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DnaK/DnaJ-assisted recombinant protein production in *Trichoplusia ni* larvae.

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Since bacterial folding modulators proved to have a positive effect on recombinant proteins produced in cultured insect cells, we were interested in determining whether this would be maintained in a larvae system of use as a biofactory but where protein yields are usually reduced due to aggregation of the target protein.

For that purpose, we infected insect larvae with our baculovirus vectors for expression of recombinant GFP either alone or coproduced with the bacterial chaperones DnaK and DnaJ. Soluble yields (but not total) were increased in presence of the bacterial chaperones, which in turn resulted in enhanced solubility of the recombinant protein. Although conformational quality declined, this was not unexpected as it was again in agreement with the proposed principle for bacterial systems by which enhancing protein yields results in quality impairment. Thus, this principle may prove a general rule for protein production in recombinant systems.

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DnaK/DnaJ-assisted recombinant protein production in *Trichoplusia ni* larvae

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Abstract The DnaK/DnaJ *Escherichia coli* chaperone pair, co-produced along with recombinant proteins, has been widely used to assist protein folding in bacterial cells, although with poor consensus about the ultimate effect on protein quality and its general applicability. Here, we have evaluated for the first time these bacterial proteins as folding modulators in a highly promising recombinant protein platform based on insect larvae. Intriguingly, the bacterial chaperones enhanced the solubility of a reporter, misfolding-prone GFP, doubling the yield of recombinant protein that can be recovered from the larvae extracts in a production process. This occurs without negative effects on the yield of total protein (extractable plus insoluble), indicative of a proteolytic stability of the chaperone substrate. It is in contrast with what has been observed in bacteria for the same reporter protein, which is dramatically degraded in a DnaK-dependent manner. The reported data are discussed in the context of the biotechnological potential and applicability of prokaryotic chaperones in complex, eukaryotic factories for recombinant protein production.

Keywords Protein folding · Chaperones · Insect larvae · Protein quality · DnaK · Protein factories

Mónica Martínez-Alonso and Silvia Gómez-Sebastián have equally contributed to this study.

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Introduction

Recombinant protein production is a routine practise in structural and functional genomics and the methodological source of many biotechnological and biomedical products, both at laboratory and industrial scales. The use of microbial cell systems (namely bacteria, yeast and filamentous fungi) encounters several obstacles during production processes, including conformational stress (Gasser et al. 2008), diverse metabolic responses that compromise productivity (Hoffmann and Rinas 2004; Mattanovich et al. 2004; Martinez-Alonso et al. 2008a), lack of post-translational modifications and poor solubility and stability of many heterologous proteins (Baneyx and Mujacic 2004; Rosano and Ceccarelli 2009) and the affectation of the whole cell integrity as response to protein production (Villa et al. 2009). Among the most critical points, many

recombinant proteins are produced as insoluble versions, and in particular, in bacterial systems, it is commonly observed the occurrence of inclusion bodies, namely large clusters of aggregated polypeptides that are not suitable for most of biomedical or biotechnological applications (Villaverde and Carrió 2003). These bottlenecks have pressed the exploration of new biological systems as alternative platforms to obtain higher quality proteins, especially under the urgent need of new protein pharmaceuticals (Leader et al. 2008) for which microbial cells might be inefficient as factories. In this context, mammalian and insect cell lines and transgenic plants and animals are being progressively incorporated to production activities (Ferrer-Miralles et al. 2009). Many recombinant therapeutics (that are subjected to highly restrictive quality controls) produced in mammalian cells have already been approved for use, competing in number to those obtained in microbial factories (Ferrer-Miralles et al. 2009). Among these alternative platforms for protein production, baculovirus-based expression systems show a highly promising potential, and insect larvae have several advantages compared with the insect cell line version, scaling-up of which is both methodologically complex and costly. While keeping the glycosylation abilities offered by the insect system, insect larvae do not require sterile conditions or growth media, importantly reducing production costs (Shi and Jarvis 2007). Also, larvae factories render biologically safe products either for diagnostic or vaccine purposes (Gomez-Sebastian et al. 2008; Ferrer et al. 2007; Lopez et al. 2005; Perez-Filgueira et al. 2007; Perez-Filgueira et al. 2006; Barderas et al. 2000). Furthermore, scaling up is straightforward once optimal larval infection conditions are defined. In contrast to protein production in mammals' milk, that requires the establishment of transgenic animal lines, the batch-based protein production in larvae skips complex cell and DNA manipulation procedures and clone selection.

However, yields of heterologous proteins in larvae may be reduced due to protein aggregation (Ailor et al. 1999) that could be at least partially accounted for by the use of strong viral promoters, namely polyhedrin or p10 promoters in the expression systems. Compared with microbial cells, much fewer approaches to improve recombinant protein production and solubility have been described in insect larvae. In bacteria, one of the most common strategies to increase solubility is the co-production of homologous chaperones (usually as sets of cooperating chaperones or chaperone-co-chaperone pairs) along with the recombinant protein, as folding modulators are believed to be limiting in recombinant cells (Kolaj et al. 2009; de Marco et al. 2007; de Marco 2007). We have here explored for the first time the potential of the main *E. coli* chaperone DnaK and its co-chaperone DnaJ to modulate the quality of a reporter misfolding-prone GFP (mGFP) when produced in insect larvae. In *E. coli*, mGFP forms highly visible, fluorescent inclusion bodies rapidly after induction of gene expression (Garcia-Fruitos et al. 2007). The proposed approach has resulted in the doubling of the yield of extractable mGFP, proving that prokaryotic proteins act efficiently in complex eukaryotic systems for protein production. A moderate reduction in mGFP conformational quality (estimated through its specific fluorescence) is in agreement with a general concept proposed for bacterial systems under which protein yield and conformational quality cannot be gained simultaneously (Martinez-Alonso et al. 2008a). These results indicate the applicability of prokaryotic chaperones DnaK/DnaJ as folding modulators in complex, whole body protein factories, and opens possibilities of expanding the catalogue of platforms, in which largely characterized folding modulators (such as those from *E. coli*) can be successfully used.

Material and methods

Vectors and virus stocks

mGFP (Garcia-Fruitos et al. 2007) has been used as a reporter to study the effect of a set of *E. coli* chaperones when produced in a baculovirus expression system. This protein consists of the aggregation-prone VP1 capsid protein of foot-and-mouth disease virus fused to the amino terminus of the Green Fluorescent Protein (GFP). mGFP has been produced either alone or with the *E. coli* chaperone pair DnaK/J. For this purpose, two different transfer vectors based on pAcAB4 (Belyaev and Roy 1993) were constructed. The *mGFP* gene was cloned alone for single expression, and for chaperone co-expression a second vector containing both mGFP and the DnaK/J chaperone pair was constructed. Kozak sequences were added to each gene. The p10 promoter was used to drive the expression of *mGFP* and *dnaJ* genes, and *dnaK* expression was driven by the polyhedrin promoter.

Recombinant baculoviruses were obtained by co-transfection of each transfer vector with Bsu36I-linearized viral DNA BAC10:KO1629 (Zhao et al. 2003) into *Sf9* cells. Individual clones were plaque purified after 5 days, amplified once and screened for protein expression by Western blot analysis. Recombinant baculoviruses expressing the desired proteins were further amplified in Sf9 cells by infecting them at MOI=0.1 when cell density was 1×10^6 cells/ml. After 4 days, the culture supernatant was harvested, cleared by centrifugation at $9{,}500\times g$ for 10 min, and titered by standard plaque assay. This virus stock was amplified once more to produce a larger high titer stock. This time, *Sf9* cells were grown to a density of 2×10^6 cells/ml and infected at a MOI of 0.1. Culture

supernatants were harvested after 6 days, processed as described above and titered. These virus stocks were used directly to infect cells for protein expression.

Cells were maintained in Insect Xpress medium (Lonza, #12-730) and supplemented with 2% Foetal Calf Serum at the time of infection. Both cells and infected cultures were kept at 27°C and 110 rpm. Cell density was determined by cell counting using a haemocytometer, and Trypan Blue was used to assess viability. All virus stocks were stored in dark at 4°C.

Insect growth conditions and inoculation

Trichoplusia ni (Cabbage looper) larvae (*T. ni*) were reared under level-2 biosafety conditions following previously described methodology (Perez-Filgueira et al., 2006). For all experiments, fifth-instar larvae (last instar larvae before pupation) of about 300 mg weight, were injected with the recombinant baculoviruses (budded virus forms) in the body cavity near the proleg using different pfu/larva doses, as indicated on each experiment. Infected larvae were kept in growth chambers at 28°C and collected at indicated times. Larvae were then immediately frozen and kept at −20°C until processed.

Preparation of protein extracts

Total protein extracts from *T. ni* larvae were obtained as previously described (Perez-Filgueira et al. 2006). Briefly, frozen insect material was homogenised using an extraction buffer containing phosphate buffered saline (PBS) pH7.2, 0.01% Triton X-100, 1% sodium dodecyl sulphate, 2.5 mM, dithiothreitol, 10 mM β-mercaptoethanol and a protease inhibitor cocktail (Complete, Roche, Germany). Such material was directly used for the analysis of the total recombinant protein, while for the evaluation of the soluble fraction the extract was clarified by centrifugation at 10,000×*g* for 10 min and the pellet discarded. The supernatant was filtered through Whatman papers and centrifuged once again as described above. In both cases, total concentration of extracted protein was quantified by Bradford assay (Bradford 1976).

Protein analysis

To check for protein expression of the plaque-purified individual clones, *Sf9* cells were seeded on 6-well plates (1×10^6 cells/well) and infected with the individual clones. At least five clones were checked for either single expression of mGFP or expression of mGFP together with the DnaK/J pair. Cells were harvested 72 hpi, washed and resuspended in cold PBS. Three volumes of cells in PBS were mixed with one volume of sample buffer (50 mM Tris-HCl pH6.9, 10% sodium-dodecyl sulphate (SDS), 10% β-mercaptoethanol, 25% glycerol, 0.02% bromophenol blue) and loaded onto 12% acrylamide sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for protein analysis. Proteins were transferred to a nitrocellulose membrane and probed with the appropriate antibodies. mGFP was detected using a commercial rabbit polyclonal antibody against GFP (Santa Cruz Biotechnology, Inc., # sc-8334). DnaK was detected using a hyperimmune rabbit serum, and for DnaJ a commercial rabbit polyclonal antibody (Stressgen, #SPA-410) was used. In all cases, goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad, #172-1019) was used as second antibody.

For the analysis of protein production in larvae, samples were prepared by mixing three volumes of extracts with one volume of sample buffer for protein analysis in 12% acrylamide SDS-PAGE gels followed by Western blotting. For that, proteins were transferred to a nitrocellulose membrane and GFP was detected as described above. Bands were scanned at high resolution and quantified by Quantity One Software (Bio-Rad). Experiments were carried out in duplicate.

Fluorescence determination

The emission of 40 µg of the larvae extracts (lysates or soluble extracts) was measured at 535 nm after being excited at 485 nm using a GENIOS fluorimeter. Each value was taken by measuring four times each extract. Fluorescence values were referred to mGFP amounts to obtain specific fluorescence of mGFP, defined as fluorescence units per microgram of mGFP.

Results

The amounts of mGFP released from larvae extracts (namely soluble mGFP species) were monitored by Western Blot upon viral infection at different pfu, when produced alone or with simultaneous production of *E. coli* DnaK/DnaJ chaperones. As observed (Fig. 1a), mGFP bands were hardly visible at 24 and 48 hpi in the absence of the bacterial proteins, but the co-production of DnaK/DnaJ dramatically increased the amount of soluble mGFP in all viral doses, making the product clearly immunodetectable already at 48 hpi.

At 10^5 pfu, chosen as a model infection dose, quantification of Western blot analyses revealed a twofold increase of the releasable mGFP at both 48 and 72 hpi, when referred either to larvae extract volume (Fig. 1b) or total protein (Fig. 1c; $p=0.003$ at 72 hpi). Importantly, the bacterial chaperones had no significant effects on the total amount of produced mGFP (soluble+retained in the

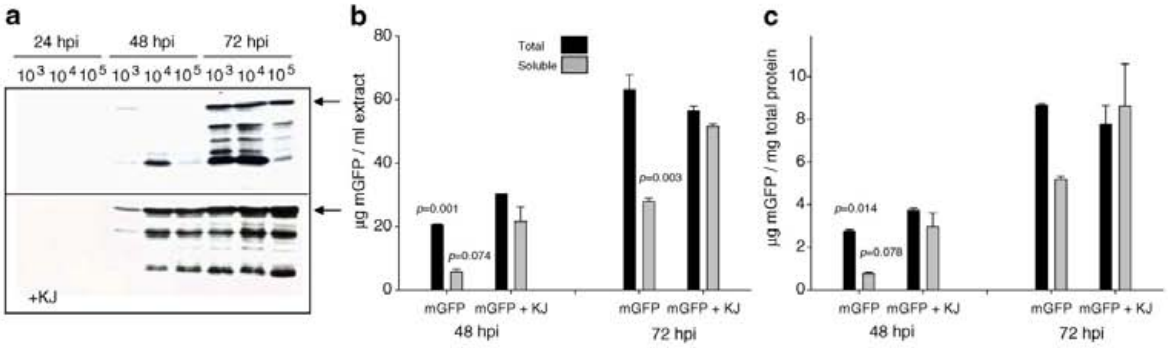

Fig. 1 (**a**) Western blot kinetic analysis of soluble mGFP production in larvae infected with different viral doses (pfu values per larva are indicated, in absence (*top*) and presence (*bottom*) of bacterial chaperones DnaK/DnaJ (*KJ*). *Arrows* indicate the position of the full-length protein. Quantitative analysis from several infection experiments with 10^5 pfu are shown in panels **b** and **c**, indicating the total and soluble mGFP referred to extract volume (**b**) or total protein (**c**). Significantly different values (always comparing data with and without chaperones for a given fraction) are indicated by the *p* parameter obtained in an ANOVA test. Only $p<0.1$ values are shown. For clarity, further analyses were done only on larvae infected with 10^5 pfu. *Bars* here and in the following figures indicate standard errors

insoluble extract fraction). In summary, the solubility of mGFP expressed as the percent of releasable mGFP over the total recombinant protein was significantly enhanced in co-expressing larvae up to around twofold, again at 48 and 72 hpi (Fig. 2; $p=0.001$ at 72 hpi).

We were interested in the analysis of protein quality, as we have recently shown that solubility of recombinant proteins is not necessarily linked to their conformational quality (Gonzalez-Montalban et al. 2007; Martinez-Alonso et al. 2009). Co-production of DnaK/DnaJ along with mGFP promoted a reduction of fluorescence in larvae extracts, particularly evident at 48 hpi ($p=0.005$ at 48 hpi and $p=0.041$ at 72 hpi), and a null effect on the fluorescence of the soluble fraction (Fig. 3a). As a consequence, the average specific fluorescence of mGFP in the whole cell extracts resulted negatively influenced (between around 40% and 60%) by the expression of bacterial genes ($p<0.001$ at 48 hpi and $p=0.026$ at 72 hpi) and this effect was also observed, although less robustly, in the soluble fraction ($p=0.017$ at 48 hpi and $p=0.107$ at 72 hpi; Fig. 3b). This is indicative of a moderate reduction in the conformational quality of mGFP irrespective of the protein fractioning. Such inactivation is in agreement with previous observations derived from systems level analysis of recombinant *E. coli*, indicating that up-modulation of protein productivity by genetic or process approaches is detrimental regarding conformational and functional protein quality (Garcia-Fruitos et al. 2007; Martinez-Alonso et al. 2009; Martinez-Alonso et al. 2008a).

The mentioned reduction in the fluorescence emission observed in whole cell extracts (Fig. 3a) was confirmed by a direct observation of infected, mGFP-producing larvae (Fig. 4), which were less fluorescent when bacterial proteins were also produced.

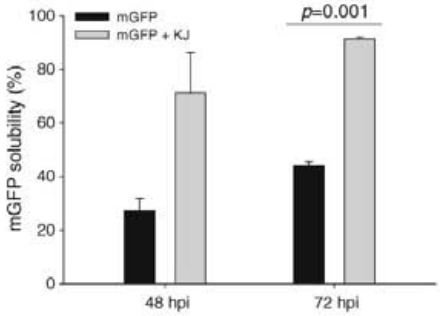

Fig. 2 mGFP solubility as influenced by DnaK/DnaJ (*KJ*) co-production, determined at two times post-infection. Significantly different values (always comparing data with and without chaperones for a given fraction) are indicated by the *p* parameter obtained in an ANOVA test. Only $p<0.1$ values are shown

Discussion

Protein production in recombinant hosts is severely compromised by protein misfolding and poor solubility (Baneyx and Mujacic 2004), host stress responses (Gasser et al. 2008) and different host-specific bottlenecks (Ferrer-Miralles et al. 2009), including the absence of many post-translational modifications in prokaryotic cells (de Marco 2009). The urgent need of new recombinant products, including protein drugs for molecular therapies, has strongly pushed the incorporation of new cell types and organisms into the protein production arena, including mould, filamentous fungi, methilotrophic yeasts and insect larvae, among others.

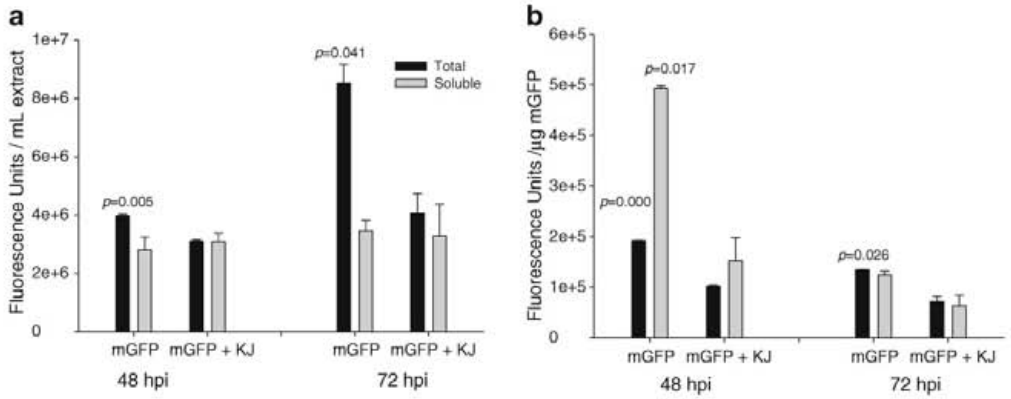

Fig. 3 Fluorescence emission of mGFP, produced in absence or presence of bacterial chaperones DnaK/DnaJ (*KJ*), referred to extract volume (**a**) or given as mGFP specific fluorescence (**b**). Significantly different values (always comparing data with and without chaperones for a given fraction) are indicated by the *p* parameter obtained in an ANOVA test. Only $p<0.1$ values are shown

In bacteria, chaperone DnaK and its co-chaperone DnaJ have been observed as promising tools to improve solubility (de Marco et al. 2007; de Marco 2007). They act in a sequential network of folding assistant proteins that involves trigger factor and GroEL/GroES proteins among others (Deuerling et al. 1999; Langer et al. 1992; Krueger and Walker 1984; Gragerov et al. 1992) and have a wide spectrum of protein substrates that expose hydrophobic

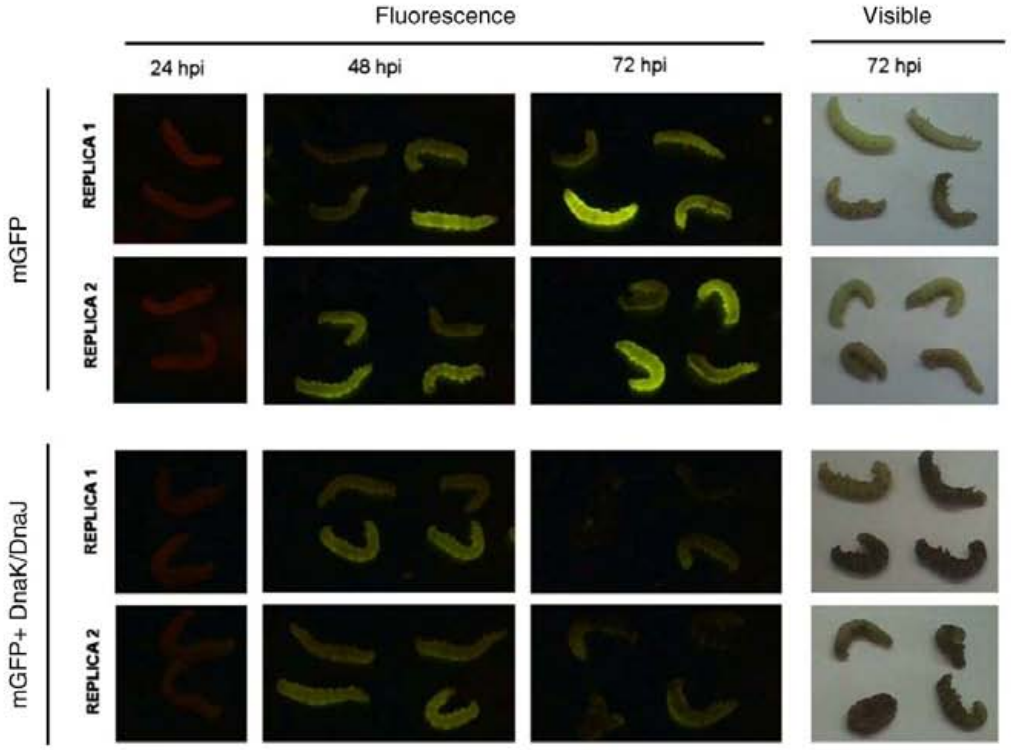

Fig. 4 Fluorescence and visible imaging of infected larvae (two replicas) producing mGFP in absence or presence of bacterial chaperones DnaK/DnaJ (*KJ*), at different post-infection times

regions to the solvent (Mayer et al. 2000; Rodriguez et al. 2008). Although this fact enables these proteins to be used as folding tools in recombinant processes, co-production of DnaK/DnaJ might eventually occur at the expense of productivity since in the bacterial quality control network, DnaK can reduce the proteolytic stability of recombinant proteins (Martinez-Alonso et al. 2007; Garcia-Fruitos et al. 2007). In an attempt to evaluate how the well-studied bacterial chaperone DnaK, could promote protein folding in a complex eukaryotic platform we have expressed the encoding *dnaK* and *dnaJ* genes in insect larvae producing an aggregation prone GFP.

While the total yield of mGFP was not significantly affected by these folding assistant proteins (Fig. 1b), both the yield of soluble mGFP (the fraction that can be recovered from larvae extracts) and the solubility ratio were significantly improved (Fig. 1b, c and 2). Contrarily to what occurs in *E. coli* cells, in which the inactivation of the *dnaK* gene or its plasmid-driven overexpression increases or decreases the recombinant protein yield, respectively (Carrio and Villaverde 2003; Martinez-Alonso et al. 2007; Garcia-Fruitos et al. 2007), the results presented here indicate that in larvae, DnaK has quality-restricted, yield-independent effects on protein production.

On the other hand, the folding assistant proteins moderately reduced the specific fluorescence of the soluble mGFP (Fig. 3b). This fact could be accounted for by a recently proposed concept for bacterial platforms (in particular *E. coli*) in which protein yield and quality are observed as antagonistic parameters that evolve divergently when modifying the production conditions or tuning the cell's genetic background (Garcia-Fruitos et al. 2007; Martinez-Alonso et al. 2008a). The results obtained here in a completely different biological system prompt to consider such principle as not restricted to a specific production platform and probably as a general rule in the recombinant protein production arena. Importantly, the soluble versions of aggregation-prone proteins consist of molecular isoforms that can adopt a wide conformational spectrum (de Marco and Schroedel 2005; Martinez-Alonso et al. 2008b; de Marco 2008) and exhibit a gradation in their biological activity (Gonzalez-Montalban et al. 2007). The relative proportion of such soluble, but biologically diverse protein species seems to be strongly influenced by the in vivo protein concentration (Martinez-Alonso et al. 2007), what in the production process context results in an incapability to simultaneously favour soluble yield and protein quality (Martinez-Alonso et al. 2008a).

In few previous studies, several human chaperones have been individually tested on insect larvae producing recombinant proteins (Nakajima et al. 2009), being the human chaperone Bip the only that had positively affected protein solubility and functionality in a significant way (Nakajima et al. 2009; Hsu and Betenbaugh 1997). Here we have proved, for the first time, the utility of DnaK/DnaJ as folding modulators in complex eukaryotic platforms, increasing the extractable amounts of a model protein. The functional performance of bacterial chaperones in insect larvae largely expands the catalogue of folding modulators, beyond chaperones of eukaryotic origin, which can be used in this platform, by the incorporation of deeply characterized proteins and protein teams of bacterial origin. In particular, the cooperative mode of action of DnaK/DnaJ, through their simultaneous binding to distinct target sites within the same substrate protein, has been recently determined using the σ^{32} transcription factor as a model (Rodriguez et al. 2008). Furthermore, DnaK/DnaJ, IbpA/IbpB and ClpB cooperate in the physiological removal of misfolded species from protein aggregates (Mogk et al. 2003; Schlieker et al. 2004; Weibezahn et al. 2005). The positive results presented here with DnaK/DnaJ, and the absence of undesired side effects on the stability of the target protein prompt to explore, in insect larvae, appropriate combinations of bacterial proteins that have been reported as highly successful in *E. coli* (de Marco et al. 2007; de Marco 2007).

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4. Discussion

The aim of any research and industrial process involving the production of recombinant proteins is obtaining the highest possible yield of soluble and functional product. Since the cost of the process is always intended to be kept at minimum, expression systems based on prokaryotic hosts are usually the first choice, and *Escherichia coli* is without a doubt the most widely used organism for heterologous expression of recombinant proteins [15]. However, heterologous proteins produced in *E. coli* are often obtained, partially or totally, as insoluble inclusion bodies [189], which represents one of the major drawbacks of the system. Although soluble protein can be recovered from inclusion bodies [219], the required procedures are complex and inefficient and therefore many IB-forming proteins are often excluded from the market and produced in other expression systems. Nonetheless, production in *E. coli* still has many advantages, such as the high protein yields obtained from fast and easy to handle procedures, and for this reason great effort has been made to overcome the formation of inclusion bodies and promote the soluble version of the target proteins. While the deep knowledge of *E. coli* genetics has allowed approaches such as host strain engineering or tight control of mRNA stability and codon usage, other common strategies are based on adjusting process parameters like growth media or cultivation temperature [27;28]. Folding modulators have also been broadly tested to enhance protein folding since they are thought to be limiting in recombinant cells [75;112;237].

The success of these strategies is usually assessed by measuring solubility, yield or functionality of the recombinant product, and because soluble species have traditionally been considered to be properly folded and fully functional, solubility has often been used as a universal indicator of protein quality. Likewise, inclusion bodies were regarded as insoluble deposits of misfolded and inactive protein but this view is no longer supported by the current data that pictures inclusion bodies as rich in functional polypeptides with native-like secondary structure [201;203;212]. Moreover, soluble aggregates have also been found in the soluble population of recombinant proteins [354]. All these data compromise the assumption that solubility is linked to quality of recombinant proteins. In addition, the non coincidence between solubility and quality may explain the inconsistent results obtained from the different strategies used to minimise inclusion body formation, where solubility, yield and functionality have been used as equivalent parameters to monitor the procedure.

On this background, we decided to explore the conditions that would enhance simultaneously these three relevant parameters (yield, solubility and protein quality) and further investigate the functionality of the soluble version of recombinant proteins.

Moreover, we were also interested in testing whether our findings would be applicable to eukaryotic systems. For this purpose, we have used a model protein consisting of the aggregation-prone VP1 capsid protein of foot-and-mouth disease virus fused to the amino terminus of GFP as a reporter of protein aggregation and conformational quality. This protein, termed VP1GFP or mGFP, forms fluorescent inclusion bodies when produced in *E. coli* (annex II) and also resulted in protein deposition as insoluble and fluorescent clusters when produced in a eukaryotic insect cell system (article 4).

4.1. Independent control of protein yield and quality

The observations that point out solubility and conformational quality as non coincident events (annexes I, II, [208;242]) also suggest that these parameters may be engineered independently. Thus, we chose a DnaK-deficient genetic background that favoured the deposition of our mGFP model protein in the form of large but highly fluorescent inclusion bodies (article 1, Table 1) to explore the effects of growth temperature and expression rate on both the distribution and functionality of the protein.

The results obtained show divergent effects of temperature on the yield of functional protein depending on its soluble or insoluble status. While insoluble but functional protein is enhanced at higher temperatures, the yield of soluble and functional protein remains fairly constant along the range of physiological temperatures tested, showing only a slight decrease parallel to the temperature rise (article 1, Figure 1A). Regarding protein production, although both soluble and insoluble protein amounts were increased with temperature, the effect was much more pronounced in the protein present in the insoluble fraction, which resulted in solubility being highly dependent on the growth temperature (article 1, Figure 1B). Since growth temperature correlates with the total yield of mGFP, solubility is then ultimately dependent on the protein's own yield, which is in agreement with our previous data (annex I, Figure 4). Altogether, this translated in protein quality being differently affected by temperature depending on the soluble or insoluble status of the protein. Although conformational quality of the insoluble protein (evaluated through its specific fluorescence emission) was almost unaffected by temperature, the quality of the soluble fraction was impaired by increasing temperatures (article 1, Figure 1C). This is in contrast to the effect of growth temperature on a related recombinant GFP produced in a wild-type strain, where a parallel evolution of conformational quality in soluble and insoluble protein populations

was observed [208]. This might be explained by the fact that inclusion bodies produced in a DnaK$^-$ background are richer in correctly folded species than those produced in wild-type strains [209]. Thus, temperature effect would be much more evident on inclusion bodies with less native-like structures, the formation of which would be enhanced at lower temperatures promoting the increase in conformational quality of the protein. Besides, a parallel evolution of conformational quality in soluble and insoluble protein species has also been observed when mGFP is produced in wild-type cells, which in fact is impaired by a DnaK excess (annex I, Figure 5). Finally, our data indicates that gene expression rate strongly affected the yield of the recombinant protein (article 1, Figure 2B) but not its fluorescence (article 1, Figure 2A), which resulted in protein quality being highly dependent on expression rate and favoured by medium levels (article 1, Figure 2C).

Our results show how solubility can be effectively enhanced by selecting the appropriate growth temperature, in agreement with previous reports [355-357], and then protein quality can be regulated by adjusting the expression levels, which is in agreement with our previous work (annex I, Figure 5). The success of this two-step approach proves that solubility and protein quality are independently controlled, which is in agreement with a recent genetic study that linked mutations promoting solubility with a decrease in conformational quality of the protein (annex II). This is relevant in the context of protein production in industry, as obtaining high yields of functional protein should be more desirable than high solubility values corresponding to poor yields. Of course, the need of soluble proteins for crystallographic procedures or as therapeutics for the biopharmaceutical industry is also evident, and therefore enhancing simultaneously protein yield, solubility and quality would be the most appealing scenario. However, a global analysis of our data indicates that conditions favouring total yield of the recombinant protein result in more aggregation and decreased conformational quality of the soluble protein (article 1, Figure 3A and 3B). In the same way, enhancing the yield of soluble protein seems not to be possible without impairing its quality (article 1, Figure 3C). Accordingly, different protein species in the soluble protein population of mGFP produced in *E. coli* display a functional profile that does not match the protein distribution, and where higher protein amounts again result in impaired protein quality (article 2, Figure 1A and 1B). Moreover, when mGFP was produced in insect cells, conditions favouring higher protein yields (in this case, coproduction of bacterial chaperones) resulted in impaired conformational quality of the recombinant protein (article 4, Figure 2B and 2C), and the same was observed for mGFP production in insect larvae (article 5, Figures 1B, 1C and 3B).

Therefore, we propose a principle stating that yield, solubility and quality of soluble proteins cannot be favoured simultaneously during recombinant protein production. This has already been proven both for prokaryotic (*E. coli*) and eukaryotic systems (cultured insect cells and larvae) suggesting that it is not restricted to specific production platforms, and thus we suggest that it should be regarded as a general event during recombinant protein production.

Again in the context of industrial protein production, our results indicate that the unfeasibility of promoting simultaneous yield, solubility and quality of the target protein must be taken into account when designing the strategy of the process, which should be clearly addressed to the most relevant feature for the intended use of the recombinant product. Also in this line, the indistinct use of any of these three parameters to evaluate the success of a production process is probably the reason for many of the inconsistent results reported from the multiple studies aimed to improve protein production [12;75;83;84;230;231;234;358;359].

In summary, while it is now clear that solubility is not a good indicator of protein quality because aggregation does not split the protein population into active and inactive species (articles 1, 2, 3, 4, 5, annexes I and II, [209;214;217;242;360]) it is also evident that the ideal outcome of protein production (high yield, solubility and protein quality) is unrealistic. Therefore, production processes should be monitored through the most relevant parameter for the final use of the protein.

4.2. Functional status of soluble protein

Evidences that solubility and quality of recombinant proteins are divergent parameters (annex II) compromise the traditional view by which soluble proteins are pictured as highly functional species folded in their native conformation. Moreover, the presence of aggregates in the soluble version of recombinant proteins has also been reported [354;361-363]. When we explored the molecular organisation of the soluble population of our recombinant mGFP by subjecting it to density gradient centrifugation, we found that the protein dispersed along the gradient indicating the presence of different aggregation states (article 2, Figure 1A). In addition, the fluorescence profile of the gradient was not coincident with the protein distribution (article 2, Figure 1A). This indicated that the soluble protein population displayed a variable conformational quality, which was confirmed by determining the specific fluorescence along the

gradient (article 2, Figure 1B). This variable functional status of the soluble protein is against the conventional model that assumes soluble species to be folded in a unique and fully functional native conformation. In fact, the soluble fraction shows a heterogeneous functional status that depends on the size of the aggregates. Since protein species are separated according to mass along the density gradient, the most functional species would then correspond to the smaller aggregates (article 2, Figure 1B). This is in agreement with the observation that soluble species usually display specific activities higher than those of their insoluble counterparts (article 1, annexes I and II) [208;209;214;217]. The protein fraction displaying the highest conformational quality was isolated from the gradient, further purified and analysed by transmission electron microscopy. Soluble GFP aggregates of different sizes and morphologies were still present in this fraction (article 2, Figure 2A), again evidencing the heterogeneity of the soluble protein population. Moreover, the soluble aggregates were also heterogeneous regarding their secondary structure (article 2, Figure 1C). While mGFP inclusion bodies are rich in intermolecular β-sheets and only contain native-like structures to a minor extent, soluble aggregates display a wide set of native-like conformations rich in α-helix and native-like β-sheets, as well as non-native intermolecular β-sheets and unfolded conformations. Thus, the higher extent of native-like structures that characterises the soluble aggregates also accounts for the better conformational quality observed in soluble species.

These data now evidence that the soluble protein population is in fact comprised of a wide set of differently functional species that are not free from aggregation. Then, recombinant proteins can be pictured as “a continuum of forms” [354] where inclusion bodies represent only a minor subset of the population, which is also the most homogeneous regarding functionality and structural features. Moreover, the structural homogeneity of inclusion bodies is further enhanced by the fact that protein species can complete their folding once trapped in inclusion bodies [364]. In this model, quality of soluble protein forms can no longer be defined by the presence of a homogeneous population of protein species folded in their native conformation, but is rather seen as an average resulting from the prevalence of active over inactive species found in the soluble form, or *vice versa*. This scenario also explains reports of inclusion bodies displaying higher specific activities than their soluble versions [217], and would also account for the enhancement in protein quality observed for different GFP derivatives produced at suboptimal growth temperatures (article 1, Figure 1C) [208]. By producing the protein at reduced temperatures, inactive GFP species present in the soluble fraction are allowed to maturate, thereby increasing the conformational quality of the protein.

Accordingly, different production conditions or genetic backgrounds for protein production would act on protein quality by altering the balance of active and inactive protein species, irrespective of their presence in the soluble or insoluble virtual cell compartments [212].

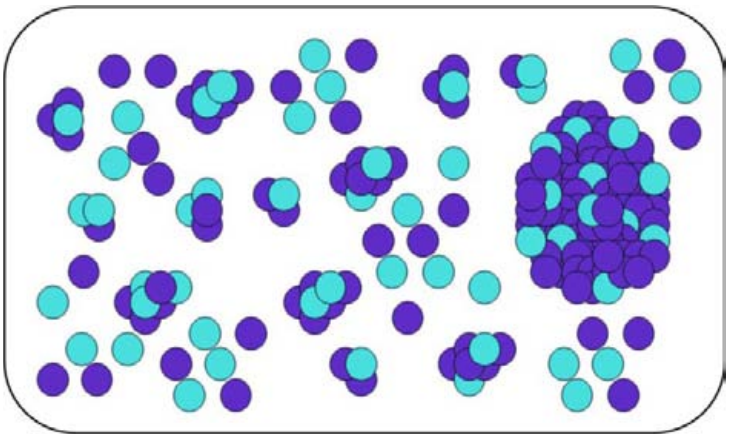

Figure 20. Distribution of protein forms in a recombinant cell. Soluble species, including differently sized soluble aggregates, and inclusion bodies are depicted in the image. Conformational quality is determined as the average of active (light blue) and inactive (dark blue) protein species.

Adapted from *Nat Biotechnol*. 2007 Jul;25(7):718-20.

4.3. Bacterial folding modulators for eukaryotic systems

The use of folding modulators in bacteria has been a common approach to improve protein production in recombinant cells, but this strategy has been scarcely explored in other production platforms such as the insect cell-baculovirus system, even when aggregation has been reported to be a problem that reduces the yield of soluble protein that can be recovered [19;20]. In fact, the high protein levels obtained in the baculovirus system by using strong promoters comes together with host protein production being essentially shut down following baculovirus infection [365;366]. Since this may result in reduced availability of molecular chaperones, coproduction of folding modulators could help to overcome this limitation. Indeed, moderate enhancement of soluble protein production has been reported upon coproduction of human Hsp70 and Hsp40, homologs of the DnaK and DnaJ cytosolic chaperones [367-369].

In *E. coli*, the chaperone DnaK has been found to promote gains in solubility upon coproduction with target proteins [370]. However, this results from cooperation of DnaK with bacterial proteases Lon and ClpP, which degrade aggregated polypeptides to increase solubility at expenses of total protein yields (annex II). Because it would be

interesting to keep the folding activities of DnaK but at the same time exclude the associated proteolysis, we devised a system to uncouple DnaK from the bacterial proteases by rehosting the chaperone to insect cells. Moreover, since DnaK belongs to the Hsp70 family, which is present in all kingdoms of life and highly conserved in terms of evolution, the chaperone was expected to maintain its folding activity when produced in insect cells. In addition, the baculovirus system provides a certain flexibility regarding the choice of host, which can be either cultured cells or living insects. Therefore, our approach allowed us to test the effect of bacterial folding modulators in a eukaryotic system both *in vitro* and *in vivo*.

Baculovirus vectors were constructed to produce our mGFP model protein either alone or together with the bacterial chaperone pair DnaK and DnaJ upon infection of cultured insect cells or larvae. The ability to encode all three proteins in the same baculovirus vector for coproduction experiments ensures that every infected cell will be producing all the required proteins, and eliminates the need of superinfection with chaperone-encoding vectors to guarantee their presence in all cells producing the target protein [17]. Although expression of all three proteins is controlled by strong promoters (polyhedrin or p10) in our recombinant baculovirus, the temporal expression of genes encoded under the control of these promoters is not simultaneous because in the baculovirus life cycle polyhedrin expression precedes that of P10 protein. Thus, by placing the *dnaK* gene under the control of the polyhedrin promoter we ensure that DnaK will already be available by the time that mGFP is synthesised, this one being under control of the p10 promoter. Guaranteeing chaperone availability before target proteins are synthesised has proven to be an effective strategy to improve production of soluble target proteins in recombinant *E. coli* cells [371] and overall activities in insect larvae [372]. Moreover, since DnaJ is under control of the p10 promoter its expression is delayed respect to that of DnaK, and therefore the higher DnaK to DnaJ ratio at any set time mimics (at least partially) that observed in their natural *E. coli* host [373].

Cell culture experiments allowed us to observe the distribution of mGFP in infected *Sf9* cells (article 4, Figure 1). Production of mGFP resulted in aggregation of the protein as insoluble but fluorescent clusters, remarkably similar to the inclusion bodies that it forms in bacterial cells (annex II, Figure 3). The presence of active polypeptides in the insoluble deposits is in agreement with reports of active inclusion bodies obtained in bacterial systems [217;243;245] and again points out solubility as an inadequate parameter to evaluate successful protein production [242]. Chaperone coproduction resulted in a reduction in the number of insoluble deposits observed in cells (article 4,

Figure 1), as well as an increase in fluorescence (article 4, Figure 1B and 2A) that was more homogeneously distributed in the cytoplasm (article 4, Figure 1) and parallel to the significant rise in protein yield (article 4, Figure 2B). However, although soluble protein was enhanced about two-fold, most mGFP still remained insoluble (article 4, Figure 2B). The yield increase for mGFP was associated to enhanced stability of the protein when it was coproduced with DnaK and DnaJ (article 4, Figure 2B), which indicates improved folding mediated by the chaperone pair without the linked proteolysis, as well as protection of mGFP from degradation by host cell proteases. This contrasts with the negative role of DnaK on mGFP stability when produced in *E. coli*, where it drives a reduction of the protein half-life by delivering misfolded species to Lon and ClpP proteases (annex II). Thus, this divergent effect of DnaK in bacteria and insect cells confirms the success of our strategy to promote protein folding without activating proteolysis.

Protection from proteolysis in presence of the chaperones was also evident when mGFP was produced *in vivo* by injecting *T. ni* insect larvae with our recombinant baculoviruses (article 5, Figure 1A). Yield of soluble protein increased parallel to the infection dose, so the highest dose was chosen as a model for the rest of the experiments. When mGFP was produced alone, between one and two thirds of the total protein was obtained as insoluble (article 5, Figure 1B and 1C). Once more, active protein was deposited in the insoluble fraction, as evidenced by fluorescence levels being higher in the total than in the soluble fraction (article 5, Figure 3A). When DnaK and DnaJ were coproduced with mGFP total protein levels were not significantly affected, but soluble protein amounts represented almost the total of the produced protein (article 5, Figure 1C). Indeed, upon chaperone coproduction solubility was higher than 90% by three days post-infection (article 5, Figure 2). Harvest at earlier times reduced significantly the amounts of both soluble and total protein, as well as solubility (article 5, Figures 1B, 1C and 2), highlighting the importance of selecting the optimal harvest time.

Evaluation of the conformational quality of the protein was in agreement with our proposed principle by which an increase in protein yield necessarily results in quality impairment (article 1). However, especially for the soluble fraction obtained in cultured cells, this effect seemed to be much more moderate than the quality impairment observed in bacterial cells (compare Figures 2B and 2C in articles 1 and 4 and Figure 3B in article 5). Although in insect larvae overall fluorescence levels decreased in presence of DnaK and DnaJ (article 5, Figures 3A and 4), this only affected the insoluble version of

mGFP (article 5, Figure 3A) and thus did not result in an impairment of soluble protein quality, which was reduced only as a consequence of the higher amounts of soluble protein obtained upon chaperone coproduction. Although soluble protein of very high quality can be obtained already without chaperone coproduction (article 5, Figure 3B), this would represent about an eight-fold reduction of the production yield (article 5, Figure 1C). This stresses the importance of taking into account the final use of the protein when designing the production process, as although higher protein activity could be preferred to elevated yields for reducing therapeutic doses of protein-based drugs, these reduced yields would be a clear disadvantage for applications such as protein crystallography, where very high amounts of protein are needed.

Since the bacterial chaperones proved to have positive effects on mGFP production in the baculovirus system, we decided to explore whether this could be extended to other proteins expressed in *Sf9* cultured cells. Coexpression of the DnaKJ chaperone pair with either foot-and-mouth disease virus VP1 capsid protein or human α-galactosidase did not reduce protein yields significantly, indicative of absence of DnaK-associated proteolysis (article 4, Figure 3A and 3B). For these proteins, both soluble yield and solubility were enhanced. Degradation of the protease sensitive α-galactosidase was also minimised, which indicated protein stabilisation promoted by DnaKJ coproduction. For a third protein, VP2 from the capsid of foot-and-mouth disease virus, there was no improvement in protein yield or solubility, but the quality of the protein was clearly enhanced. VP2 has a tendency to form unwanted oligomers, and these were cleared almost completely in presence of the bacterial chaperones (article 4, Figure 3C).

The case of α-galactosidase deserves further consideration, since the baculovirus vector encoding this protein was designed for protein secretion to the culture medium. Although our protein remained intracellular due to the limited secretion capability of *Sf9* cells [374], it would still have been directed to the secretion pathway. Proteins entering the secretion pathway are translocated across the endoplasmic reticulum (ER) membrane either co- or post-translationally. Thus, the positive effect of cytosolic chaperones such as the DnaKJ pair would not be a straightforward assumption. However, the improvement of solubility and soluble yield that we have observed for α-galactosidase upon coproduction of cytosolic chaperones is in agreement with a previous report where human Hsp70 promoted an increase in soluble intracellular levels of IgG [367]. In this case, coproduction of the ER resident Bip chaperone, another member of the Hsp70 family, also promoted a solubility enhancement. However, while Bip acts on processed heavy and light immunoglobulin chains, the effect of cytosolic

Hsp70 is on the light chain precursor. Therefore, in the case of α-galactosidase the chaperone pair DnaKJ seems to be promoting solubility by binding to the protein prior to its translocation to the ER, probably keeping it in an unfolded or translocation competent state and thus reducing protein aggregation in the cytosol.

Taken together, our results prove that bacterial chaperones are functional in the baculovirus eukaryotic system. We have shown positive effects of DnaK and DnaJ chaperones on protein yield, solubility, proteolytic stability and overall biological activity, as well as a reduction of the negative effects on protein quality that are usually observed in bacterial systems. This has been achieved as a result of the function selection accomplished by rehosting the chaperones to a eukaryotic host where the undesirable effects of the multifunctional chaperone pair have been excluded. Therefore, bacterial chaperones can expand the catalogue of folding modulators available for eukaryotic systems by including well characterised chaperones or chaperone sets of bacterial origin for high quality protein production.

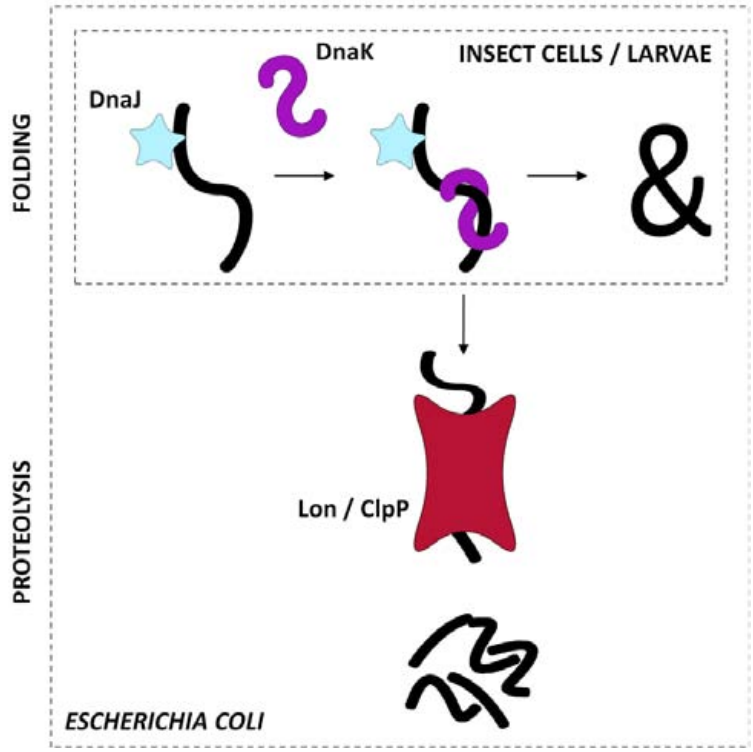

Figure 21. Model of DnaK and DnaJ differential action in bacterial and eukaryotic systems. By rehosting the chaperones to a system lacking orthologs of the bacterial proteases Lon and ClpP proteolysis can be avoided while keeping the conserved foldase activity of the DnaKJ pair.

5. Conclusions

1. Protein yield and quality are antagonistic parameters which cannot be favoured simultaneously in production processes.
2. Conditions promoting high total or soluble protein yield are adverse for the conformational quality of recombinant proteins.
3. Production processes should be targeted to yield, solubility or functionality of the recombinant protein depending on the final use of the product.
4. The soluble protein population of mGFP does not present a homogeneous molecular organisation, but is instead characterised by the presence of soluble aggregates.
5. Soluble protein species are highly heterogeneous in terms of structure and conformational quality.
6. Recombinant proteins can be regarded as a “continuum of forms” where inclusion bodies represent only a narrow subset of protein species that is also the most homogeneous regarding structure and functionality.
7. Protein quality is defined statistically by the relative abundance of the active and inactive protein species rather than by the prevalence of a canonical native structure.
8. Functional polypeptides are deposited in insoluble clusters both in prokaryotic and eukaryotic systems.
9. Undesired proteolytic activity linked to bacterial chaperones can be avoided by rehosting them to insect cells or larvae by means of the baculovirus expression system.
10. The conserved foldase activity of bacterial chaperones allows high quality recombinant protein production in insect cells or larvae.
11. Bacterial folding modulators are functional in eukaryotic systems.
12. Bacterial chaperones can be included in (and therefore expand) the catalogue of folding modulators which can be used in eukaryotic systems.

6. Annex I

Role of the chaperone DnaK in protein solubility and conformational quality in inclusion body-forming *Escherichia coli* cells

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Keywords
DnaK; misfolding; aggregation; solubility; inclusion bodies.

Abstract

Misfolding-prone proteins produced in bacteria usually fail to adopt their native conformation and aggregate. In cells producing folding-reluctant protein species, folding modulators are supposed to be limiting, a fact that enhances protein deposition. Therefore, coproducing DnaK or other main chaperones along with the target protein has been a common approach to gain solubility, although with very inconsistent and often discouraging results. In an attempt to understand the reason for this inconsistency, the impact of exogenous DnaK (encoded in an accompanying plasmid) on two protein features observed as indicators of protein quality, namely solubility and functionality, has been analysed here through the specific fluorescence emission of a reporter Green Fluorescent Protein (GFP). Intriguingly, GFP solubility is strongly dependent on its own yield but poorly affected by DnaK levels. On the contrary, the specific fluorescence of both soluble and insoluble GFP populations is simultaneously modulated by the availability of DnaK, with a profile that is clearly dissimilar to that shown by protein solubility. Therefore, solubility, not being coincident with the biological activity of the target protein, might not be a robust indicator of protein quality.

Introduction

Protein solubility is a main target during the bacterial production of recombinant proteins (Baneyx & Mujacic, 2004), because most protein species of eukaryotic or viral origin do not fold properly and aggregate as inclusion bodies (Villaverde & Carrio, 2003). Among others, lowering either the growth temperature or the transcription rate (Baneyx & Mujacic, 2004; Sorensen & Mortensen, 2005a) generally minimizes aggregation. On the other hand, since folding modulators are believed to be limiting in recombinant cells, increasing their availability by the coproduction of individual chaperones or chaperone sets should support better folding and result in higher yields of properly folded protein (Thomas & Baneyx, 1996; Thomas *et al.*, 1997; Baneyx & Palumbo, 2003; Schultz *et al.*, 2006). However, this strategy has rendered rather inconsistent results and there is no consensus about its utility and general applicability (Baldwin, 1986; Thomas *et al.*, 1997; Baneyx, 2004). The reasons for the observed experimental variability remain essentially unexplored, in general assumed to be dependent on intrinsic protein features and particular requirements of cell modulators.

On the other hand, it has lately been recognized that properly folded, functional polypeptides can aggregate as inclusion bodies (Ventura & Villaverde, 2006), and that such protein deposits, when rich in native-like secondary structure, exhibit the natural biological properties (fluorescence or enzymatic activities) of the embedded polypeptides (Garcia-Fruitos *et al.*, 2005; Arie *et al.*, 2006). Therefore, since functional polypeptides are not excluded from aggregation, it was speculated whether solubility would actually be a robust indicator of protein quality or instead, the conformational state of a given protein, reflected by its biological activity, would be more appropriate.

A misfolding-prone green fluorescent protein (GFP) fusion protein (VP1GFP) was selected as a convenient reporter to explore the extent of coincidence between solubility and conformational quality through its specific fluorescence emission. Since GFP is only fluorescent if the α-helix-rich fluorophore is well formed and the whole protein is properly structured, the specific emission of

GFP is then an excellent indicator of the conformational quality (de Groot & Ventura, 2006). Interestingly, when analysing the folding of this reporter protein under different production conditions and availability of exogenously supplied DnaK, it was observed that the solubility and conformational quality of VP1GFP were highly uncoincident features. Furthermore, while DnaK amounts dramatically influence protein quality, irrespective of its soluble or insoluble status, this chaperone is less relevant regarding solubility, which is strongly dependent on VP1GFP yield. These results indicate a divergent effect of DnaK on solubility and conformational quality, and point to solubility as a weak indicator of conformational quality. This could explain, at least partially, the noncoincident evaluations of the effect of chaperone coproduction on recombinant protein properties.

Materials and methods

Strains and plasmids

Escherichia coli streptomycin-resistant MC4100 [*araD139*Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] (Sambrook *et al.*, 1989) was used for all the experiments. Plasmid pTVP1GFP (Garcia-Fruitos *et al.*, 2005) encodes a derivative GFP fusion containing the misfolding-prone VP1 capsid protein of the foot-and-mouth disease virus. The expression of the *VP1GFP* gene is under the control of a Trc promoter, and it was induced by the addition of isopropyl-β-D-1-thiogalactoside (IPTG). The chaperone-encoding plasmid pBB535 has been described previously (Tomoyasu *et al.*, 2001). Briefly, it contains the *dnaK* and *dnaJ* genes under the control of the IPTG-inducible $P_{A1/Lac\text{-}O1}$ promoter.

Culture and gene expression conditions

Bacterial strains were cultured at 37 °C and 250 r.p.m. in shake flasks in Luria–Bertani (LB)-rich medium (Sambrook *et al.*, 1989) up to an $OD_{550\,nm}$ of 0.4, and the expression of both the recombinant protein and the chaperone genes was triggered by the addition of IPTG at different final concentrations (otherwise indicated, 1, 0.1 and 0.02 mM). For plasmid maintenance, ampicillin was used in pTVP1GFP-carrying cultures and spectinomycin was added to cultures bearing pBB535, both at 100 μg mL^{-1}. Samples were taken before and 3 h after the induction of gene expression. All experiments were performed in triplicate.

Protein fractioning and analysis

For the determination of DnaK, samples of bacterial cultures (1 mL) were centrifuged to harvest the cells (15 min at 6000 ***g***), and pellets were directly resuspended in denaturing buffer (Sambrook *et al.*, 1989) and boiled for 20 min for Western blot analysis. DnaK was immunodetected using a rabbit immune serum. Blots were scanned at a high resolution and bands were quantified using QUANTITY ONE software from Bio Rad, using different amounts of commercial DnaK protein as standards, always within the linear range.

For the analysis of VP1GFP, culture samples (10 mL) were centrifuged (15 min at 6000 ***g***) to harvest cells, and pellets were resuspended in phosphate-buffered saline (PBS). Samples were ice jacketed and sonicated for a minimum of 5 min at 50 W under 0.5 s cycles, or longer when required for total disruption of the cells, as described previously (Feliu *et al.*, 1998). After centrifugation for 15 min at 15 000 ***g***, the soluble fraction was collected and the pellet corresponding to the insoluble fraction was washed twice in lysis buffer containing 0.5% Triton and resuspended in PBS. Both soluble and insoluble material was mixed with Laemmli denaturing buffer (Sambrook *et al.*, 1989), boiled for 20 min and loaded onto denaturing gels. VP1GFP was immunodetected using a rabbit polyclonal antibody against GFP (Santa Cruz Biotechnology Inc.). Blots were scanned at a high resolution and bands were quantified using QUANTITY ONE software from Bio Rad, using different amounts of commercial GFP as standards. Determinations were always performed within the linear range.

Western blot under nondenaturing conditions was done on nonboiled samples of the soluble fraction obtained as mentioned above. One volume of sample was mixed with two volumes of nondenaturing loading buffer (250 mM Tris-HCl pH 6.8, 30.5% glycerol, 0.06% bromophenol blue) and the mixture was loaded onto gels. Duplicate gels were run in parallel for the simultaneous detection of VP1GFP (using a rabbit polyclonal antibody against GFP, Santa Cruz Biotechnology Inc.) and DnaK (using a rabbit immune serum).

Immunoprecipitation

Samples from the soluble cell fraction were incubated overnight in the presence of rabbit polyclonal antibody against GFP, at a ratio of 5 μg of antibody per 2 mg of the total protein in 0.5 mL. Protein G-Sepharose (from Sigma, 30 μL) was washed twice in PBS by centrifugation for 10 min at 8333 ***g***, and resuspended to a final volume of 300 μL. Then, 50 μL of the diluted Protein G-PBS was added to the samples. The mixture was incubated for 2 h at room temperature in agitation and then centrifuged at 8333 ***g*** for 10 min. Pellets were washed twice in PBS, resuspended in Laemmli denaturing buffer and boiled for 6 min. Samples were centrifuged at 14 083 ***g*** for 15 min and the supernatants were recovered and boiled for 20 min before loading them

onto denaturing gels for immunodetection of both DnaK and VP1GFP (in separate gels) as described above.

Fluorescence determination

Soluble cell extracts were appropriately diluted in PBS and their fluorescence was measured in triplicate, without any further treatment. The insoluble fraction was also resuspended in PBS for direct fluorescence determination. Measures were taken at 510 nm, using 450 nm as an excitation wavelength, in a Cary Eclipse Fluorescence Spectrophotometer, under continuous stirring. To evaluate the eventual fluorescence scattering in samples from the insoluble fraction containing inclusion bodies, the fluorescence of soluble GFP at 0.5 mg L^{-1} was determined in the presence of variable amounts of nonfluorescent VP1LAC (Carrio & Villaverde, 2003) inclusion bodies. Within the concentration working range, measures were only affected up to $\pm 8.5\%$. The resulting data were used to determine the specific fluorescence of both soluble and insoluble VP1GFP in combination with VP1GFP protein amounts obtained from the immunoassays.

Results

Coproduction of VP1GFP and DnaK

Using a dual expression system and different concentrations of IPTG, it was planned to generate different intracellular pools of VP1GFP and DnaK for further numerical analysis. In the first instance, the extent of the metabolic burden eventually caused in the cells by the production of either VP1GFP or VP1GFP plus DnaK was determined, within the range of IPTG concentrations commonly used for recombinant gene expression (namely 0.02, 0.1 and 1 mM). By monitoring cell growth under these conditions, moderate interferences were observed only at 1 mM IPTG in MC4100/pTVP1GFP cells, while in MC4100/pTVP1GFP/pBB535, growth under all conditions was very similar to that observed in the absence of IPTG (Fig. 1a and b). The production of both VP1GFP and DnaK proteins in MC4100/pTVP1GFP/pBB535 evolved in parallel (Fig. 1c), proving the good maintenance of both plasmids. The accumulation of both proteins slowed down after 2 h. This was especially dramatic in the case of VP1GFP, which was partially proteolysed in the cells as shown below (see Fig. 6c). This fact accounted, in addition to the occurrence of endogenous DnaK, for the higher concentration of this chaperone compared with that of VP1GFP.

On the other hand, since heat-shock genes (including *dnaK*) are expressed as a consequence of recombinant protein production (Rinas *et al.*, 1990; Allen *et al.*, 1992; Rinas, 1996; Jurgen *et al.*, 2000; Lesley *et al.*, 2002), the production of VP1GFP itself could have a positive impact on the endogenous expression of *dnaK*. As can be observed in Fig. 2, in 1 mM IPTG-treated cultures the levels of DnaK were slightly lower in the presence of pTVP1GFP than in its absence (MC4100/pTVP1GFP/pBB535 vs. MC4100/pBB535), which could be accounted for by a gene dosage effect of plasmid coexistence as shown previously for other plasmid pairs (de Marco & de Marco, 2004). However, upon IPTG addition, DnaK amounts in MC4100/pTVP1GFP/pBB535 were still threefold higher than in MC4100/pTVP1GFP. In this last case, the heat-shock response promoted by the production of VP1GFP itself doubled the background intracellular levels of the chromosomal-encoded DnaK found in plasmidless MC4100.

Effect of DnaK on VP1GFP solubility

When produced in 1 mM IPTG-treated cultures (Fig. 3a), a fraction of VP1GFP aggregated as refractile inclusion bodies (not shown), and this protein was then immunodetected in both the soluble and insoluble cell fractions to similar

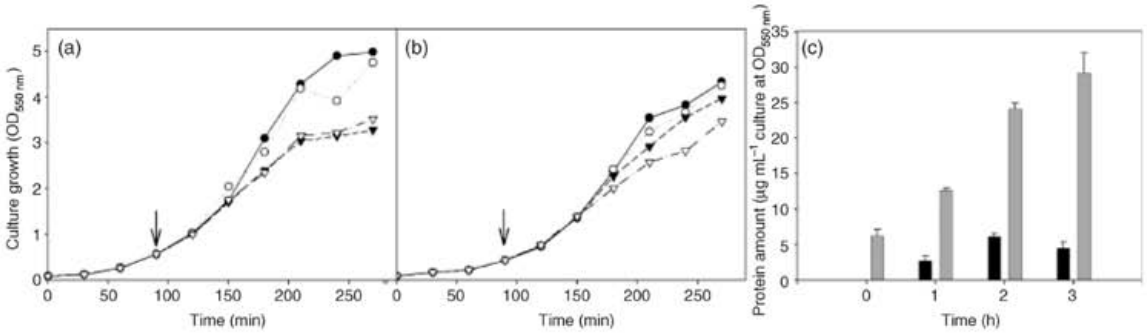

Fig. 1. Growth of MC4100/pTVP1GFP (a) and MC4100/pTVP1GFP/pBB535 (b) cultures. IPTG was added at min 90 (indicated by an arrow), at 0.02 mM (white circles), 0.1 mM (black triangles) or 1 mM (white triangles) final concentration. Cultures without IPTG are also shown as controls (black circles). (c) Accumulation of both DnaK (grey bars) and VP1GFP (black bars) in MC4100/pTVP1GFP/pBB535 cultures exposed to 0.1 mM IPTG.

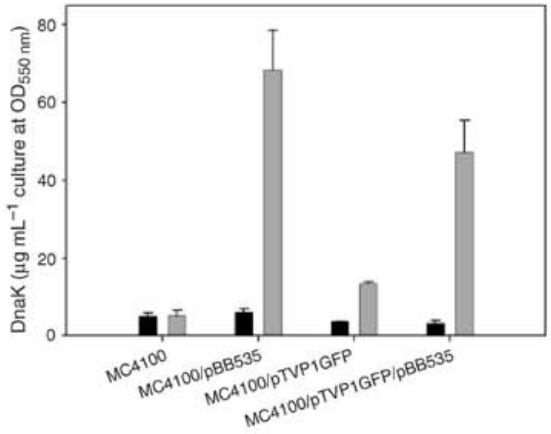

Fig. 2. Intracellular DnaK amounts determined by Western blot analysis before (black bars) and 3 h after (grey bars) 1 mM IPTG addition.

extents (42.2 ± 1.6% and 57.8 ± 1.6%, respectively) (Fig. 3b). When DnaK was overproduced along with VP1GFP, a moderate shift in the fractioning of VP1GFP was observed, but it still occurred in the insoluble cell fraction at a considerable level (48.2% ± 3.6). In the same context, the specific fluorescence of VP1GFP was not modified by the additional input of DnaK (Fig. 3c). Therefore, the coexpression approach did not result in clear improvements of protein quality.

To explore whether less strong gene expression conditions could allow a detectable and positive effect of plasmid-encoded DnaK on VP1GFP folding, the fusion protein was produced alone and with the chaperone at 0.1 and 0.02 mM IPTG. Also, the background production in the absence of the inducer was determined. Interestingly, the yield of both VP1GFP and DnaK increased concomitantly with the IPTG concentration in cells bearing both encoding plasmids (Fig. 3a). However, the endogenous levels of DnaK in MC4100/pTVP1GFP reached a plateau at 0.02 mM IPTG, indicating that low amounts of VP1GFP already represent a stress stimulus strong enough to trigger the maximum production of endogenous DnaK. The yield of VP1GFP was not modified, in any case, by the coproduction of DnaK (Fig. 3a).

On the other hand, the solubility of VP1GFP was almost absolute at basal levels of gene expression (without IPTG) and progressively decreased down to around 60% at 1 mM IPTG, irrespective of the presence of exogenous DnaK (Fig. 3b). Interestingly, the specific fluorescence of VP1GFP (although not its solubility) was moderately enhanced by the presence of pBB535 at 0.02 mM IPTG ($P = 0.021$) (Fig. 3c). This indicates that the conformational quality of the GFP fusion variant does not necessarily correlate with its solubility, and that the coproduction of DnaK has a different impact on both parameters.

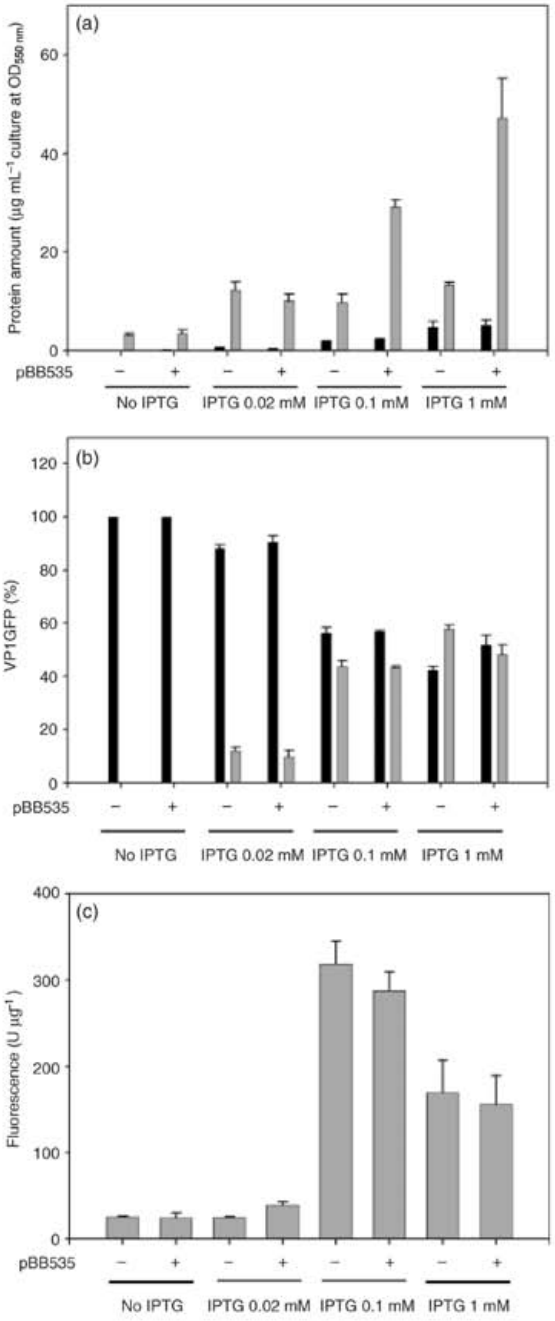

Fig. 3. Yield of both VP1GFP (black bars) and DnaK (grey bars). (a) Percentage of soluble (black bars) and insoluble (grey bars) VP1GFP (b) and total specific fluorescence (c), after addition of different amounts of IPTG. Symbols + and – indicate the presence or absence of pBB535, respectively.

Dependence between VP1GFP yield and solubility

To better understand the potential role of DnaK in modulating solubility and conformational quality, the specific fluorescence emission was determined in both soluble and insoluble VP1GFP, and these parameters and VP1GFP solubility vs. VP1GFP and DnaK concentration, and the resulting molar DnaK/VP1GFP ratio were analysed. This analysis was performed using the complete set of available data irrespective of the source of DnaK. As shown in Fig. 4, only one pair-wise comparison (VP1GFP solubility vs. VP1GFP concentration) rendered a good linear correlation. Instead, no clear dependence of VP1GFP solubility on either the DnaK amounts (although a decreasing tendency was observed) or the DnaK/VP1GFP ratio was detected. Therefore, the solubility of a misfolding-prone protein seems to be unaffected (or very poorly affected) by DnaK but by its own concentration. This is not unexpected because while protein deposition in growing inclusion bodies could follow a first-order kinetics, the seeding events triggering the formation of the aggregates probably occur under higher order kinetics (Hoffmann *et al.*, 2001; Carrio *et al.*, 2005).

Coincidence between the conformational quality of soluble and insoluble VP1GFP

The specific emission of soluble and insoluble VP1GFP was determined and these data were plotted, separately, vs. VP1GFP and DnaK concentration, and the resulting molar DnaK/VP1GFP ratio (Fig. 5). As shown, an intermediate protein yield (around 2 μg VP1GFP mL^{-1} OD^{-1}, achieved at 0.1 mM IPTG) appeared to be optimal for a proper conformational status of VP1GFP, resulting in the highest fluorescence values in both protein fractions. Note that the specific fluorescence emission ranged within 10- and 80-fold factors for the soluble and insoluble cell fractions, respectively. Also, while no clear dependence on DnaK concentration was observed, the fluorescence of both soluble and insoluble VP1GFP dramatically declined at a given DnaK/VP1GFP ratio. Interestingly, although soluble VP1GFP was around four times more fluorescent than that of the insoluble version, the fluorescence profiles of both soluble and aggregated protein were extremely coincident in front of both VP1GFP concentration and DnaK/VP1GFP ratio.

Status of soluble VP1GFP and interaction with DnaK

The diversity in the specific activities (or specific fluorescence) of proteins forming inclusion bodies has been associated with the different extents of the native-like structure found in these particles (Ventura & Villaverde, 2006). However, there are few studies investigating the presumable conformational variability in soluble protein versions. To better understand the variable specific fluorescence observed in soluble VP1GFP (Fig. 5), the presence of oligomer versions in the soluble cell fraction was explored. It was aimed to discriminate between the possibility of wrongly folded protein species kinetically trapped in alternative native-like conformations, or oligomeric species on the pathway to larger aggregate formation. As shown in Fig. 6a, a considerable fraction of VP1GFP was observed as sized oligomers, and some of them were still observed even after electrophoresis under denaturing conditions, reaching up to around 155 kDa in mass (Fig. 6c). Those oligomers might then be intermediates in the formation of inclusion bodies and they occur as 'soluble aggregates', as observed previously in other GFP-derived fusions (de Marco & Schroedel, 2005).

Interestingly, the coproduction of DnaK up-shifted low-molecular-weight forms of soluble VP1GFP to larger complexes, some of them apparently comigrating with DnaK (Fig. 6a and b). Since in addition, high molar DnaK/VP1GFP ratios minimized the specific fluorescence of VP1GFP (Fig. 5), potential interactions between VP1GFP

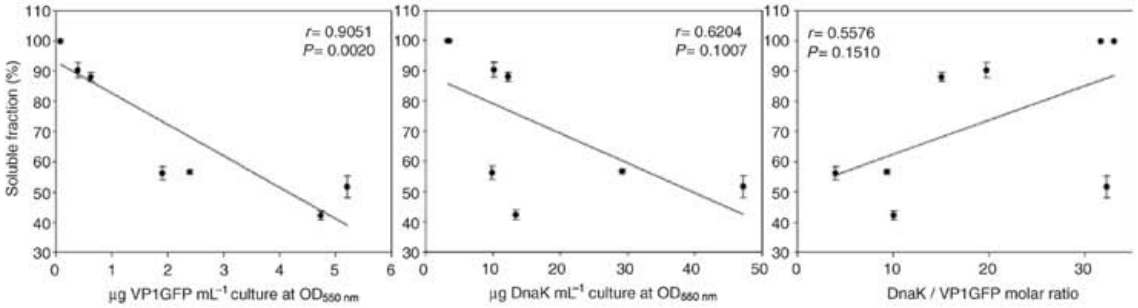

Fig. 4. Pair-wise comparisons between VP1GFP solubility and VP1GFP concentration, DnaK concentration and estimated DnaK/VP1GFP molar ratio. Correlation parameters are indicated in each panel. Data from all tested production conditions were used for the analysis, including those obtained in the absence of IPTG.

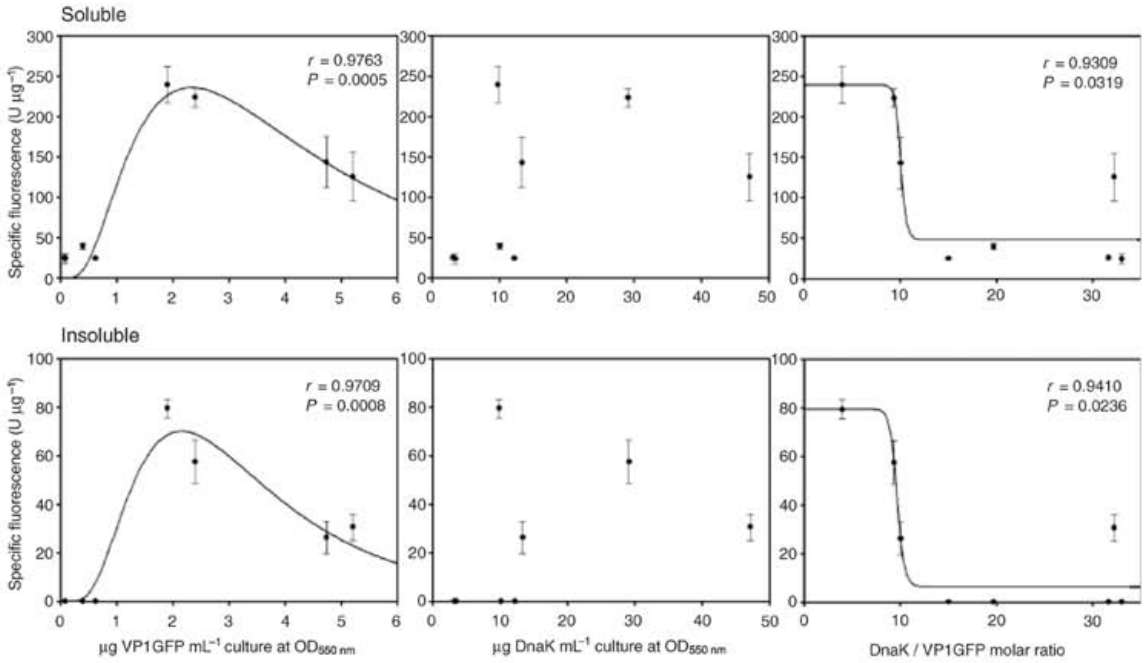

Fig. 5. Pair-wise comparisons between the specific fluorescence of soluble (top) and insoluble (bottom) VP1GFP and VP1GFP concentration, DnaK concentration and estimated DnaK/VP1GFP molar ratio. Data from all tested production conditions were used for the analysis, including those obtained in the absence of IPTG. Data in the left panels fitted a logarithmic equation while those in the right panels fitted a logistic curve equation.

and DnaK were investigated in more detail. This chaperone was found to be associated with soluble VP1GFP after immunoprecipitation with an anti-GFP polyclonal antibody (Fig. 6d), indicating a direct interaction between both proteins that could account for the described effects. The amount of coprecipitated DnaK increased in the presence of the DnaK-encoding vector, indicating that the DnaK-binding sites of VP1GFP were not saturated by the cell-encoded amounts of this chaperone.

Discussion

The solubility of recombinant proteins is obviously required for X-ray determination in structural genomics (Bussow *et al.*, 2005; Esposito & Chatterjee, 2006) and probably for most of the pharmacological uses of microbially produced polypeptides used as drugs. Under this general view, solubility has been universally assumed to be a main indicator of protein quality in production bioprocesses (Baneyx & Mujacic, 2004; Sorensen & Mortensen, 2005b). In the context of coproduction of folding modulators, the success of the explored strategies has been mainly measured by analysing solubility (Blum *et al.*, 1992; Amrein *et al.*, 1995; Thomas & Baneyx, 1996; Thomas & Baneyx, 1997; Nishihara *et al.*, 1998; Wu *et al.*, 1998; Nishihara *et al.*, 2000; Levy *et al.*, 2001; Lee *et al.*, 2003; Hoffmann & Rinas, 2004; Kim *et al.*, 2005; Heo *et al.*, 2006; Ying *et al.*, 2006). The yield of functional protein (Schaffner *et al.*, 2001) and overall enzymatic activity or fluorescence emission (but not true specific activities) (Thomas & Baneyx, 1996; Thomas & Baneyx, 1997; Levy *et al.*, 2001; Kim *et al.*, 2005; Zhang *et al.*, 2005; Heo *et al.*, 2006; Ying *et al.*, 2006) have been more rarely examined, and rough comparisons between solubility and activity, when reported, are poorly consistent (Zhang *et al.*, 2005; Heo *et al.*, 2006). However, in recombinant cells, functional protein is also found embedded in both cytoplasmic (Garcia-Fruitos *et al.*, 2005) and periplasmic (Arie *et al.*, 2006) inclusion bodies, indicating that aggregation does not necessarily imply inactivity (Ventura & Villaverde, 2006). Therefore, the biological activity should be, in principle, a better indicator of the protein-folding status than solubility, and therefore a most convenient reporter of the conformational quality (Gonzalez-Montalban *et al.*, 2007).

Here, both solubility and biological activity (fluorescence emission) of the misfolding-prone VP1GFP reporter protein have been independently explored under different production conditions, and how these parameters are influenced by DnaK. This chaperone plays major roles in regulating aggregation, refolding and proteolysis, and in general, managing the whole conformational *E. coli* stress response (Schlieker *et al.*, 2002; Hengge & Bukau, 2003; Mogk &

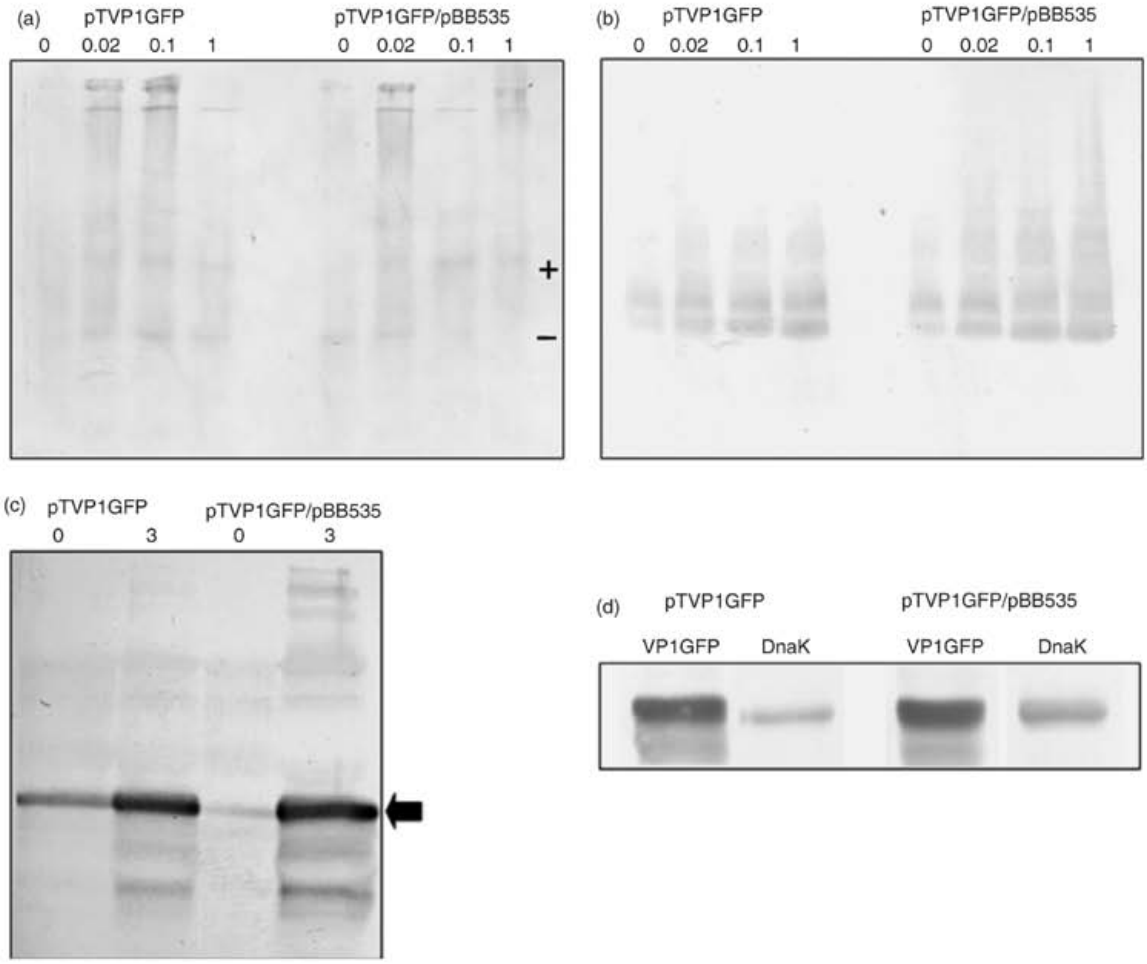

Fig. 6. Immunodetection of soluble VP1GFP (a) and DnaK (b), 3 h after treating the cultures with 0.02, 0.1 or 1 mM IPTG. Samples from cultures growing without IPTG are also shown (0). Labels indicate VP1GFP forms minimized (−) or enhanced (+) by DnaK coproduction. (c) Western blot detection of soluble VP1GFP under denaturing conditions, before (0) and 3 h after (3) addition of 0.1 mM IPTG. The arrow indicates the full-length protein band. Upper and lower bands correspond to oligomers or proteolytic fragments, respectively. (d) Immunodetection of both VP1GFP and DnaK after immunoprecipitation of soluble VP1GFP with anti-GFP polyclonal antibodies is shown. Results are shown for MC4100 strains bearing both pTVP1GFP and pBB535 plasmids, or pTVP1GFP alone.

Bukau, 2004; Weibezahn *et al.*, 2004; Gonzalez-Montalban *et al.*, 2006). Interestingly, VP1GFP solubility is not significantly affected by DnaK concentration or availability (estimated by the DnaK/VP1GFP molar ratio) (Fig. 4), but is strongly dependent on its own yield (Figs 3 and 4). Being rather irrelevant regarding solubility, DnaK moderately enhances the specific fluorescence emission, but only under mild gene expression conditions such as those promoted by 0.02 mM IPTG (Fig. 3c). In fact, increasing the DnaK/VP1GFP ratios suddenly impairs the conformational status of the reporter protein (Fig. 5, right panels). This cannot be directly attributed to a DnaK-mediated down-regulation of the heat-shock response as described previously in other systems (Petersson *et al.*, 2004), since the straightforward concentration of DnaK does not have any detectable influence on the fluorescence emission (Fig. 5, central panels). The optimal ratios for enhanced quality can be observed at medium induction conditions within the tested range (Fig. 5, left panels), when the solubility starts declining (Fig. 3). The extremely sharp reduction in fluorescence emission reported here suggests a threshold-based DnaK-assisted VP1GFP folding. In this regard, it has been shown previously that the *in vivo* release of a misfolding-prone β-galactosidase from bound DnaK molecules results in its progressive refolding to the native state (Thomas & Baneyx, 1996). Hence, a molar excess of DnaK could prevent proper

protein folding by binding to target unfolded stretches and thus may be, paradoxically, deleterious regarding protein quality. Along this line of discussion, binding of significant amounts of DnaK to soluble VP1GFP has been proven here (Fig. 6). Obviously, this fact does not exclude the possibility of indirect effects of DnaK on VP1GFP folding, but it strongly invites investigation of the possible impairment in the folding of misfolding-prone proteins when bound to a large molar excess of DnaK.

Finally, in agreement with a very recent finding indicating that the growth temperature influences in parallel the quality of soluble and insoluble recombinant proteins (Vera *et al.*, 2007), the specific emission of soluble and inclusion body VP1GFP evolves with an exquisite correspondence along protein yield and DnaK availability ranges (Fig. 5). Again, this indicates that conditions favouring proper folding act on both soluble and insoluble protein species, and that aggregation determinants are distinct from protein features supporting their biological activity.

The divergent effect of DnaK and other parameters on protein solubility and functionality prompts disregarding solubility as a straightforward indicator of protein quality, and partially accounts for the nonconsistent analytical evaluation of chaperone coproduction strategies.

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7. Annex II

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Divergent Genetic Control of Protein Solubility and Conformational Quality in *Escherichia coli*

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In bacteria, protein overproduction results in the formation of inclusion bodies, sized protein aggregates showing amyloid-like properties such as seeding-driven formation, amyloid-tropic dye binding, intermolecular β-sheet architecture and cytotoxicity on mammalian cells. During protein deposition, exposed hydrophobic patches force intermolecular clustering and aggregation but these aggregation determinants coexist with properly folded stretches, exhibiting native-like secondary structure. Several reports indicate that inclusion bodies formed by different enzymes or fluorescent proteins show detectable biological activity. By using an engineered green fluorescent protein as reporter we have examined how the cell quality control distributes such active but misfolded protein species between the soluble and insoluble cell fractions and how aggregation determinants act in cells deficient in quality control functions. Most of the tested genetic deficiencies in different cytosolic chaperones and proteases (affecting DnaK, GroEL, GroES, ClpB, ClpP and Lon at different extents) resulted in much less soluble but unexpectedly more fluorescent polypeptides. The enrichment of aggregates with fluorescent species results from a dramatic inhibition of ClpP and Lon-mediated, DnaK-surveyed green fluorescent protein degradation, and it does not perturb the amyloid-like architecture of inclusion bodies. Therefore, the *Escherichia coli* quality control system promotes protein solubility instead of conformational quality through an overcommitted proteolysis of aggregation-prone polypeptides, irrespective of their global conformational status and biological properties.

Keywords: protein folding; protein solubility; DnaK; *E. coli*; quality control

Introduction

In bacteria, overproduction of different proteins results in their deposition as amyloid-like aggregates called inclusion bodies.[1] Inclusion body formation is a sequence-specific, seeding-driven aggregation process[1,2] in which misfolded protein species, folding intermediates and presumably oligomeric species, cross-react in amorphous protein deposits rich in a pleated β-sheet architecture.[3] Such aggregates are stained with amyloid-tropic dies, such as thioflavin-T and Congo red,[1] and when exposed to mammalian cells show a toxicity profile indistinguishable from that caused by mammalian amyloids.[4]

However, bacterial inclusion bodies also contain important extents of native-like secondary structure.[5–7] Therefore, when they are formed by enzymes or fluorescent proteins they show catalytic properties or are highly fluorescent.[8] Therefore, in inclusion body-forming proteins aggregation determinants and properly folded regions coexist in single polypeptides. This is obvious when observing the core of green fluorescent protein (GFP) inclusion bodies rich in fluorescent species.[9] The occurrence of

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Abbreviations used: GFP, green fluorescent protein; mGFP, misfolding-prone GFP fusion protein; FTIR, Fourier transform infrared.

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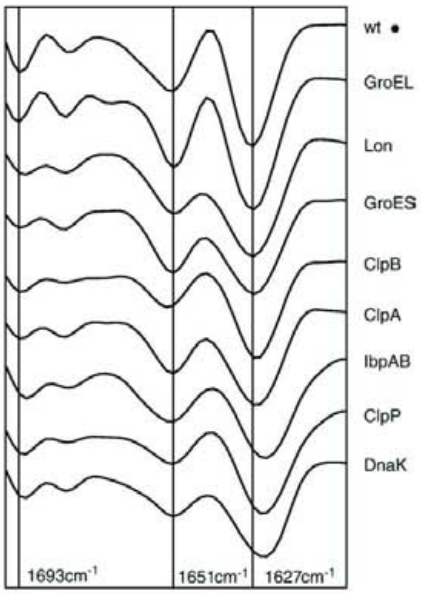

Figure 1. Second derivative spectra in the amide I region of inclusion bodies formed in either the parental strain or the derivative mutants. Vertical lines correspond to the observed extended β-sheet (1627 cm^{-1}), α-helix and/or unfolded structure (1651 cm^{-1}) and antiparallel and/or loop structure peaks (1693 cm^{-1}). Labels at the right indicate the protein whose function is totally or partially lost, and the wild-type strain is highlighted by a dot for reference, here and in the other Figures.

functional protein in inclusion bodies[3] indicates that, contrary to what has been generally believed, protein solubility and conformational quality are not coincident properties.[10] In fact, the soluble fraction of inclusion body-producing cells contains "soluble aggregates", observed, among others, in glutathion transferase,[11] β-galactosidase[12] and maltose-binding protein.[13,14] Such oligomeric protein species might be eventually less functional than the equivalent "true" soluble species. The variability in the specific activities observed in different soluble enzymes when produced under different environmental conditions strongly supports this presumption.[7,15–18] How the amyloidal deposition of conformationally hybrid, misfolded but functional proteins is regulated by the cell is not known.

In *Escherichia coli*, an evolutionarily conserved quality control apparatus (essentially composed of chaperones and proteases) is believed to survey the conformational status of the cell protein pool.[19–21] The bacterial protein DnaK, which is analogous to the eukaryotic Hsp70, regulates the whole set of quality control genes by modulating the levels of active σ 32 heat-shock transcription factor.[22–24] Being itself a chaperone, DnaK cooperates with DnaJ and GrpE for protein holding and folding,[25–30] and in association with ClpB and the small heat shock proteins IbpA and IbpB, removes individual polypeptides from aggregates for eventual refolding and activation.[31,32,63] Conventionally, solubility and conformational quality are considered to ne unequivocally connected, enhancing the cellular levels of DnaK and other chaperones through plasmid-encoded expressible genes that should result in higher solubility and biological activity.[33,34] However, such experimental approaches, generally improving solubility,[35] have often yielded inconsistent results.[36,37]

In the light of recent observations indicating that conformational quality and solubility are distinguishable protein properties,[10] it is not clear how these two parameters are genetically regulated by the cell and how misfolded but active polypeptides are selected from the soluble cell fraction for deposition. Here, we present evidence that the bacterial quality control system acts coordinately to promote solubility at the expense of conformational

Table 1. Fluorescence emission per cell biomass observed in mGFP-producing cells

	Fluorescence per cell biomass							
	Conventional fluorometry				Flow cytometry			
Phenotype	Units/OD	%[a]	Units/ng mGFP	%[a]	Mean FL1 units/cell	%[a]	Function of the missing protein	Relevant references
wt (MC4100)[b]	405.6±13.1	100	1027.4±89.2	100	176.5±10.1	100		
DnaK⁻	547.3±77.8	134.9±19.1	1506.9±89.2	146.6±8.6	443.2±15.8	250.9±8.9	Heat-shock system regulator, foldase, disagregase	22,27–29, 31,57,63,73
GroEL140[c]	342.8±35.3	84.529±8.7	1062.9±119.8	103.4±11.6	237.5±2.6	134.5±1.4	Foldase subunit	27,28,30
ClpB⁻	515.6±24.3	127.10±6.0	1628.6±78.7	158.5±7.6	290.2±7.8	164.3±4.4	Disagregase	31,57,63
ClpA⁻	543.0±45.4	133.87±11.2	1756.5±401.1	170.9±39.0	284.2±9.4	160.9±5.3	ATPase, foldase	43,74–77
GroES⁻	440.2±5.2	108.52±1.3	1408.4±62.7	137.0±8.3	218.6±11.7	123.7±5.4	Foldase subunit	27,28,30
IbpAB⁻	303.2±16.1	74.74±3.9	1036.2±123.5	100.8±12.0	150.1±2.4	85.0±1.4	Disaggregase	31,57,63,67,78
ClpP⁻	522.3±31.1	128.77±7.6	1821.5±144.8	177.2±14.0	377.4±2.5	213.7±1.1	Protease	43,74–77,79
Lon⁻	686.4±40.9	169.22±10.0	2168.0±463.9	211.0±45.1	295.3±15.4	168.4±5.1	Protease	80–84

The columns display, from left to right, fluorescence per total biomass (OD), fluorescence per specific GFP protein, and fluorescence per single cell.

[a] Fluorescence values in % are referred to those observed in MC4100.

[b] MC4100 is used as wild-type cells regarding the protein quality control system.

[c] Protein GroEL140 is partially active at 37 °C, allowing cell growth at this temperature (although not at 43 °C).

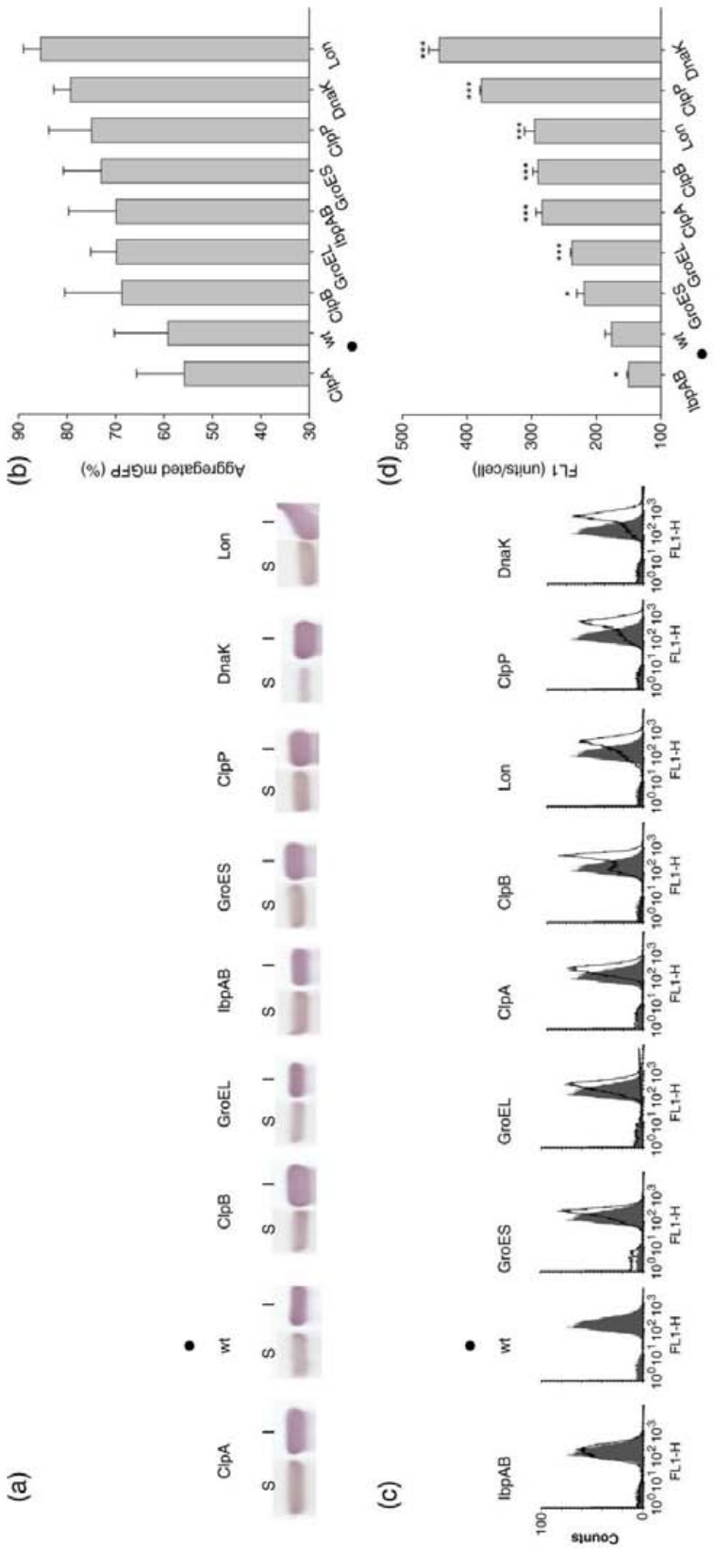

Figure 2. (a) Immunodetection of mGFP in the soluble (S) and insoluble (I) cell fractions; (b) extent of aggregation (percentage of aggregated mGFP with respect to total mGFP) in different mutant strains. (c) Flow cytometry analysis of mGFP-producing cells (black lines), showing the wild-type strain for reference (filled symbols); (d) fluorescence emission/cell in all these strains. Values significantly different from those of wild-type cells are indicated by the p parameter obtained in an ANOVA test as follows: $^{***}p<0.01$ $<^{**}p<0.05<^{*}p<0.1$. Data are ordered numerically following an increasing pattern, here and in the other Figures.

quality, and that high levels of solubility are achieved by a powerful proteolysis of target proteins in the bacterial cytosol under a complex regulation. At least Lon and ClpP proteases, directed by DnaK, degrade aggregation-prone but functional protein species by an over-committed activity that, while minimizing aggregation, dramatically reduces the cellular amounts of functional versions of misfolded protein species.

Results

Fluorescence emission of GFP requires the presence of the chromophore attached to the inner α-helix,[38] and the proper folding of the β-barrel and the complete maturation of the whole polypeptide through late folding steps that eliminate the residual water and the resulting quenching.[38–40] Therefore, the specific emission of GFP has been used successfully as a marker of the conformational quality of GFP-containing misfolding-prone proteins.[8,20,41,42] To explore the eventual divergent control of solubility and conformational quality we have analysed both parameters in a reporter, misfolding-prone mGFP fusion protein when produced in a set of mutants devoid of functional chaperones and proteases critical for the cytoplasmic quality control. In all these strains, as well as in the parental wild-type MC4100, the reporter mGFP formed cytoplasmic inclusion bodies with a consistent pattern of secondary structure characteristic of amyloid deposits (Figure 1). Considering the functions disrupted in these strains (Table 1), we envisaged a dramatic decrease of both mGFP solubility and fluorescence.

In this context, the proportion of insoluble mGFP increased in mutant cells showing that, as expected, total or partial inactivation of different arms of the quality control results in more protein aggregation (Figure 2(a) and (b)). A progressive downshift in the β-sheet peaks in all the mutants indicated mild variations in the molecular organization of the aggregates, tending to a more compact cross β-sheet architecture when comparing with the wild-type (Figure 1). However the fluorescence emission per cell was also higher in the absence of relevant chaperones (except IbpAB) or proteases than in wild-type cells (Figure 2(c) and (d)). This unexpected observation was confirmed by conventional fluorimetry (Table 1). It was especially surprising that cells lacking a functional DnaK, which strongly enhanced aggregation, were also highly fluorescent (Figure 2(c) and (d)). In such a DnaK$^-$ background, fluorescence returned to wild-type levels when producing supplementary amounts of DnaK from a DnaK-encoding plasmid (Figure 3).

When checking the soluble cell fraction and purified inclusion bodies separately, we observed that both soluble and aggregated mGFP were more fluorescent in most of the tested mutants than in the wild-type cells (Figure 4). The higher level of specific fluorescence occurred in genetic backgrounds in which the aggregation of the reporter protein was more intense (compare with Figure 2). To check if the enhanced emission of the mGFP produced in these cases could be due to less saturation of the folding machinery and, consequently, to more opportunities for proper folding, we determined the intracellular concentration of mGFP. However, much more mGFP was observed in most of the mutant strains (Figure 5(a)).

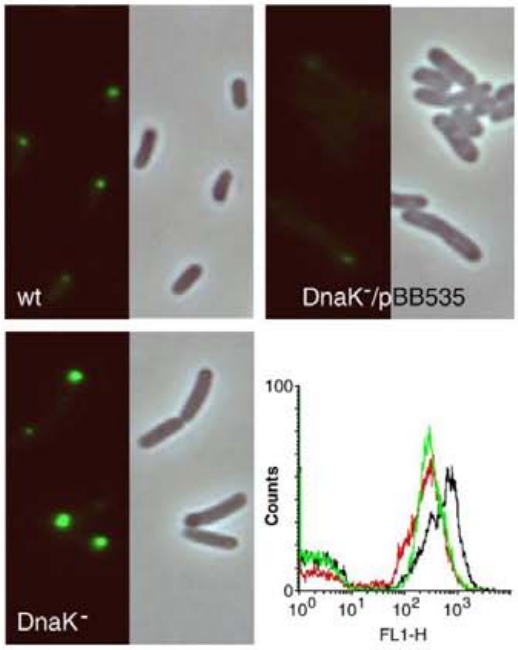

Figure 3. Fluorescence microscopy images of wild-type, DnaK$^+$ and DnaK$^-$ cells overproducing DnaK (encoded in pBB535). Flow cytometry of these strains is comparatively shown in red, black and green, respectively.

We hypothesized that protein stability could be the major contributor to the different GFP pools observed in the mutant set. While a lower degradation rate of misfolding-prone species would be expected in protease-deficient cells (in either Lon$^-$ or ClpP$^-$ cells), such a phenotypic trait would not be evident in the other genetic backgrounds tested. Therefore, degradation of mGFP was monitored in the absence of protein synthesis and its half-life was determined in several independent experiments. Intriguingly, proteolysis of mGFP was clearly inhibited in all the tested mutants except in IbpAB$^-$ cells (Figure 5(b)). Furthermore, the stability of mGFP in the absence of DnaK or ClpB was close to that observed in the absence of Lon, ClpP, or the ClpP ATPase subunit ClpA,[43] proving that both DnaK and ClpB are positive regulators of mGFP degradation. Again, and in agreement with complementation data shown in Figure 3, the transformation of DnaK$^-$ cells with an expressible, DnaK-encoding plasmid accelerated the degradation of mGFP to wild-type rates. Therefore, chaperone-modulated digestion of misfolding-prone but functional polypeptides appears as the

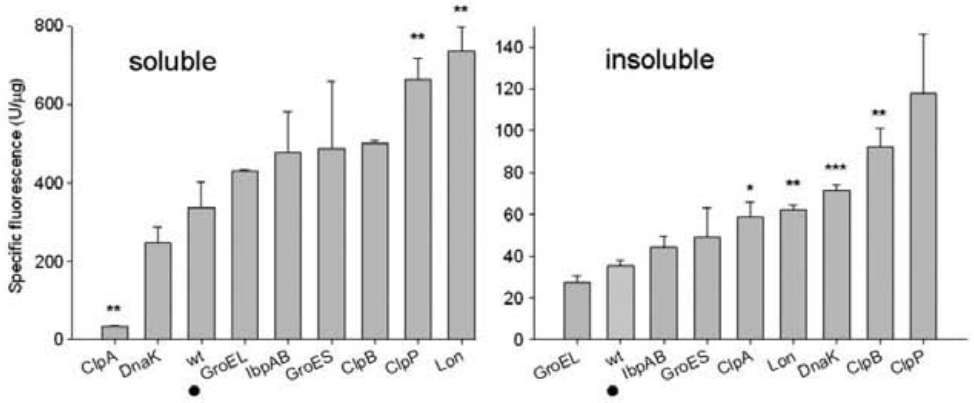

Figure 4. Specific fluorescence of mGFP in the soluble and insoluble cell fractions.

main determinant of the relatively small amounts of fluorescent mGFP in wild-type cells.

Discussion

The functional meaning of protein aggregation is still controversial. Intriguingly, many examples of bacterial inclusion bodies formed by highly functional enzymatic forms have been reported, including those formed by β-galactosidase,[44] endoglucanase-D,[45] β-lactamase,[46] rHtr1 aliase[46] and dihydrofolate reductase,[8] and by fusion proteins containing β-galactosidase,[8] β-lactamase and alkaline phosphatase. In a similar way, GFP,[20,47] GFP fusion versions[8,20] and BFP[8,42] form highly fluorescent inclusion bodies. This indicates that solubility is not matching conformational quality, and the connection between the occurrence of aggregation determinants and the average folding status of the protein is still far from being solved. Numerous pieces of evidence point to defined protein patches as drivers of the aggregation process,[48] which can occur without necessarily compromising the biological activity of the protein deposited.[8,49] The progressive characterization of individual genes expressed during the *E. coli* heat-shock response, and the identification of the functions displayed by the encoded proteins, pictured the existence in *E. coli* and in higher organisms, of a complex protein quality system that mediates the elimination of folding-reluctant, potentially cytotoxic species. The quality control is executed through the combined action of chaperones and proteases and is especially relevant under conformational stresses such as those caused by high temperatures or high rates of protein synthesis. However, as discussed below, the physiological mechanics of the quality control, at the system level, might have been largely misunderstood.

Recombinant bacteria are one of the best models to investigate protein quality control, since many of the foreign proteins over-produced therein tend to form a special type of amyloid aggregates known as inclusion bodies.[1,50] It has been generally believed that aggregation is promoted by a high substrate load of the quality control system. Therefore, the

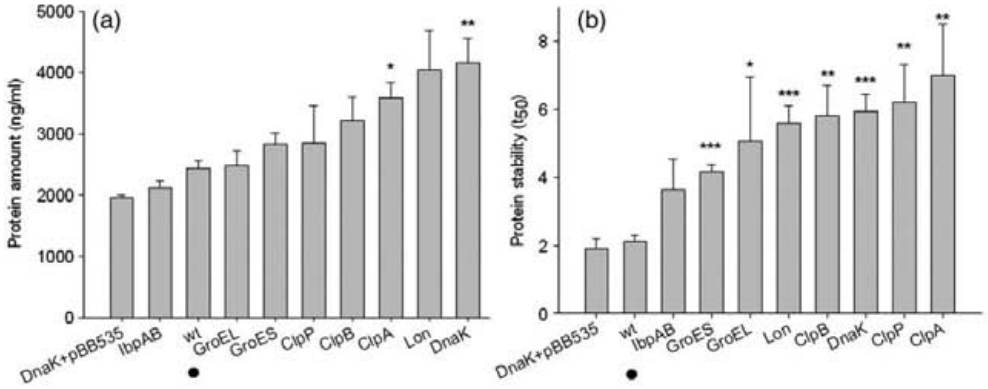

Figure 5. (a) Total intracellular amounts and (b) proteolytic stability of mGFP in the different producing strains. Values significantly different from those of wild-type cells are indicated by the p parameter obtained in an ANOVA test as follows: $^{***}p<0.01<^{**}p<0.05<^{*}p<0.1$. Labels indicate the protein whose function is lost, and the wild-type strain is highlighted by an asterisk. The presence of pBB535 is indicated. Total mGFP amounts and half-life correlated within the limits of statistical significance ($p=0.052$).

insufficient availability of folding modulators and proteases would fail in preventing the accumulation of misfolded species. Therefore, the additional supply of chaperones from encoding plasmids has been explored repeatedly as a way to gain solubility in production processes,[33,37] and solubility has been taken as universal indicator of conformational quality. In this context, functional deficiencies in the main chaperones such as DnaK or GroEL were expected to result in lower conformational quality (therefore less functional recombinant protein) than when the quality control system is completely active.

When analyzing the fluorescence of different mutant strains (deficient in GroEL, ClpA, ClpB, ClpP, Lon and DnaK activities) producing a misfolding-prone version of GFP, we surprisingly noted more fluorescence emission per cell than in the wild-type (Figure 2(d)). This was due to a generally higher specific emission of both soluble and insoluble mGFP populations (Figure 4). However, solubility in these genetic backgrounds was not concomitantly high. On the contrary, the aggregation of the reporter GFP was enhanced (Figure 2(b)) and the Fourier transform infrared (FTIR) analysis of the inclusion bodies showed a band down-shift from 1627 cm^{-1} to around 1623 cm^{-1} (Figure 1), indicative of a more condensed, amyloid-like cross molecular β-sheet architecture. In particular, when observing aggregation in the absence of DnaK, the high level of fluorescence exhibited by the aggregates returned to wild-type levels when complemented by a *dnaK*-carrying expression vector (Figure 3). In summary, the disruption of different arms of the *E. coli* cytosolic quality control resulted, as expected, in less solubility but, contrary to what is generally presumed, in much more functional proteins in both soluble and insoluble populations (Figure 4). Note that, for instance, the big aggregates formed in the absence of DnaK were extremely fluorescent (Figure 3).

Since the cellular amounts of mGFP were also enhanced in most of the strains tested (Figure 5(a)), and this fact correlated with proteolytic stability (Figure 5(b)), it was reasonable to infer that in cells with a fully functional quality control system, proteolysis acts largely on aggregation-prone but functional (or suitable to be activated) polypeptides. Such over-committed activity must remove insoluble protein species thus increasing solubility in relative terms, but resulting in less cell fluorescence. This is illustrated by the negative role of DnaK in protein stability. In the absence of this chaperone, GFP half-life increases dramatically to levels comparable with those observed in the absence of Lon (Figure 5(b)), one of the main cytosolic proteases degrading recombinant proteins.[51] However, the expression of plasmid-harboured copies of *dnaK* reduced fluorescence (Figure 3), minimized accumulation of mGFP by lowering its half-life to wild-type levels (Figure 5(a) and (b)), and reduced aggregation to around 30 % (not shown).

While Lon and ClpP (and its ATPase subunit ClpA) are known to be involved in the degradation of most recombinant proteins in *E. coli*,[52–54] the roles of DnaK and ClpB (and other chaperones identified here) in positively mediating proteolysis are less apparent. In this respect, it could be considered that greater stability could be an indirect consequence of the enhanced aggregation observed in the absence of these chaperones. Although it is generally believed that aggregation as inclusion bodies prevents proteolysis,[36] *in situ* or release-associated digestion of inclusion body-forming proteins has been described.[51,55,56] In fact, the absence of main cell proteases results in oversized aggregates,[51] indicating that, on the contrary, inclusion body formation is regulated strongly by proteolysis. In addition, the absence of a negative correlation between solubility and the half-life of mGFP ($p=0.5907$, data not shown) is further evidence that aggregation as inclusion bodies is not the cause of proteolytic resistance but *vice versa*.

Both DnaK and ClpB act as a disaggregase complex[31,57,58] that remove aggregated polypeptides for eventual refolding. In inclusion body-forming cells, DnaK accumulates on the inclusion body surfaces,[59] where disaggregation is expected to occur. When either DnaK or ClpB are absent, the spontaneous disintegration of inclusion bodies is largely delayed,[60] while the co-production of both chaperones favors aggregate dissolution.[61] On the other hand, physiological removal of inclusion body proteins is temporarily associated with protein cleavage, and the mathematical modelling of inclusion body dissolution repeatedly indicated that protein digestion is intimately associated with protein removal.[55,62] How DnaK and ClpB could mechanistically promote Lon- or ClpP-mediated digestion during protein extraction from aggregates is not clear and requires further investigation. Interestingly, the small heat shock proteins IbpA and IbpB, which act in cooperation with DnaK and ClpB in protein refolding,[57,63] do not seem to be positively involved in proteolysis, as their absence does not result in stabilization of mGFP (Figure 5). On the contrary, these small chaperones display protease-protective roles on misfolded proteins,[64] which could antagonistically regulate *the in situ* proteolysis promoted by DnaK-ClpB.

Intriguingly, the divergence between solubility and conformational quality recently proposed in the context of recombinant protein production is clearly illustrated by the negative relationship between the relative occurrence of soluble species and their specific emission (Figure 6(a)).[10] This fact supports the suggestion that insoluble but functional polypeptides are preferred targets for DnaK-controlled proteolysis, and indicated that biological activity is not compromised by the occurrence of aggregation determinants. In this respect, it has been shown recently that factors favouring protein folding, such as low temperature,[20] or appropriate levels of DnaK,[65] activate soluble and inclusion body protein versions simultaneously. We determine here that the specific fluorescence of soluble and insoluble GFP correlate well when these parameters are compared pair-wise in different strains, although the level of

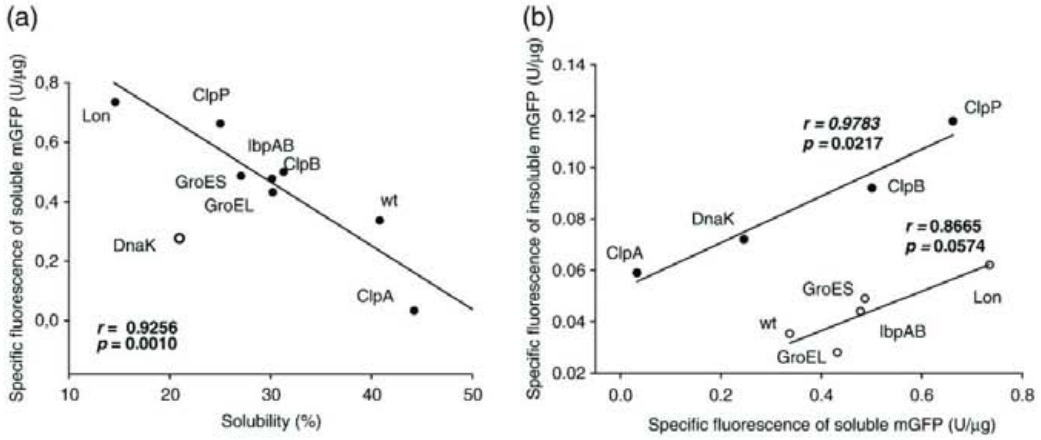

Figure 6. (a) Pair-wise comparison of solubility and the specific emission of soluble mGFP in different producing strains. The data from DnaK$^-$ cells has been excluded from the analysis but the coordinate is indicated by a white circle. When including this value, the correlation is still significant ($r = 0.698$; $p = 0.036$). (b) Pair-wise comparison of the specific fluorescence of soluble and insoluble mGFP in all the different producing strains. Correlation parameters are shown in each panel. The slopes are 0.0906 and 0.0767 for the upper and lower lines, respectively.

fluorescence of aggregated protein is higher in the set lacking DnaK, ClpA, ClpB or ClpP than in others (Figure 6(b)). This fact indicates that these four proteins participate in the discrimination of functional species between soluble and insoluble fractions. It also supports the hypothesis that inclusion bodies are not excluded from conformational control, which acts irrespective of protein solubility. Interestingly, the slopes of both correlation lines are numerically very similar (Figure 6(b)), indicating again a high level of regularity in the genetic control of the conformational quality of soluble and aggregated species. The similarity between the specific activity of soluble and inclusion body versions of a recombinant β-galactosidase (structurally and functionally different from GFP) in the absence of DnaK,[7,15] indicates that the genetics of quality control described here is not limited to the model protein used in the present study.

Therefore, solubility, resulting from the combination of the protein folding process and an overcommitted, highly selective proteolysis should be reconsidered as a universal indicator of conformational quality.

Materials and Methods

Strains and plasmids

The *Escherichia coli* strains used in this work were MC4100 (*araD139* Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*),[66] used as a wild-type, and its derivatives JGT3 (Δ*clpB::kan*), JGT4 (*clpA::kan*), JGT6 (*zjd::Tn10 groES30*), JGT17 (Δ*ibp::kan*), JGT19 (*clpP::cat*), JGT20 (*dnak756 thr::Tn10*),[67] BB4564 (*groEL140 zjd::Tn10 zje::*Ω*Spc*^{r}/*Str*^{r}),[30,30,68] and BB2395 (Δ*lon146::miniTn10*).[53] These strains were transformed with pTVP1GFP,[8] encoding a misfolding-prone fusion version of GFP (mGFP), in which the VP1 capsid protein of foot-and-mouth disease virus is joined at the amino terminus and produced under the control of the *trc* promoter. The chaperone-encoding plasmid pBB535 (Spec^R), has been described.[53] Briefly, it drives the expression of the *dnaK* and *dnaJ* genes under the control of the IPTG-inducible $P_{A1/Lac\text{-}O1}$ promoter and it is fully compatible with pTVP1GFP.

Culture conditions

All the production processes were performed in shake-flask cultures growing at 37 °C and 250 rpm in LB rich medium,[66] plus 100 μg/ml of ampicillin and eventually 100 μg/ml of spectinomycin for plasmid maintenance. Expression of VP1GFP gene was induced when the absorbance at 550 nm reached 0.4, by adding IPTG to 1 mM. Cell samples were taken at 2 h after induction of gene expression for further analysis. For the *in vivo* determination of proteolytic stability, protein synthesis was arrested by adding chloramphenicol to 200 μg/ml, and the cultures were further incubated at 37 °C and sampled at different times. Data were obtained from three or more independent experiments.

Flow cytometry

Flow cytometric analyses were performed after sample fixation with 0.1 % (v/v) formaldehyde overnight at 4 °C, on a FACSCalibur system (Becton Dickinson) with 488 nm excitation from a 15 mW air-cooled argon-ion laser. Fluorescence emission was acquired through a 530/30 nm bandpass filter (FL1) in logarithmic mode. For every sample, the fluorescence emission in channel FL1 was analyzed with a FACSCalibur and converted in a spreadsheet by the WinMDI 2.8 software. During the elaboration of the data, we calculated the geometric mean for every sample.

Determination of total and specific fluorescence

Culture samples of 1 ml were jacketed in ice, disrupted by sonication for 4 min at 50 W under 0.5 s cycles as described,[69] and centrifuged at 4 °C for 15 min at 15,000*g*. The supernatant was used directly for the analysis as the soluble cell fraction. Inclusion bodies were purified by a

detergent-washing protocol as described,[70] and used in suspension for activity analysis. Fluorescence at 510 nm was recorded with a Cary Eclipse fluorescence spectrophotometer (Variant) by using 450 nm as the excitation wavelength. Fluorescence was measured, in triplicate, in 1 ml samples using dilutions when necessary, and corrected by either biomass or amounts of mGFP protein determined as described below.

Quantitative protein analysis

Samples of bacterial cultures (10 ml) were centrifuged at low speed (15 min at 15,000*g*) to harvest the cells and resuspended in 2 ml of PBS with one tablet of Protease Inhibitor Cocktail (Roche, ref. 1 836 170) per 10 ml of buffer. These samples, once jacketed in ice, were sonicated for 5 min (or longer when required to achieve a complete disruption) at 50 W under 0.5 s cycles as described,[69] and centrifuged for 15 min at 15,000*g*. The supernatant was mixed with denaturing buffer at the appropriate ratios.[71] For the determination of inclusion body protein, these aggregates were purified by repeated washing with detergent as described,[70] and resuspended in denaturing buffer.[71] For the analysis of proteolytic stability, protein synthesis was arrested by adding chloramphenicol at 200 μg/ml 2 h after of induction of gene expression, and the cultures were further incubated at 37 °C. Samples taken at different times were then centrifuged for 15 min at 15,000*g* and pellets were resuspended in denaturing buffer.[71] After boiling for 15 min (for the soluble fraction) or 25 min (for inclusion bodies), appropriate sample volumes were loaded onto denaturing gels. For Western blot, a polyclonal serum directed against GFP was used. Dried blots were scanned at high resolution and bands were quantified by using the Quantity One software from Bio Rad, with the appropriate protein dilutions of known concentration as controls. Slight bands compatible partially with proteolysed mGFP and with full-length GFP were observed in some cases, representing always <4% of the total immunoreactive material and were included in all the analysis. Determinations were done within a linear range and they were used to calculate the specific activity values. We performed a nested ANOVA to analyse the different samples.

FTIR spectroscopy

Purified inclusion bodies were dried for 2 h in a Speed-Vac system before analysis to reduce water interference in the infrared spectra. The FTIR spectrum of the dry samples was analysed directly into a Bruker Tensor FTIR spectrometer. All processing procedures were carried out so as to optimize the quality of the spectrum in the amide I region, between 1600 cm^{-1} and 1700 cm^{-1}. Second derivatives of the amide I band spectra were used to determine the frequencies at which the different spectral components were located. A general description of FTIR procedures can be found elsewhere.[72]

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