE31, the periplasmic receptor for the high affinity transport of maltose. This variant carries a double substitution G32D, I33P in a turn of the N-domain [4]. In vitro, unfolding–refolding experiments showed that a folding intermediate of MalE31 is kinetically trapped in an off-pathway folding reaction leading to its aggregation [21]. In vivo, the MalE31 precursor was correctly exported based on the kinetics of signal sequence processing, but the defective folding of the mature protein in the periplasm led, at high levels of production, to the formation of inclusion bodies [4]. At low levels of production, the misfolded MalE31 polypeptide chain is completely degraded by DegP, a heat-shock periplasmic protease [14,24]. A kinetic competition between folding, aggregation and degradation was proposed to explain the different fates of newly translocated proteins in the bacterial periplasm [6]. One major physiological consequence for the cells overproducing MalE31 was an increase in the σ^E-dependent response but not that of the general heat shock sigma factor, σ^{32} [3]. These results indicated that *E. coli* senses the presence of misfolded MalE31 in the periplasm and responds to it in a manner that is distinct from the heat shock response induced by protein misfolding in the cytoplasm.

In this study, we have examined the expression of MalE31 carrying altered signal sequences to determine the consequences of a decreased export efficiency on both

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MalE31 folding and on its ability to induce a cellular stress response.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli K12 strain pop6499 (F^- *araD139* Δ*lacU169 rpsL150 relA1 flbB5301* Δ(*srlR-recA*)*306*::Tn*10 deoC1 ptsF25 rbsR* Δ*malE444 malT*^{c}) was used as a host strain for plasmids encoding the various MalE proteins [4]. This strain carries a nonpolar deletion of the chromosomal *malE* gene [10] and harbors the *malT*^{c} allele which confers constitutive expression on the maltose regulons. Expression of P*lon*::*lacZ* and P*degP*::*lacZ* fusions was monitored in strains SR1364 and SR1458, respectively [3]. Plasmids p1H and p31H carrying the wild-type *malE* and *malE31* genes, both under the control of their natural promoters, were previously described [3]. Plasmid pΔ709, which carries a deletion of the ribosome binding site and the first 256 codons of the *malE* gene [5], was used as a negative control.

2.2. Site-directed mutagenesis

Signal peptide mutations were constructed by oligonucleotide mutagenesis with single-stranded p1H and p31H DNA containing uracil as the template for primer extension and ligation [13]. The mutagenic oligonucleotides used for the deletion of the signal peptide (ΔSS) and for creating the M-18R (M-18) substitution were, respectively: 5′-TACCTTCTTCGATTTTCATAATCTATGG-3′, 5′-GGAAAACCTCATCGTC-3′. Both mutations were confirmed by dideoxynucleotide sequencing, and subcloned into parental nonmutagenized p1H and p31H plasmids.

2.3. Subcellular fractionation of MalE

Cultures were grown in LB medium supplemented with 0.1 mg/ml ampicillin at 30 °C. Cells were harvested at $A_{600} = 1.5$, and fractionated by spheroplast preparation as previously described [3]. Cell pellets, normalized to the same A_{600}, were resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M sucrose. After adding lysozyme (0.2 mg/ml) and EDTA (10 mM), the suspensions were incubated for 15 min at 4 °C. Then, the samples were centrifuged for 5 min at 14 000 rpm, and supernatants containing the periplasmic fractions were withdrawn. The spheroplast pellets were washed, freeze-thawed, sonicated and centrifuged at 14 000 rpm for 15 min. Supernatants were collected to obtain the cytoplasmic fractions. Pellets were washed with Tris-HCl buffer containing 0.1% (v/v) Triton and resuspended in Tris-HCl buffer to obtain the membrane fractions. Whole cell extracts and the three subcellular fractions were each mixed with SDS-PAGE sample buffer, boiled, and analyzed on 12.5% SDS-polyacrylamide gels followed by Coomassie blue staining. For quantitative analyses, gels were scanned with an ImageMaster VDS camera (Amersham Biotech). MalE proteins were visualized by immunodetection using an antibody raised against MalE as a primary antibody and an alkaline phosphatase-conjugated secondary antibody, as described previously [21].

2.4. β-galactosidase assays

E. coli SR1364 or SR1458 strains, carrying the various plasmids, were grown in LB medium supplemented with 0.2% maltose and 0.1 mg/ml ampicillin at 30 °C. After 1.5 h of induction, cells were harvested and β-galactosidase activity in Miller units was determined as described previously [16]. A minimum of four independent assays were averaged to obtain the indicated activities.

3. Results

Our previous studies indicated that the MalE31 precursor was rapidly and efficiently exported to the periplasm. This conclusion was based on the findings that the kinetics of MalE31 maturation was similar to that of the wild type, and that overexpression of MalE31 could induce a stress response mediated not by the classical heat-shock sigma factor, σ^{32}, but by the alternative heat-shock sigma factor σ^E, specific to extracytoplasmic stress [3,17]. However, if an alteration in the signal sequence of the precursor decreases the efficiency of export, a significant proportion of the precursor could accumulate inside the cytoplasm.

3.1. Export, folding and aggregation of MalE variants

In order to examine the kinetic partition between export, folding and aggregation, MalE and MalE31 carrying either a complete deletion of their signal sequence (MalEΔSS and MalE31ΔSS) or a single mutation at position −18 in the signal sequence (MalEM-18 and MalE31M-18) were constructed. The latter corresponds to the original *malE18-1* mutation for which an arginine is substituted for methionine at residue −18 in the signal sequence [2]. Manson et al. [15] showed that 4% of the normal periplasmic amount of mature MalE could be found in the strain carrying the original signal sequence mutation *malE18-1*.

The steady-state production level of each variant was first analyzed by SDS-polyacrylamide gel electrophoresis to determine whether the signal sequence mutations could affect the amount of MalE found in whole cell extracts (Fig. 1A). All variants except one (MalE31 expressed from p31M-18) were detected at a yield comparable to that of the wild-type MalE expressed either from p1H or from p1ΔSS (Table 1). In the case of MalE31 expressed with the export-defective signal sequence, the protein was produced at a low level. Furthermore, its electrophoretic mobility was identical to that of the elongation factor protein EF-Tu, the

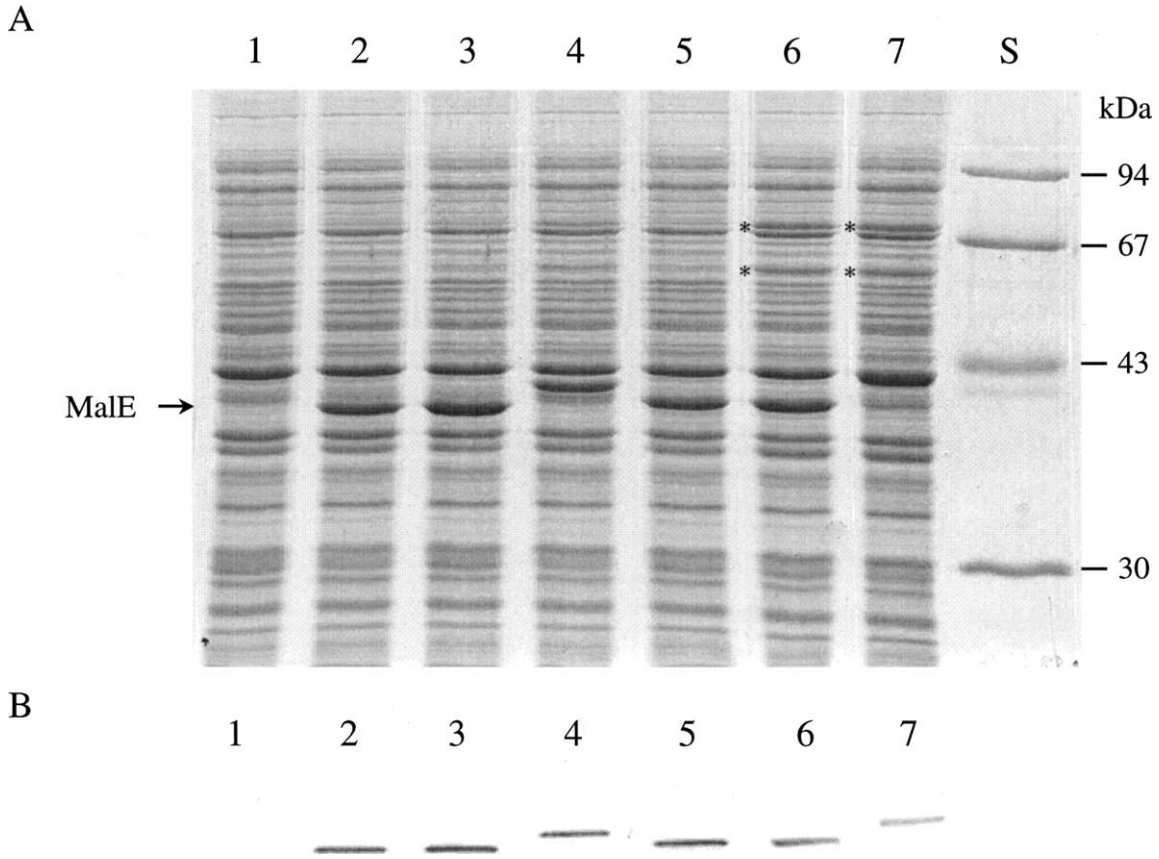

Fig. 1. Expression of MalE variants. A. Whole cell extracts were separated by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide), corresponding to 10^8 bacteria. The gel was stained with Coomassie blue. Lanes: (1) pΔ709, (2) p1H, (3) p1ΔSS, (4) p1M-18, (5) p31H, (6) p31ΔSS, (7) p31M-18. The band corresponding to MalE is indicated by an arrow, and those corresponding to GroEL and DnaK by asterisks. B. Immunoblot analysis of whole cell extracts from 10-fold diluted samples was performed using a specific anti-MalE antibody.

Table 1
Relative expression levels of MalE variants

Plasmid	Protein	Signal sequence	SDS-PAGE scan (%)	Immunoblot scan (%)
p1H	MalE	wild-type	80	85
p1ΔSS	MalE	no	100	100
p1M-18	MalE	M-18R	75	70
p31H	MalE31	wild-type	85	90
p31ΔSS	MalE31	no	80	70
p31M-18	MalE31	M-18R	20[a]	25

[a] This value was obtained by subtracting the area of EF-Tu band determined for the corresponding lane in the gel shown in Fig. 1A from the mean value of EF-Tu bands determined from the other lanes.

predominant protein of *E. coli*. Thus, its quantification was rendered less accurate. Nevertheless, the apparent molecular weight of MalE or MalE31 expressed from p1M-18 or p31M-18 indicates that this export-defective signal sequence of both proteins is not processed, in good agreement with previous kinetics studies of export [1]. Densitometry of an immunoblot of duplicate gel, loaded with 10-fold diluted samples, with a specific anti-MalE antibody gave similar results to those from the Coomassie blue stained gel (Fig. 1B and Table 1). Close inspection of the gel shown in Fig. 1A revealed the presence of increased amounts of two bands of about 60 and 70 kDa, in the lanes corresponding to p31ΔSS and p31M-18R. These bands represent the major heat-shock proteins GroEL and DnaK (see below).

The formation of inclusion bodies, which is a consequence of a folding defect, can be biochemically determined by soluble/insoluble fractionation based on separation by centrifugation of native lysates [12]. Therefore, we determined the cellular location of the different MalE variants after spheroplast preparation (Fig. 2). The periplasmic and cytoplasmic location (both fractions define a soluble fraction) of wild-type MalE, expressed from p1H and p1ΔSS, confirmed that our subcellular fractionation procedure was correct. MalE31 expressed with a wild-type signal sequence (from p31H) is found entirely in the membrane or insoluble fraction as expected for aggregated proteins [12]. We previously demonstrated by flotation gradient centrifugation that aggregated MalE31 proteins form periplasmic inclusion bodies which co-sediment with membrane vesicles rather than membrane-associated proteins [3]. In contrast, MalE31 expressed either without a signal sequence (from p31ΔSS) or with an altered signal sequence (from p31M-18) displays different soluble/insoluble partitions.

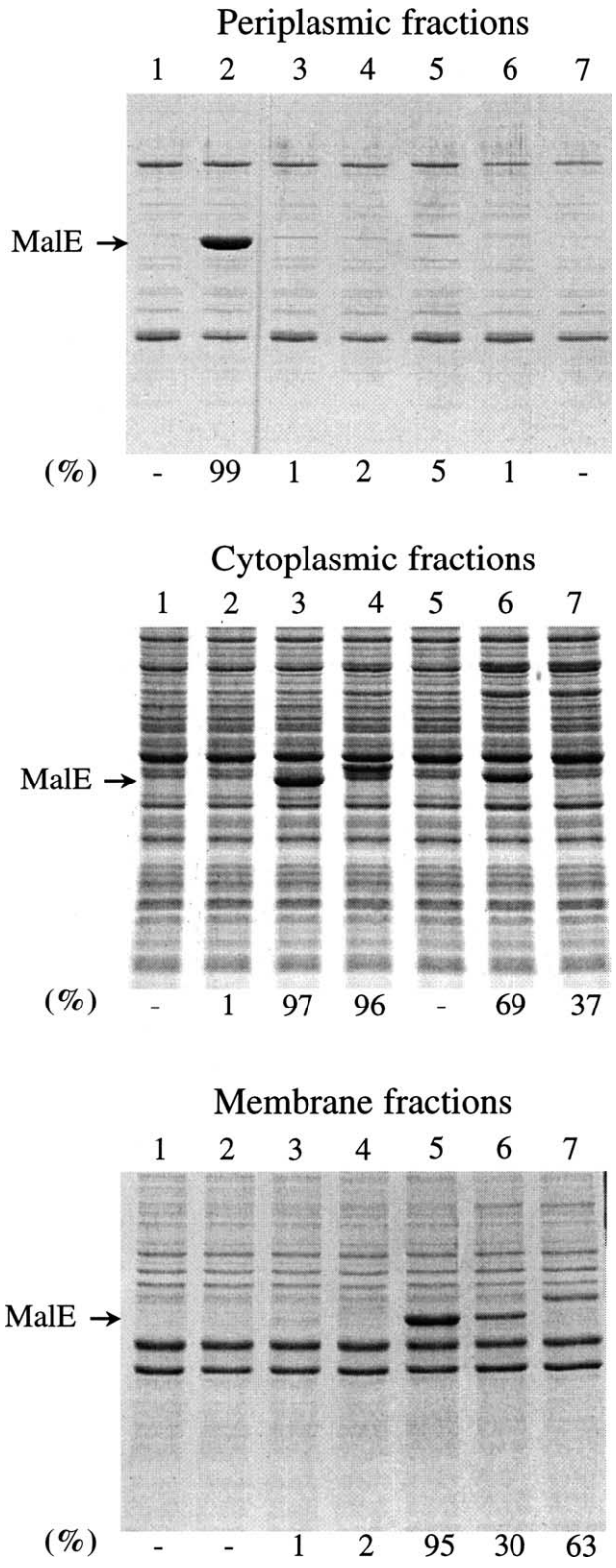

Fig. 2. Subcellular fractionation of MalE variants. Periplasmic, cytoplasmic, and membrane fractions were separated by SDS-polyacrylamide gel electrophoresis, and Coomassie stained bands were scanned by densitometry. Lanes: (1) pΔ709, (2) p1H, (3) p1ΔSS, (4) p1M-18, (5) p31H, (6) p31ΔSS, (7) p31M-18. An arrow indicates the band corresponding to MalE. The proportions were calculated by summing the area of the MalE band in the three subcellular fractions and are shown under the corresponding lanes.

3.2. Stress responses induced by expression of MalE variants

From Fig. 1 it is evident that cytoplasmic expression of MalE31 from p31ΔSS or p31M-18R increased the synthesis of GroEL and DnaK. In contrast, no such elevated level of heat-shock proteins was visible with periplasmic expression of MalE31. We quantitatively determined the extent of induction of stress response caused by these variants, using two bacterial strains carrying either a single copy of P*lon*::*lacZ* (SR1364) or P*degP*::*lacZ* (SR1458) transcriptional gene fusions. The *lon* gene encoding a heat-shock cytoplasmic protease is transcribed by the $E\sigma^{32}$ RNA polymerase, whereas *degP* encoding a heat-shock periplasmic protease is transcribed by the $E\sigma^{E}$ RNA polymerase [11]. This gene differs from the former in that its transcription is not only regulated by σ^{E} but also by the CpxRA pathway. The sigma factor σ^{E}, encoded by the *rpoE* gene, is a member of the extracytoplasmic function (ECF) subfamily sigma factors, which function as effectors molecules responding to extracytoplasmic stimuli [18]. The cytoplasmic accumulation of MalE31 expressed from p31ΔSS or p31M-18R is reflected by a more than twofold increase in β-galactosidase synthesis directed from the P*lon* promotor (Fig. 3). Surprisingly, wild-type MalE carrying the export-defective signal sequence significantly induced the *lon* promotor. The periplasmic aggregation of MalE31 from p31H increased by a factor of two the β-galactosidase activity measured from the P*degP*::*lacZ* fusion, confirming our previous data [3]. It is interesting to note that the expression of MalE31 from p31ΔSS or p31M-18R caused a decreased basal level of activity of the *degP* promotor (Fig. 3). This observation suggests that a direct competition between both heat-shock sigma factors may play a role in directing RNA

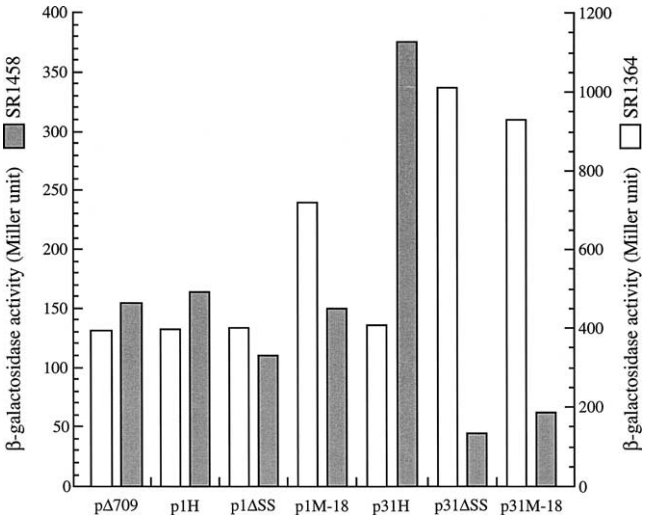

Fig. 3. Stress responses induced by MalE variants. The activities of *lon* and *degP* promoters, both fused to the *lacZ* gene, were assessed in *E. coli* strains SR1364 and SR1458, respectively, each carrying the various plasmids described in Fig. 1. Miller activities of the *lacZ* encoded β-galactosidase are calculated using the average of four independent assays.

polymerase predominantly either to σ^{32}-dependent or σ^{E}-dependent promoters.

4. Discussion

It was previously proposed that there is a kinetic partitioning of MalE precursor between the productive export and the folding pathway [23]. After the precursor has folded, it can no longer enter the export pathway. Since we never observed misfolding of MalE31 when it was expressed with its wild-type signal sequence in the cytoplasm, we concluded that the MalE31 precursor enters efficiently and rapidly into the export pathway. Because MalE31 refolds slowly in vitro [21], the export pathway could have a kinetic advantage over the aggregation pathway. The mutational deletion or substitution in signal sequences of the MalE31 precursors studied here has been shown to strongly decrease the efficiency of MalE export [2]. The presence of these altered signal sequences in combination with the defective-folding *malE31* mutation results in the accumulation of MalE31 precursors. The consequence is a doubly altered MalE31 precursor, leading to strongly reduced rates of export and folding.

In the steady state, the cytoplasmic soluble fraction of MalE31, when expressed from p31ΔSS or p31M-18 (69% and 37%), is higher than the periplasmic soluble fraction of MalE31, when expressed from p31H (5%). This observation suggests that in the former case, the increased level of GroEL and DnaK, resulting from induction of the cytoplasmic stress response by expression of MalE31ΔSS or MalE31M-18, can partially suppress the misfolding pathway of these aggregation-prone proteins. Furthermore, it appears that under these experimental conditions (temperature of growth, expression levels, bacterial strain, etc.), there are insufficient or no classical heat-shock molecular chaperones, such as cytoplasmic DnaK or GroEL, in the periplasm to prevent or reduce aggregation of MalE31 in this compartment. Indeed, aside from specific molecular chaperones such as the PapD family involved in pilus assembly, most of the known periplasmic folding helpers thus far identified are folding catalysts: The protein disulfide isomerases of the Dsb family and the peptidyl-prolyl isomerases [8]. An alternative explanation for the increased soluble yield of MalE31 in the cytoplasm could be that the conformation of nascent MalE31 polypeptide chains emerging from the large ribosomal subunit is already directed towards the productive folding pathway, in contrast to their conformation when they emerge inside the periplasm from the translocation machinery. Such beneficial associations of newly synthesized polypeptide chains with ribosomes have been recently described for the tailspike protein of phage P22 [7].

However, the MalE31ΔSS protein accumulates predominantly in the cytoplasm, whereas the MalE31M-18 precursor is mainly found in the insoluble fraction, presumably as aggregate. This result suggests that the tendency of this MalE31 precursor to aggregate is most likely related to the presence of a mutated signal sequence. In accord with this idea, it has been proposed that signal sequences maintain the export-competence conformation by interfering directly with the folding of precursors [19]. Structurally, a nascent precursor in an export-competent conformation may be similar to a partially folded protein prone to aggregate. Indeed, Wild et al. [25] showed that accumulation of protein precursors in strains lacking the SecB chaperone, the primary chaperone involved in export of many periplasmic and outer membrane proteins [9], generated a signal for induction of the σ^{32}-stress response. Obviously, the increased activity of the *lon* promotor in cells producing either the MalEM-18 or MalE31M-18 precursor agrees with this observation. However, while both proteins are encountered exclusively in their precursor sizes, the MalE precursor with the altered signal sequence (expressed from p1M-18R) is produced at a higher level than the MalE31 carrying both the altered signal sequence and the *malE31* mutation (expressed from p31M-18). This difference is likely explained by a higher degradation of the latter protein. Because this MalE31 variant displaying defective folding properties induced the strongest σ^{32}-stress response, the heat-shock cytoplasmic proteases (such as Lon, HslUV, or Clp proteases) could also degrade this misfolded protein, and its level of production is dictated by the alternative fate between folding, degradation, and aggregation.

Finally, the stress response induced by the presence of misfolded proteins in *E. coli* is compartmentalized into cytoplasmic and extracytoplasmic responses that are controlled by two different sigma factors, σ^{32} and σ^{E} respectively [11]. We showed that MalE31 misfolding in the periplasm does not induce a stress response via σ^{32} as it does when MalE31 is produced in the cytoplasm with altered signal sequences. By this criterion, we were able to discriminate between cytoplasmic and periplasmic, the exact cellular location of the folding defect of MalE31.

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